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On-line preconcentration of pharmaceutical residues from large volume water samples using short reversed-phase monolithic cartridges coupled to LC-UV-ESI-MS

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Abstract

A simplified preconcentration method for a range of ultra-trace level pharmaceuticals in natural waters has been developed. Solid phase extraction was performed on-line using a micro-reversed-phase monolithic silica column, allowing for very rapid trace enrichment from large volume (500 ml) samples with minimal sample handling. Acceptable recoveries of >70% were obtained for the majority of compounds investigated and the monolithic columns could be washed and conditioned on-line with no sample carryover and used reproducibly for up to eight extractions each. The on-line SPE-LC-UV method was coupled to electrospray ionisation ion trap mass spectrometry (ESI-MS) to increase both selectivity and specificity. Detection limits were determined in spiked river and tap water samples and found to lie in the low ng/l region using sample volumes of 500 ml, loaded at a flow rate of 10 ml/min, and therefore, were suitable for ultra trace analysis.

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1. Introduction

Within the last decade pharmaceuticals and personal care products (PPCPs) have emerged as a new class of organic micro contaminants that are frequently detected in the aquatic environment [1–2], although to date the presence of pharmaceuticals in environmental waters remains uncontrolled within Europe. As a result, the number of methods appearing in the peer-reviewed literature for the determination of these ‘new wave’ contaminants has increased. Due to the ultra trace levels of pharmaceuticals that may be present, normally within the ng/l to low µg/l range [3], a analyte preconcentration procedure is almost always necessary in order to attain the desired levels of analytical sensitivity, often requiring relatively large sample volumes of between 100 and 1000 ml. The foremost approach reported to date has been the use of solid phase extraction (SPE) in the off-line mode [4–6]. Sorbents reported in the literature for the trace enrichment of

pharmaceuticals from various matrices include C₁₈ silica [4,7], crosslinked poly(styrene divinylbenzene) [8,9], C₂ silica [10], immunosorbents [11] and hydrophilic lipophilic balanced polymers [5,6,12–21].

A drawback of off-line SPE procedures is that they can be time consuming and cumbersome to perform, often requiring many steps before reaching a concentrated extract suitable for instrumental analysis, of which only a small proportion is actually injected onto the chromatographic column. In contrast on-line SPE techniques offer increased sensitivity with reduced sampling handling as all the extracted components can easily be introduced to the analytical column. Reported detection limits in the pg/l range are not uncommon [22,23]. One of the driving forces for on-line SPE methods is higher sample throughput and faster analysis [24] and the format of on-line SPE can vary from column switching techniques [25–33] to automated SPE instruments coupled to liquid chromatographs [23,34–36]. Although on-line SPE is an attractive prospect it is not without limitation. Extraction columns are generally quite small and therefore can only contain a limited quantity of sorbent leading to problems with analyte retention [25]. Sorbents are generally

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particulate in nature, often having relatively large particle sizes but with large active surface areas required to achieve acceptable recoveries. Applications of on-line SPE and automated SPE for PPCPs are fairly limited in number, but include the preconcentration of pharmaceuticals [25,33], estrogens and endocrine disruptors [23,35] and nonylphenol [29], with subsequent liquid chromatography and photodiode array or MS detection.

Monolithic silica and polymer materials have recently found much application in the field of separation science, particularly for rapid separations. Commercially available silica based short monolithic reversed-phase columns (Merck Chromolith range), which have a bimodal pore structure (macropores of $\sim 2\ \mu\text{m}$ and mesopores of $\sim 13\ \text{nm}$), are both highly porous and high in active surface area, and can produce typical separation efficiencies equivalent to those seen with 3–5 μm particle packed columns [37]. Such porous structure also allows monolithic columns to be used at elevated flow rates without the generation of excessive backpressure, while sorbed analytes remain within sharp bands within the column, undergoing less band broadening than seen with many traditional particle packed columns. These characteristics make short monolithic phases potentially very suited to rapid sample extraction at high loading flow rates, while maintaining relatively high extraction efficiencies due to the large surface area, and also maintaining the sharp adsorbed analyte bands required for the on-line elution onto an analytical separation column.

In the following study, for the first time the use of short (10.0 mm $\times$ 4.6 mm i.d.) reversed-phase silica monolithic columns for the on-line rapid preconcentration of ultra-trace levels of pharmaceuticals from large volume natural water samples has been investigated. The effects of sample loading conditions such as sample pH and loading rates were investigated in relation to analyte recoveries for a range of acidic, basic and neutral pharmaceuticals. The precision and reusability of the short monoliths was also investigated. The on-line monolithic preconcentrator column was connected to a longer reversed-phase monolithic column of the same type, used for the analytical separation applying a previously reported ‘dual gradient’ method of simultaneous solvent and flow rate increase to reduce run times and increase the efficiency of the separation [38]. The high loading rate possible with the monolithic concentrator column meant 500 ml samples could be loaded within the time required for the chromatographic separation of the previous sample. Coupling the on-line SPE-LC-UV procedure with ESI-MS detection increased the selectivity and sensitivity of the complete method.

2. Experimental

2.1. Chemicals and reagents

Reagent water used throughout this study was obtained from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) and was 18.2 M Ω or greater. Methanol and acetonitrile were received from Labscan (Dublin, Ireland), acetone and ethyl acetate were obtained from Aldrich (Gillingham, UK). All solvents used were HPLC grade. Dichlorodimethylsilane, ammonium formate, ammonium acetate, 4-morpholine ethane

sulphonic acid hydrate (MES), *N*-2-hydroxyethyl piperazine-*N*-2-ethane sulphonic acid (HEPES) and formic acid were also purchased from Aldrich. BDH Analar grade sulphuric acid was used for sample pH adjustment (Poole, UK). Pharmaceuticals chosen for investigation included those that showed prevalence in both the published literature and also the list of the most prescribed pharmaceuticals in Ireland for the year ending 2003 [39]. Metformin hydrochloride, acetaminophen, salicylic acid, propranolol hydrochloride, clofibric acid, ketoprofen, diclofenac sodium salt and clotrimazole were obtained from Aldrich (Steinheim, Germany). Trimethoprim, caffeine, naproxen, ciprofloxacin and triclosan were received from Fluka (Buchs, Switzerland). Bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, mefenamic acid, gemfibrozil, atenolol, salbutamol, amoxicillin, sulfamethoxazole, sulfamethazine sodium salt, furosemide, carbamazepine, nimesulide, fluoxetine hydrochloride and ivermectin were all obtained from Sigma (Steinheim). All pharmaceuticals were of a purity >95%. Table 1 lists the range of pharmaceuticals investigated, together with reported pK_a values and both experimentally determined and theoretical log P data.

Stock 1000 mg/l solutions of each pharmaceutical were prepared in methanol with the exception of ciprofloxacin, which was prepared in water and were stored as described on the

Table 1
Pharmaceuticals, pK_a and log P values

Analyte	pK_a [41]	log P , experimental data and theoretical (Xlog P) [42]
Metformin	12.40 [43]	–, (0.56)
Atenolol	9.16 [44]	0.16, (0.46)
Salbutamol	5.90 [45]	–, (1.44)
Paracetamol	9.50 [46]	0.46, (0.45)
Salicylic acid	3.50	2.36, (2.43)
Amoxicillin	2.40	0.87, (0.03)
Trimethoprim	6.60	0.91, (0.65)
Sulfamethazine	2.65	0.89, (0.46)
Caffeine	14.00 [47]	–0.07, (–0.50)
Sulfamethoxazole	5.70	0.89, (0.68)
Ciprofloxacin	6.38	–, (1.94)
Furosemide	3.90 [44]	2.03, (1.41)
Propranolol	9.49	3.48, (3.03)
Clofibric acid	–	2.58, (2.58)
Carbamazepine	13.90	2.45, (2.30)
Ketoprofen	4.45	3.12, (3.22)
Bezafibrate	3.60	–, (3.85)
Nimesulide	6.50 [48]	–, (2.51)
Naproxen	4.20	3.18, (2.84)
Warfarin	5.05 [49]	2.60, (2.96)
Fluoxetine	8.70 [50]	–, (3.82)
Flurbiprofen	4.33 [44]	4.16, (3.76)
Diclofenac	4.15	–, (3.91)
Indomethacin	4.50 [43]	4.27, (4.18)
Ibuprofen	4.51	3.97, (3.64)
Meclofenamic acid	–	–, (4.32)
Mefenamic acid	4.20 [51]	5.12, (4.16)
Clotrimazole	6.12	–, (6.14)
Gemfibrozil	–	–, (3.56)
Triclosan	8.10 [52]	4.76, (4.96)
Ivermectin	–	–, (3.41)

material safety data sheets (MSDS) for maximum stability, i.e. refrigerated and in the dark. In the case of salts, standards were prepared in terms of the parent analyte. These standards were periodically replaced on a monthly basis. Working standards were prepared from these stock standards by appropriate dilution using methanol.

Buffer solutions used for the optimisation of the extraction pH were prepared by mixing the appropriate amounts of acid and its conjugate base for formate and acetate, respectively. In the case of MES and HEPES weighted proportions of salt were dissolved in ~900 ml Milli-Q water and titrated to the desired pH using either 1 M HCl or 1 M NaOH.

2.2. Glassware preparation

Prior to use all glassware was silanised to prevent adsorption of pharmaceutical residues by rinsing thoroughly with a 10% (v/v) solution of dichlorodimethylsilane in dichloromethane, followed by rinsing twice with dichloromethane and twice with methanol.

2.3. On-line SPE

On-line SPE was performed using a Rheodyne 7000 six-port column-switching valve (Cotati, CA, USA). The extraction column was a Merck Chromolith C₁₈ monolithic silica guard cartridge, 10.0 mm × 4.6 mm i.d. (Merck, Darmstadt, Germany).

A Merck Hitachi LaChrom L-7100 pump was used for sample delivery. Prior to extraction the short monolithic column was conditioned with 20 ml of acetonitrile and 20 ml of water at flow rates of 5 ml/min. Samples were filtered through Whatman GF/C glass fibre filters to remove suspended matter and a 500 ml aliquot was transferred to a silanised Duran bottle and adjusted to pH 4.0 using concentrated sulphuric acid. Samples were pumped through the extraction column at a rate of 10 ml/min. Following complete extraction the column was washed with 20 ml of 10% (v/v) acetonitrile in water at a rate of 10 ml/min. Elution was performed by back flushing with mobile phase for 25 min of the chromatographic dual gradient run. After such time the valve was switched back to the load position for the next extraction.

2.4. LC-UV and LC-UV-MS analysis

For method development a Hewlett Packard HP 1050 series high performance liquid chromatograph was used and consisted of a quaternary pump with on-line vacuum degasser and a variable wavelength detector, model 78953C. Agilent ChemStation Version A.09.03 was used for instrument control and data analysis (Agilent Technologies, Palo Alto, CA, USA). LC-ESI-MS analysis were performed using an Agilent 1100 series high performance liquid chromatograph consisting of a binary pump with on-line vacuum degasser and a photodiode array detector, model G1315B. The LC system was coupled to a Bruker Daltonics esquireLC ion trap MS complete with an atmospheric pressure electrospray ionisation interface. Agilent ChemStation Version A.06.01 (Agilent Technologies) and Bruker Daltonics esquire control Version 6.08 (Bruker Daltonics, Coventry, UK)

were used for LC-UV-ESI-MS system control. Bruker Daltonics DataAnalysis Version 2.0 was used for data processing. For electrospray ionisation optimisation solutions of analyte were directly infused into the mass spectrometer using a Cole Parmer 74900 series syringe pump at a rate of 300 µl/h (Cole Parmer, Vernon Hills, IL, USA).

Analytical separations were performed using two 100 mm × 4.6 mm i.d. Merck Chromolith Performance RP-18e monolithic silica columns joined in series using a Chromolith zero dead volume column coupler (Merck KGaA, Darmstadt, Germany). Details of the dual gradient separation parameters were previously reported [38] and were as follows; the initial % methanol was 10% held isocratically for 8 min followed by a linear increase to 52.5% methanol in 7 min with an isocratic hold for 20 min, after such time the % methanol was again linearly increased to 65% in 5 min and held for 15 min, the final linear increase to 90% methanol was performed in 5 min with a further isocratic hold for 10 min. A simultaneous flow gradient was used in conjunction with the solvent gradient whereby the flow was linearly raised from 1.00 to 2.30 ml/min in 55 min and then held isofluently for the remainder of the chromatographic run. Fig. 1 shows overlaid chromatograms for standard mixtures of pharmaceuticals separated using the above dual gradient method. Prior to entering the electrospray interface the flow was split ~1/20 using an UpChurch Scientific graduated micro splitter valve, model P-470 (UpChurch Scientific, Oak Harbor, WA, USA).

3. Results and discussion

3.1. On-line SPE—optimisation of the sample loading rate

The unique porous structure of monolithic silica allows for the use of elevated flow rates without the generation of excessive backpressure normally encountered when using particulate-based media under the same conditions. Here, the use of a short monolith as an on-line concentrator column with LC was investigated. On-line SPE using the monolithic preconcentrator offers the following potential advantages over using an off-line SPE approach. Firstly, despite the surface area of the short monolith being ~350 m²/g compared to higher surface areas for commercial packed SPE cartridges (Oasis XLB = 700 m²/g, LiChrolut = 1000 m²/g), it was envisaged the relatively high efficiency of the monolithic phase, comparable to 3–5 µm particle packed phases, and the comparatively small flow through channels would result in sharp preconcentration bands and therefore equal or improved extraction efficiencies for analytes exhibiting some reasonable degree of hydrophobicity. Secondly, the system would permit rapid sample loading, equivalent to the loading rates used with off-line SPE using commercial cartridges, but also permit automated washing and elution steps. Thirdly, the elution of the retained analytes would be possible using the LC mobile phase and the direct back flushing of the analytes as sharp sample bands onto the analytical column possible, without any analyte loss. Finally, the system allowed for simultaneous loading of the next sample during the chromatographic separation of the previous sample.

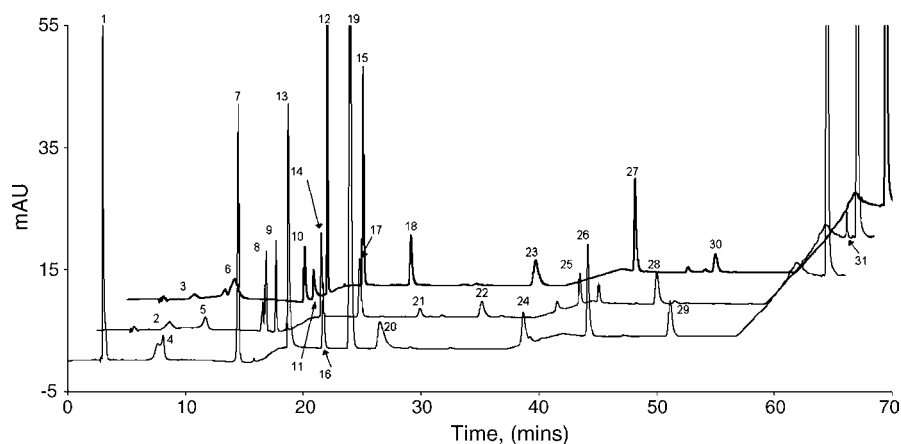


Fig. 1. Standard separation of chosen pharmaceuticals. *Peak identification:* 1, metformin; 2, atenolol; 3, salbutamol; 4, acetaminophen; 5, amoxicillin; 6, salicylic acid; 7, trimethoprim; 8, sulfamethazine; 9, sulfamethoxazole; 10, caffeine; 11, ciprofloxacin; 12, furosemide; 13, propranolol; 14, clofibrac acid; 15, carbamazepine; 16, ketoprofen; 17, bezafibrate; 18, nimesulide; 19, naproxen; 20, fluoxetine; 21, warfarin; 22, flurbiprofen; 23, diclofenac; 24, indomethacin; 25, ibuprofen; 26, meclofenamic acid; 27, mefenamic acid; 28, clotrimazole; 29, gemfibrozil; 30, triclosan; 31, ivermectin. The middle trace is offset by 2.5 min in time and 5 mAU, the top trace is offset by 5 min in time and 10 mAU for the purposes of clarity.

A brief study of the backpressure across the short monolith using Milli-Q water was carried out at increasing flow rates from 2 to 10 ml/min. It was found, as expected, that the backpressure generated increased in a linear fashion with flow rate to a maximum of ~ 115 bar at 10 ml/min. The recommended operating pressure according to the product literature is not more than 200 bar [40], and therefore it is estimated that the maximum possible flow rate could be as high as ~ 19 ml/min (although in this

work instrumental restrictions limited sample pump flow rates to 10 ml/min). The effect of sample loading flow rate on analyte recovery was determined through extracting 500 ml portions of 200 ng/l mixed analyte standards prepared in Milli-Q water (adjusted to pH 4.0 with sulphuric acid) [38]. This concentration level, i.e. 200 ng/l, was selected as it could be accurately prepared from a low mg/l standard and provided clearly distinguishable peaks in the UV traces thereby facilitating integration and quan-

Table 2
The effect of sample loading flow rate upon analyte recovery

Analyte	% Analyte recovery at different sample loading flow rates (ml/min)				
	2.00	4.00	6.00	8.00	10.00
Metformin, atenolol, salbutamol, paracetamol, salicylic acid, amoxicillin	0	0	0	0	0
Trimethoprim	4	3	4	5	3
Sulfamethazine	8	4	3	5	3
Caffeine	0	9	5	5	4
Sulfamethoxazole	8	5	4	8	7
Ciprofloxacin	0	38	12	16	15
Furosemide	20	19	29	16	20
Propranolol	24	34	49	50	48
Clofibrac acid	6	57	53	30	10
Carbamazepine	105	33	75	75	74
Ketoprofen	>130	98	108	102	105
Bezafibrate	90	130	101	124	80
Nimesulide	>130	90	116	119	77
Naproxen	99	93	109	105	109
Warfarin	>130	>130	123	>130	94
Fluoxetine	69	92	97	101	92
Flurbiprofen	64	126	95	>130	82
Diclofenac	30	28	85	90	65
Indomethacin	103	85	100	96	112
Ibuprofen	106	118	83	99	130
Meclofenamic acid	57	103	75	83	63
Mefenamic acid	80	84	87	84	92
Clotrimazole	43	98	28	14	20
Gemfibrozil	75	109	87	72	69
Triclosan	54	55	61	60	66
Ivermectin	—	—	—	—	—

titation. Flow rates investigated were 2, 4, 6, 8 and 10 ml/min and all extractions were performed in duplicate. Table 2 lists the calculated average recovery values at each of the investigated sample loading flow rates. Recovery was calculated based upon peak area comparison (using LC) with the direct injection of a 5 mg/l standard.

From Table 2, it can be observed that there was little or no recovery of a number of the more polar analytes from the extraction column due to insufficient retention of these polar compounds on the reversed-phase sorbent. However, for the majority of the pharmaceuticals investigated recoveries of >50% were readily achieved, with a large number showing almost quantitative recovery even at loading flow rates of as high as 10 ml/min. No significant trends were observed which would indicate a loss in extraction efficiency with increased flow rate for the majority of analytes, although clearly some variation in the optimum loading rate was seen. Based upon a mass balance approach, a maximum 25,000-fold preconcentration of target analytes was possible using this procedure, however the non-selective nature of the C₁₈ monolith meant trace levels of all hydrophobic organic species and impurities were also retained. Such effects were particularly prevalent at lower flow rates even for standard solutions. It was also observed that due to the increased path length through the on-line SPE system, retention times slightly increased when compared to those recorded by direct standard injection. In most instances such an effect caused no problems, except in the case of ivermectin, which eluted just before a system peak during standard injection and was observed to co-elute with the system

peak in the chromatograms of on-line SPE extracts. Recovery data for ivermectin is therefore not included in Table 2.

3.2. On-line SPE—optimisation of the sample pH

In an attempt to increase the retention of the more polar pharmaceuticals the extraction pH was optimised. As the extraction column is a silica-based material and according to the product literature is only stable within the range of pH 2–7.5 [40], pH values in the range of three to seven were investigated using appropriate buffer solutions prepared in Milli-Q water. Table 3 following lists analyte recovery at each of the experimental pH values.

As was noted during the flow rate study, no appreciable recovery of very polar analytes was observed. These analytes may require more extreme acidic or alkaline conditions in order to exhibit retention on the C₁₈ phase. However, because of the chemical diversity of the analytes, extractions at acidic pH would promote the protonation of basic analytes, thereby reducing retention on the reversed-phase sorbent, with the opposite expected at more alkaline pH values. An example of such an effect is demonstrated by clotrimazole, the imidazole ring of the compound is protonated at acidic pH values but as the pH is increased the percentage recovery is also observed to increase due to the conversion of clotrimazole to a neutral moiety.

From results obtained it was decided to extract all samples at pH 4.0 as at this pH an acceptable balance between all analyte recovery was observed. Obviously the more non-polar com-

Table 3
Analyte recovery as a function of sample pH

Analyte	% Analyte recovery				
	pH 3 (formate)	pH 4 (formate)	pH 5 (acetate)	pH 6 (MES)	pH 7 (HEPES)
Metformin, atenolol, salbutamol, paracetamol, salicylic acid, amoxicillin	0	0	0	0	0
Trimethoprim	4	6	9	25	33
Sulfamethazine	5	3	3	3	1
Caffeine	5	8	7	7	0
Sulfamethoxazole	7	9	6	4	2
Ciprofloxacin	36	77	40	15	0
Furosemide	48	36	16	5	0
Propranolol	58	40	49	82	71
Clofibric acid	40	11	4	0	0
Carbamazepine	88	97	84	84	75
Ketoprofen	93	82	98	51	28
Bezafibrate	92	94	87	83	100
Nimesulide	83	104	123	85	32
Naproxen	73	78	73	16	11
Warfarin	73	112	99	90	0
Fluoxetine	46	58	77	58	70
Flurbiprofen	92	90	98	0	0
Diclofenac	100	105	98	46	0
Indomethacin	70	67	107	110	79
Ibuprofen	110	93	101	35	51
Meclofenamic acid	58	70	90	85	0
Mefenamic acid	68	68	85	87	71
Clotrimazole	24	35	48	93	60
Gemfibrozil	75	96	92	86	87
Triclosan	59	57	59	91	74
Ivermectin	–	–	–	–	–

pounds exhibited a greater tolerance to changes in the loading pH. For example, the recovery of gemfibrozil varied only slightly over the pH range investigated. As was previously observed ivermectin could not be distinguished due to co-elution with the system peak.

3.3. Matrix removal

For any on-line extraction technique preconcentration of matrix components is an important factor. In an attempt to examine the suitability of the method for sample solutions, a tap water sample was spiked with 200 ng/l of the chosen analytes and extracted at pH 4.0. However, elution of the retained species directly onto the analytical column resulted in a large matrix peak (~20 min wide), which masked a large number of the smaller pharmaceutical peaks and also caused suppression of the electrospray signal during initial MS analyses. This peak was attributed to preconcentration of sample components such as natural organic matter, humic and fulvic acids, tannins, etc. Previously reported methods for matrix removal in the literature have focused upon changing the pH at which the extraction is performed [53], sample dilution [54] or the addition of cationic surfactants to the sample prior to extraction [55]. However, such approaches are impractical in this instance. Traditionally, in

off-line SPE methods the sorbent is washed with a weak wash solution prior to elution, and so such a step was investigated here.

Upon complete sample introduction (500 ml) the extraction column was linked to the UV–vis detector and analytes were desorbed using a linear gradient of 100% water to 100% solvent over 30 min. Both acetonitrile and methanol were investigated but acetonitrile removed a larger proportion of the matrix in a smaller period of time than methanol and was therefore preferred for further development. Upon examination of the solvent profile it was observed that at 20% acetonitrile the majority of the matrix components had been desorbed, see Fig. 2(a) and (b) shows the elution profile of the wash step under isocratic conditions, in this case using only 10% (v/v) acetonitrile/water at 1 ml/min. From this investigation, it was observed that the majority of adsorbed components were removed directly upon the introduction of the wash solution and that after 20 min of washing nearly all the matrix components are completely removed from the extraction column. The effect of the washing procedure on the retention of the analytes was also investigated by preconcentrating a 200 ng/l standard prepared in Milli-Q water. Again the column was washed with 10% (v/v) acetonitrile/water for 20 min, after such time the percentage of acetonitrile was increased in a linear fashion to ensure complete analyte elution. Fig. 2(c) shows the resulting UV trace.

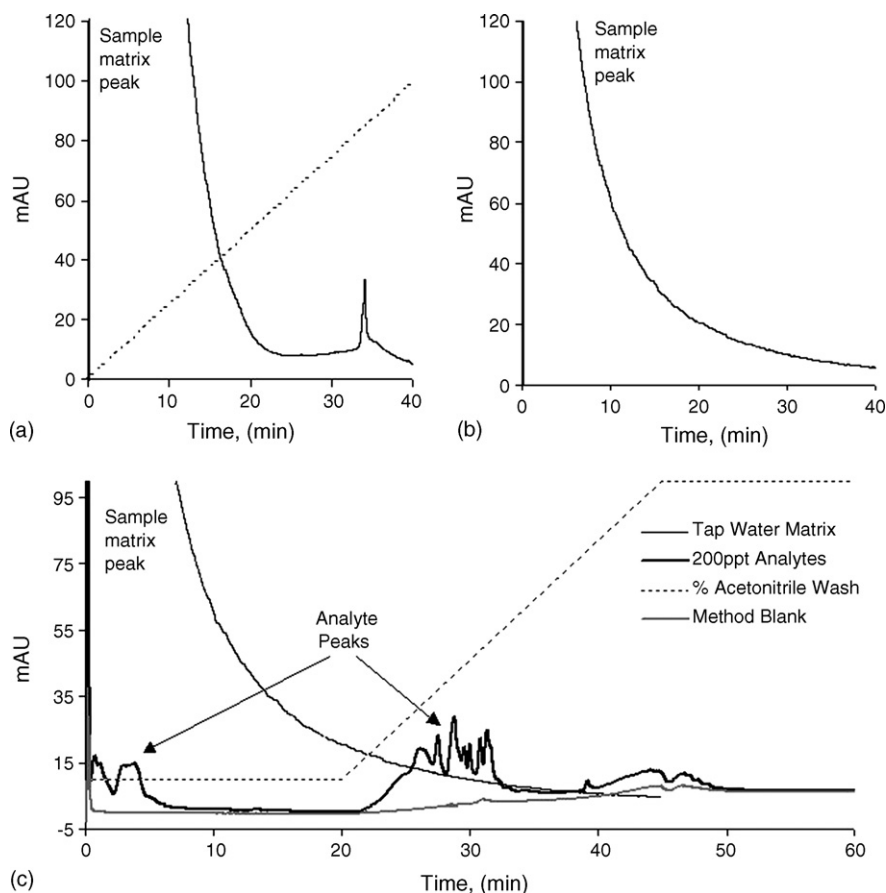


Fig. 2. Matrix removal using acetonitrile washes/on-line SPE wash development. (a) Depicts the determination of the % MeCN required for the removal of the sample matrix. (b) Depicts the removal of the preconcentrated matrix under the chosen isocratic solvent wash conditions (10% MeCN). (c) Shows the effect of the matrix removal wash procedure upon the retention of the analytes on the short monolithic extraction column, a MeCN gradient, depicted by the dashed line, was used to completely remove the retained components from the extraction column.

From Fig. 2(c), it can be observed that the retention of some early eluting analytes was indeed affected by the washing procedure with peaks present at the beginning of the elution profile. However, a significant proportion of the analytes were completely retained under the 10% MeCN conditions and were only observed to elute as the percentage of organic solvent was increased. The volume of the wash solvent required can also be determined from Fig. 2(c). It was clear that for the above retained analyte species retention was unaffected for at least 20 min at a rate of 1 ml/min. However, considering the porosity of the monolithic extraction column it was possible to perform the washing procedure at an elevated flow rate of 10 ml/min to facilitate a more practical amount of wash time.

3.4. Extraction to extraction carryover

The possibility of sample carryover from repeat preconcentration steps was investigated through the extraction of a 500 ml portion of tap water without a matrix removal wash step, i.e. a ‘worst case scenario’ approach. The sample was eluted by mobile phase back flush onto the analytical column and gradient separation carried out (see Section 2.4). At the end of the chromatographic run, the analytical column was analysed for residual matrix using the gradient method without sample injection, while the extraction monolith was conditioned as normal and then examined for any residual contamination also using the gradient method. Fig. 3 shows an overlay of the resulting UV traces. The figure shows firstly, the large matrix peaks evident prior to the additional column washing step, and secondly, the lack of any significant traces of the matrix peaks in the subsequent runs. This was as expected as during the separation step the percentage methanol is eventually increased to 90% at a flow rate of 2.3 ml/min, while the extraction column is conditioned with 20 ml of acetonitrile prior to extraction. This indicates both

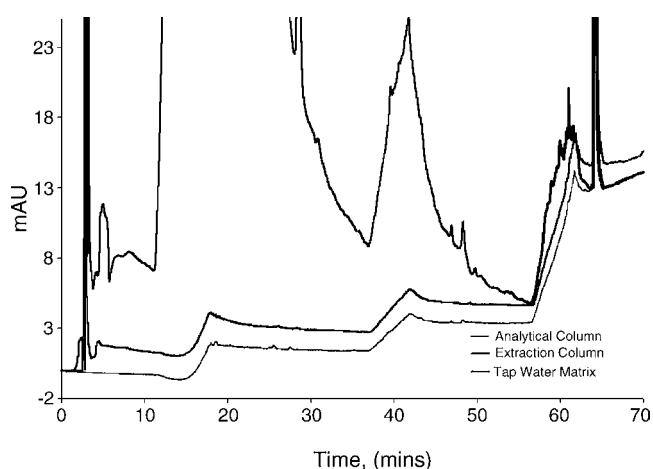


Fig. 3. Extraction to extraction carryover checking, the upper trace depicts the elution of the ‘unwashed’ sample matrix from the extraction and analytical columns. No washing procedure was incorporated so as to simulate the worst-case scenario. The middle trace depicts the resulting UV trace and shows that there is no residual matrix contamination on the analytical column. The lower trace is the resulting UV trace of the reconditioned extraction column also demonstrating that any residual matrix carryover is completely removed during the conditioning procedures.

sets of solvent conditions are sufficient to completely remove any adsorbed sample or matrix from both the C₁₈ phases.

3.5. Method performance

The performance of the on-line SPE-LC-UV method was evaluated using two distinct sample matrices, river water taken from the River Boyne in Co. Meath, Ireland, and potable water obtained from the Dublin laboratory. The levels of natural organic matter present in these sample types was expected to be quite different with a greater proportion expected in the river water samples. Overall method detection limits for those species amenable to the developed preconcentration procedure were determined in each sample matrix, limits of detection (LOD) were determined as three times the peak-to-peak baseline noise, while the limits of quantitation (LOQ) are quoted as 10 times the peak-to-peak baseline noise. The values quoted are determined from UV–vis detection using peak heights and for both river water and tap water samples spiked at the 200 ng/l concentration level. These data are shown in Table 4.

The calculated detection and quantitation limits were acceptably low in comparison to previous work for similar pharmaceutical analytes in similar matrices using optical detection [56–58], and comparable if not superior to many limits quoted using LC-MS and LC-MS/MS methods with off-line SPE [59]. This suggested that the on-line SPE procedure imparts greater sensitivity to the method due to the higher levels of preconcentration obtainable and complete sample introduction. However, it can be observed that the matrix of the sample exhibited an observable effect on sensitivity, with limits being lower in potable water compared to river water. In addition, components of the river water matrix were observed to co-elute and mask the responses of carbamazepine, fluoxetine and warfarin, making their detection at such ultra trace levels impossible by UV alone.

Overall method precision was determined by performing 10 replicate extractions of 200 ng/l analyte spiked samples, pre-

Table 4
Overall method sensitivity using on-line SPE

Values in µg/l	Boyne River water		Lab tap water	
	LOD	LOQ	LOD	LOQ
Carbamazepine	–	–	0.009	0.031
Ketoprofen	0.051	0.171	0.046	0.155
Bezafibrate	0.064	0.213	0.021	0.070
Nimesulide	0.072	0.239	0.021	0.071
Naproxen	0.008	0.027	0.004	0.012
Fluoxetine	–	–	0.054	0.179
Warfarin	–	–	0.037	0.123
Flurbiprofen	0.204	0.681	0.059	0.196
Diclofenac	0.113	0.378	0.037	0.122
Indomethacin	0.167	0.556	0.028	0.094
Ibuprofen	0.091	0.302	0.025	0.082
Meclofenamic acid	0.028	0.094	0.010	0.033
Mefenamic acid	0.031	0.105	0.012	0.039
Clotrimazole	0.145	0.482	0.081	0.271
Gemfibrozil	0.138	0.461	0.051	0.170
Triclosan	0.087	0.291	0.032	0.108
Ivermectin	0.317	1.055	0.165	0.551

Table 5

Overall method precision and analyte recovery from two different sample matrices ($n = 10$)

Analyte	River water		Potable water	
	Precision (%R.S.D.)	Recovery	Precision (%R.S.D.)	Recovery
Carbamazepine	–	–	14	9 ± 1
Ketoprofen	23	71 ± 15	16	18 ± 3
Bezafibrate	16	64 ± 11	21	42 ± 9
Nimesulide	18	96 ± 17	7	107 ± 8
Naproxen	13	88 ± 11	48	41 ± 19
Fluoxetine	–	–	38	52 ± 20
Warfarin	–	–	26	100 ± 26
Flurbiprofen	16	65 ± 10	9	86 ± 8
Diclofenac	22	60 ± 13	6	75 ± 4
Indomethacin	35	56 ± 19	25	74 ± 18
Ibuprofen	17	89 ± 15	5	73 ± 3
Meclofenamic acid	30	68 ± 20	6	77 ± 5
Mefenamic acid	20	79 ± 16	3	86 ± 3
Clotrimazole	45	37 ± 17	9	58 ± 5
Gemfibrozil	11	86 ± 10	11	81 ± 9
Triclosan	11	102 ± 11	7	78 ± 5
Ivermectin	38	23 ± 9	19	19 ± 4

pared in both river and potable water samples. Method precision data is presented in Table 5 and is quoted as the percentage relative standard deviation (%R.S.D.) based upon peak area measurement for the 10 replicate extractions in each matrix. The average analyte recovery was also determined in each matrix by comparison with the direct injection of a 5 mg/l standard.

Overall method precision based upon peak area measurements showed higher variability with increased levels of sample matrix. In the majority of cases the %R.S.D. values are higher in river water when compared to those for the potable water spikes. Precision is <20% R.S.D. for most analytes in both matrices with some exceptions, notable clotrimazole in river water and naproxen in potable samples, however given the low level of the spiked samples and the large sample volume used, the precision data obtained was seen as acceptable. The levels of precision attainable with on-line SPE are expected to be dependant on the homogeneity of the sample matrix, analytes with small responses such as warfarin and clotrimazole demonstrate this fact and are more affected by the presence of small matrix peaks than analytes that exhibit a larger UV response.

The calculated rates of recovery were also acceptable for the majority of analytes at such low concentration levels. Interestingly ivermectin, which was indistinguishable from the system peak, was again completely resolved since the inclusion of the matrix removal step, allowing for its unhindered determination. There did not appear to be a trend when correlating the calculated recovery to the type of sample matrix with some analytes exhibiting higher rates of recovery in matrix rich river water than in tap water and vice versa. The recovery of naproxen from tap water was much lower with higher variability when compared to the rate calculated for the same spiked level in river water. A possible explanation for the lower recovery and variability may include the reaction of naproxen with residual chloride present from the drinking water disinfection process. Boyd et al. [60]

investigated the removal of naproxen during water chlorination and reported that naproxen is highly reactive with residual chlorine to form a range of chlorinated disinfection by-products. The recovery of ketoprofen and bezafibrate are also considerably lower from tap water than from the river samples, the reason for which is unclear but may be again sample matrix related.

From the 10 replicate extractions, the reusability of the extraction column was estimated by examining the stability of analyte peak areas. It was found that the column displayed excellent stability for the extraction of potable water samples with no significant variation of peak area for the majority of analytes over the course of the 10 extractions with the exception of naproxen as previously discussed. For the extraction of the river water samples the column displayed no great disparity for the first eight extractions but small decreases of peak area were observed for extraction numbers 9 and 10, respectively. Therefore, it was concluded that the maximum column usage for the extraction of matrix rich samples such as river water should not exceed eight extractions but for samples with lower natural organic matter content the column can be used a minimum of 10 times with no significant loss in recovery. This makes the on-line SPE method economically viable, as a single extraction column is similar in cost to 10 SPE cartridges.

3.6. Direct infusion mass spectrometry

Prior to coupling the on-line SPE-LC-UV method with mass selective detection an optimisation of the electrospray and ion trap focusing parameters was performed by directly infusing solutions of each individual analyte prepared in 50:50 methanol/ammonium formate buffer (1 mM pH 4.5) into the MS. These parameters were automatically fine tuned using the Bruker esquire software. Analytes were infused using both negative and positive ion polarity and that which provided the highest intensity was selected for further study. An average of the optimised focusing parameters under each ion polarity was then taken for use with coupled SPE-LC-UV method.

Simple spectra were recorded in most instances, under positive ion polarity the majority of analytes exhibited a $[M + H]^+$ pseudo molecular ion, e.g. carbamazepine, ketoprofen, bezafibrate, naproxen and fluoxetine, while for clotrimazole no significant pseudo molecular ion response was observed. The predominant ion for clotrimazole was recorded at m/z 277.0 suggesting the loss of the imazole moiety from the molecule. Ibuprofen also exhibited no pseudo molecular ion response, the most intense signal being present at m/z 251.1 suggesting the formation of a formic acid adduct of ibuprofen, $[M + HCOOH]^+$. The monitoring of ketoprofen, bezafibrate and naproxen using positive ion conditions is also unusual, as these compounds are acidic in nature (pK_a values of 4.45, 3.6 and 4.2, respectively) and have more usually been reported under (–) ESI conditions. The intensity of the pseudo molecular ions was higher in (+) ESI than when the same standard was infused under (–) ESI, and therefore (+) ESI was chosen for further study. Under negative ion polarity again all analytes exhibited a pseudo molecular $[M - H]^-$ ion with the exception of flurbiprofen whose most intense signal corresponded to the decarboxylation of

the molecule, $[M - \text{COOH}]^-$. For diclofenac and indomethacin the $[M - \text{COOH}]^-$ ion was significantly more intense than the $[M - \text{H}]^-$ ion and was therefore more suitable for SIM studies. Gemfibrozil produced two distinct ions corresponding to a $[M - \text{H}]^-$ molecular ion at m/z 249.1 and a $[M - \text{C}_7\text{H}_{12}\text{O}_2]^-$ ion at m/z 121.1, with the latter displaying higher intensity and therefore being chosen as more suitable for SIM studies. Both triclosan and ivermectin exhibited no response when infused under both negative and positive polarity as these analytes are neutral and non-polar. Previous reports of ivermectin analysis by LC-MS have focused upon the use of atmospheric pressure chemical ionisation (APCI) [8], which is more suitable for the determination of higher molecular weight non-polar organics. Triclosan has been determined using negative ion electrospray ionisation mass spectrometry but requires the use of high pH mobile phases ($\text{pH} > 7.5$) for significant ion formation [7,61].

Due to the flow mismatch between the on-line SPE-LC-UV method and ESI-MS a variable flow splitter was used. To optimise the flow splitter injections of a 10 mg/l mixed analyte standard were performed at various splitter settings using the dual gradient LC-UV method now complete with ESI-MS detection. The resulting SIM peak areas were monitored at each splitter setting until a maximum was recorded. At this optimum splitter setting the volume of mobile phase entering the electrospray source at both the initial and final flow rates was determined by weight and determined to be approximately 1/20 at both flow rates. The reproducibility of the combined LC-UV-ESI-MS method, incorporating the flow splitter, was also investigated by performing six replicate injections of a 10 mg/l standard at the optimised splitter settings (without sample preconcentration). The %R.S.D. for the six replicate injections was up to 26% (maximum for bezafibrate), which was possibly elevated due to the added variability of the incorporated flow splitter. Due to

this high degree of variability within the method when using MS detection, it is advisable that MS should not be used on its own for quantitative detection, but instead used in conjunction with the more quantitative UV-vis based detection.

The previously unoptimised conditions of nebuliser pressure and dry gas flow rates along with the drying temperature were then determined from values recommended by the esquire software now taking into account the approximate volumes of mobile phase entering the electrospray source. The values adopted were a nebuliser pressure of 30 psi with a dry gas flow of 8 l/min and a drying temperature of 325 °C. These parameters were used for all further LC-UV-MS analyses.

3.7. Analysis of samples using on-line SPE-LC-UV-ESI-MS.

Samples of water were collected from the River Boyne in Co. Meath in the vicinity downstream of the Navan Wastewater Treatment Centre that caters for a population equivalent of >40,000, along with samples of potable water from the laboratory. Both were analysed using the developed on-line SPE-LC-UV-ESI-MS method. Aliquots of each sample were spiked with 200 ng/l of the chosen pharmaceuticals and also analysed. None of the chosen analytes were detected in either sample type however, clear sharp distinguishable peaks are observed for most analytes in the UV chromatograms of both spiked sample types. Fig. 4 depicts the resulting UV traces for 200 ng/l spikes in both river and potable water samples. The highlighted region of the chromatogram illustrates the sensitivity of the developed method and also shows the resolution of the spiked pharmaceutical peaks from a number of unknown peaks present within the river water, and to a lesser extent the drinking water sample. The excellent retention time reproducibility between the two sample

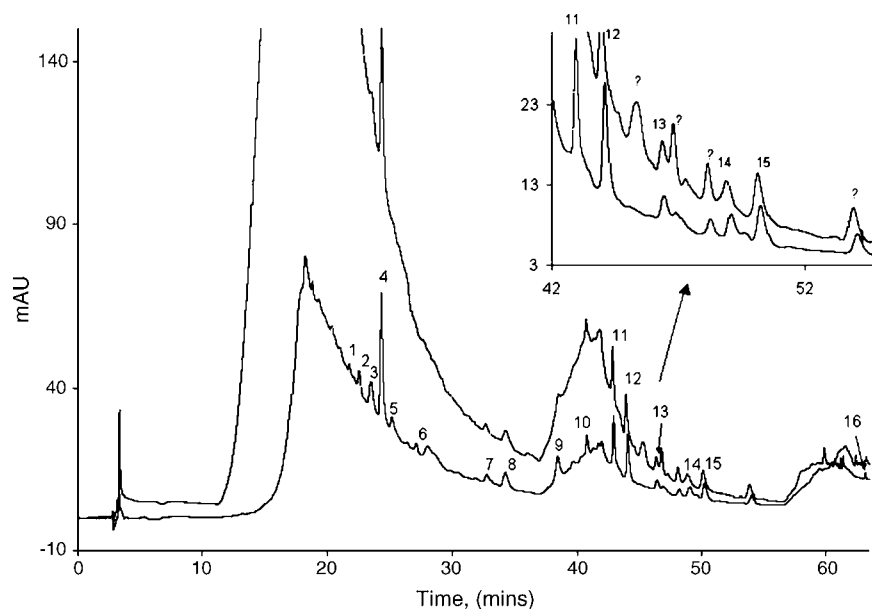


Fig. 4. Overlay chromatogram of 200 ng/l analyte spikes in river (upper trace) and potable water (lower trace). Peak identification: 1, ketoprofen; 2, bezafibrate; 3, nimesulide; 4, naproxen; 5, fluoxetine; 6, warfarin; 7, flurbiprofen; 8, diclofenac; 9, indomethacin; 10, ibuprofen; 11, meclofenamic acid; 12, mefenamic acid; 13, clotrimazole; 14, gemfibrozil; 15, triclosan; 16, ivermectin.

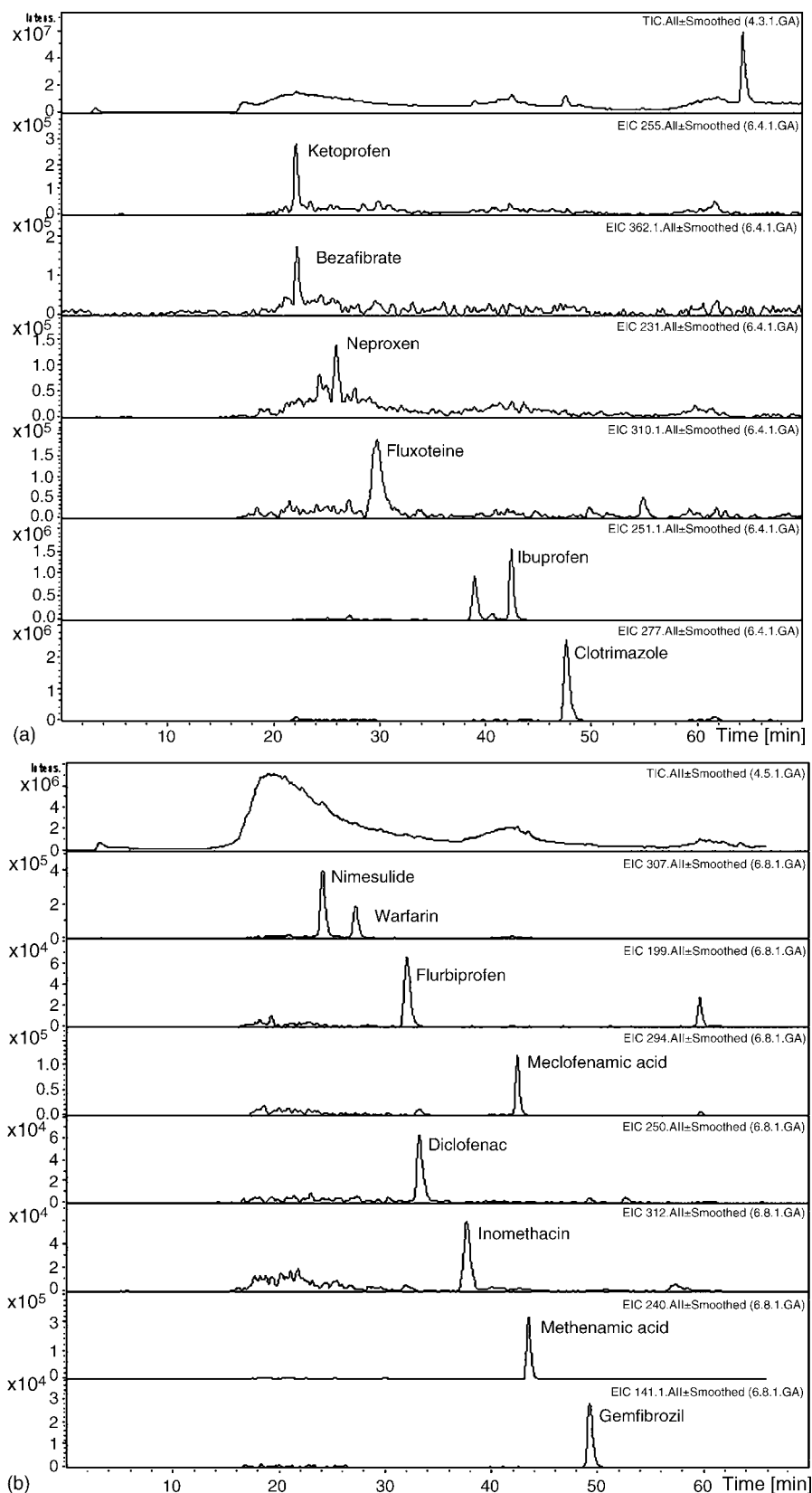


Fig. 5. TIC and SIM chromatograms of river water spiked with 200 ng/l using both (a) positive and (b) negative electrospray polarity.

types is also clear from these overlaid sample chromatograms. Fig. 5 depicts TIC and SIM chromatograms for the spiked river water. The excellent selectivity imparted by MS detection is clearly visible, with well defined analyte peaks present even in regions of high matrix interference in the UV and TIC chromatograms. For example, both fluoxetine and warfarin can be detected without matrix interference in the river water matrix unlike with UV detection where the identification of these analytes was obscured. The ability to obtain such clear mass spectra in regions of simultaneous matrix elution illustrates the success of the washing step which reduces this background to acceptable levels. The advantage of the simultaneous use of UV and ESI-MS detection, as shown in Figs. 4 and 5, in overcoming problematic background interferences incurred during ultra trace analyses is clear in this case.

4. Conclusions

A practical and simple on-line preconcentration procedure, including a selective matrix wash step (effectively reducing the matrix signal by ~90%), for large volume samples using a short reversed-phase monolithic preconcentrator column has been developed and applied. The short monolithic extraction column allows for the rapid extraction (at 10 ml/min) of such large sample volumes with acceptably high analyte recovery for ultra-trace levels of pharmaceuticals from natural and treated water samples. The method was found to be repeatable and detection limits were in the low ng/l range. Coupled on-line with LC-UV-ESI-MS the method was shown to be suitable for the screening of the above types of water sample.

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Measurements of contaminant elements of wines by inductively coupled plasma-mass spectrometry: A comparison of two calibration approaches

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Abstract

The aim of the present work was to develop and validate an accurate method by ICP-MS focalized to the measurement of contaminant elements in wines, in special those with legal importance. In addition, we intended to evaluate the suitability of ICP-MS semi-quantitative methodology in order to reduce the time and cost of analysis. Twenty-six contaminant elements of wine (Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Ag, Cd, In, Cs, Ba, Hg, Tl, Pb, Bi and U) were measured using quantitative and semi-quantitative calibration approaches, in diluted white and red wines. In an early step potential interferences caused by Cl and Ca species were evaluated, in order to establish suitable mathematical corrections. For validation of ICP-MS procedures a few elements were determined by flame and electrothermal AAS. Reference wines from 1992 year, with provisional values, were analyzed and the results showed satisfactory agreement. The semi-quantitative calibration provided slightly higher limits of detection than those obtained by the quantitative calibration, and always lower than $0.1 \mu\text{g l}^{-1}$, except for Fe and Zn. For most elements the recovery percentages (between 90 and 100%) and precision of the results (R.S.D. (%) < 4) were similar for both modes. Differences lower than 20% of concentration was obtained for most elements. Both methodologies offer valuable alternatives to wine characterization and comparison purposes. For legal requirements control purposes, with reference to the importance of accurate results, quantitative approach is the most suitable alternative.

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1. Introduction

Mineral content of wines depend on several factors, including soil, variety of grape, environmental conditions and viticultural and enological practices. The determination of some elements is of interest due to their toxicological or physiological properties, while others can lead to wine spoilage [1–5]. It should be noted that the levels of certain contaminant elements, such as Cu, Zn, As, Cd and Pb, at different stages of the winemaking process are of great concern because of legal requirements [6].

Inductively coupled plasma mass spectrometry (ICP-MS) is a multi-element technique applied to a wide range of applications in analytical chemistry, with high selectivity and sensitivity and low analytical limits, so that it is an excellent tool for isotopic

analysis [7–15] and for detailed characterization of elemental composition of wine [16–27].

The application field of ICP-MS technique to wine is large. Different methodologies have been used in preliminary studies to classify wines according to their geographic origin or grape variety, from its trace composition, as a fingerprint [17,18,24,27,28]. A number of papers have been published reporting the use of ICP-MS methods for determine trace metals and rare earth elements in wine fingerprints [16,18,21–23,25,27,29]. In the last years this technique has been a very important tool to evaluate the changes in the contents of trace elements through the winemaking process. Nicolini et al. [25] have applied the ICP-MS technique to evaluate the changes in the contents of micro and trace elements in wine due to winemaking treatments. The changes of the metal composition in German white wines through the winemaking process were evaluated by Gómez et al. [26].

ICP-MS offers different quantification procedures depending on the accuracy and precision required. Isotope dilution mode

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of analysis presents the highest quality of results. However, when a high quality of results is needed quantitative approach may be a suitable strategy as well. This procedure requires external calibration with standards of each element to be determined. This strategy is time consuming and it is not easy to have a complete set of the multi-element standards required for the calibration. The application of this mode of quantification in wines has already been reported [12,16–19,22,23,25,27,30].

The semi-quantitative approach is a versatile application of ICP-MS, which is claimed to allow the determination of 81 elements with accuracy errors lower than 20% for most elements. The semi-quantitative analysis software available for ICP-MS instrumentation (Total Quant III from Perkin-Elmer – ELAN software, for instance) automatically corrects for isotopic interferences and interfering molecular species and produces a comprehensive report, listing each element present in the sample along with its concentration. Unlike quantitative analysis methods, calibration is achieved using just a few elements distributed throughout the mass range of interest. The calibration process is used to update internal response data that correlates measured ion intensities to the concentrations of elements in a solution. During calibration, the response data is adjusted to account for changes in the instrument's sensitivity due to variations in the sample matrix. This methodology has already been applied to wine [15,18,20,21,25,29,30]. Pérez-Jordán et al. [19] and Sagrado et al. [30] have compared two ICP-MS methods for analyzing wine samples (quantitative and semi-quantitative methodologies). For the majority of elements determined by both methodologies, comparable results were found. The advantages and limitations of the semi-quantitative calibration of an inductively coupled plasma-mass spectrometer for multi-element characterization of fortified and table wines were tested by Almeida and Vasconcelos [22]. The wine samples were submitted to an UV-irradiation treatment prior to analysis. Pruszkowski [31] have applied a semi-quantitative methodology of ICP-MS technique to wine, supporting its suitability for final sample characterization, such as total metal content and fingerprint.

Although the concentrations for only few elements in wines are currently under regulation, the increasing concern for health effects and environmental exposure will probably result in a longer list of potentially toxic elements, the concentrations of which have to be monitored in a near future. As far as we know, ICP-MS methodologies are not yet used in routine wine analysis by control laboratories. Nevertheless, the establishment of an ICP-MS methodology for contaminant elements of wine will carry great advantages to these control laboratories.

The purpose of the present study was to develop and validate an accurate method by ICP-MS focalized to the determination of contaminant elements in wines, in particular those which concentration is, or could be in a near future legally controlled. In addition, we intended to evaluate the suitability of ICP-MS semi-quantitative calibration, by comparison of the results obtained by both calibration approaches, in order to reduce the time and cost of analysis relatively to those of quantitative calibration.

2. Experimental

2.1. Apparatus

The analytical measurements were carried out with a Perkin-Elmer SCIEX Elan 9000 ICP-MS (Perkin-Elmer SCIEX, Norwalk, CT, USA) apparatus, equipped with a crossflow nebulizer, a Scott-type spray chamber made of Rytan and nickel cones. A peristaltic sample delivery pump with four channels, model Gilson, was used. Autosampler Perkin-Elmer AS-93 Plus was protected by a laminar-flow-chamber clean room class 100 (Reinraumtechnik Max Petek). Application software Elan – 6100 Windows NT (Version 2.4), was used.

For validation of the ICP-MS results, some elements were also determined by flame atomic absorption spectrometry (FAAS) (AAnalyst 100, Perkin-Elmer, Norwalk, CT, USA) and electrothermal atomic absorption spectrometry (ETAAS) (4110 ZL, Perkin-Elmer, Norwalk, CT, USA).

2.2. Material and reagents

Monoelement standard solutions of Be, Co and In (1000 mg l^{-1}), from Merck, and a solution with Mg, Cu, Rh, Cd, In, Ba, Ce, Pb and U ($10 \mu\text{g l}^{-1}$), from Perkin-Elmer were used for ICP-MS optimization procedures. Ultrapure concentrated HNO_3 (J.T. Baker), $\text{C}_2\text{H}_5\text{OH}$ (Lichrosolv, Merck) and Au (Merck, 1000 mg l^{-1}) for wash, blank and standard solutions were used. ICP-MS calibration was established with a multielement standard solution with 30 elements: Li, Be, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Ag, Cd, In, Cs, Ba, Hg, Tl, Pb, Bi, U (Perkin-Elmer, 10 mg l^{-1}). For ICP-MS internal standardisation standard solutions of Rh and Re (Merck, 1000 mg l^{-1}), were used. For the determination of Cl and Ca interference free ranges and interference ratios, a monoelement standard solution of Ca (1000 mg l^{-1}) and Suprapur HCl (30%), both from Merck, were used.

For AAS analysis, monoelement standard solutions of Al, Pb, Cd (Merck, 1000 mg l^{-1}) and Cu and Fe (Perkin-Elmer, 1000 mg l^{-1}), $\text{Pd}(\text{NO}_3)_2$ and $\text{Mg}(\text{NO}_3)_2$ as matrix modifiers, both from Merck (10 g l^{-1}), were used.

Purified water was produced using a Seralpur Pro 90CN apparatus (Seral, Ransbach-Baumbach, Germany). For solution preparation plastic material was only used. All the material was soaked in 20% HNO_3 (v/v) for at least 24 h and rinsed several times with purified water, before use.

2.3. Samples

White and red table wines were used for the study and comparison of quantitative and semi-quantitative approaches. A Portuguese red wine was used for AAS analysis comparison.

Three provisionally certified wines from a BCR intercomparison trial (BCR C white, BCR D liquor and BCR E red) were analyzed for method validation. These wines are from 1992 year and showed some precipitation material. Our attempt to get

younger reference wine materials had no success, so we decided to work with these wines, in spite of their state. To the best of our knowledge, no recent reference wine materials can be found for contaminant elements analysis in wines.

A 10-fold final dilution of wine samples, without any further sample preparation, was used for ICP-MS analysis.

HNO₃, a solution of Rh and Re (1 mg l⁻¹) and a solution of Au (1 mg l⁻¹) were added to samples in order to provide a final concentration of 1% (v/v), 10 and 200 µg l⁻¹, respectively. Blank solution and standards contained 1% HNO₃ (v/v), 1% C₂H₅OH (v/v), 10 µg l⁻¹ of Rh and Re, and 200 µg l⁻¹ of Au. The Au was used to eliminate the Hg and U memory effect at nebulizer chamber. The wine samples, blank and standard solutions were prepared daily, in polyethylene tubes.

2.4. ICP-MS determinations

The operating conditions were optimized daily, by using an aqueous solution containing 10 µg l⁻¹ of Mg, Ba, Ce, Cu, Cd, Rh, In and Pb, and monitoring the intensities of the isotopes ²⁴Mg, ¹⁰³Rh, ¹¹⁴In, ²⁰⁸Pb, ¹³⁸Ba and ¹⁴⁰Ce as well as the intensities at mass 69, 156 and 220 (corresponding to species ¹³⁸Ba²⁺, ¹⁴⁰Ce¹⁶O⁺ and background, respectively).

The chosen conditions were a compromise between the highest ¹⁰³Rh ion signal and the lowest percentage of doubly charge ions (obtained by the intensities ratio Ba²⁺/Ba⁺; always ≤3%) and of oxide ions (obtained by the intensities ratio CeO⁺/Ce⁺, always ≤3%), precision better than 2% and background <30 cps. The Autolens system was optimised with a 10 µg l⁻¹ Be, Co and In solution, and dual detector calibration with a 200 µg l⁻¹ solution with 29 elements.

Operating conditions used were as follows: r.f. power of 1200 W; sample uptake rate of 0.85 ml min⁻¹; nebulizer argon flow between 0.85 and 0.95 l min⁻¹.

For both modes of analysis, in order to get signal stabilization, a sample read delay of 75 s was chosen. Between samples or standards, the sampling system was rinsed with a 2% HNO₃ (v/v), 1% C₂H₅OH (v/v) and 200 µg l⁻¹ of Au solution for 75 s.

Rh and Re were used as internal standards for elements in the mass range (*m/z*) 7–138 and 205–238, respectively.

2.5. Determination of Cl and Ca species interference ratios—an approach to improve accuracy

In an early step, potential polyatomic interferences caused by Cl and Ca, two main elements in wine, their interference free ranges and interference ratios were evaluated, using ⁴³Ca and ³⁵Cl as references. The effects of several concentrations of Cl (20–1000 mg l⁻¹) and Ca (10–250 mg l⁻¹) on ⁴⁰Ar³⁵Cl, ³⁵Cl¹⁶O, ³⁵Cl¹⁸O, ⁴³Ca¹⁶O and ⁴⁴Ca¹⁶O species signal were studied. The stability of interference ratios over time was monitored, at 20 and 10 mg l⁻¹ Cl and Ca concentrations, respectively. These concentrations have been used taking into account both the usual levels of Cl and Ca in wines and the sample dilution factor.

Table 1

Isotopes monitored and concentration range for external calibration in the quantitative approach of analysis

Isotopes monitored	Concentration range (µg l ⁻¹) for external calibration
²⁰¹ Hg; ²⁰² Hg	0.1; 0.2; 0.5
⁹ Be; ⁵⁹ Co; ⁷¹ Ga; ⁷⁵ As; ⁸² Se; ¹⁰⁷ Ag; ¹¹¹ Cd; ¹¹⁵ In; ¹³³ Cs; ²⁰⁵ Tl; ²⁰⁹ Bi; ²³⁸ U	0.05; 0.25; 0.5; 2.5
⁷ Li; ⁵¹ V; ⁵³ Cr; ⁶⁰ Ni	0.25; 0.5; 2.5; 10
⁶⁵ Cu; ¹³⁸ Ba; ²⁰⁶ Pb; ²⁰⁷ Pb; ²⁰⁸ Pb	0.5; 2.5; 10; 50
²⁷ Al; ⁵⁵ Mn; ⁵⁷ Fe; ⁶⁶ Zn; ⁸⁵ Rb; ⁸⁸ Sr	2.5; 10; 50; 200

2.6. Quantitative approach

External calibration was used and the appropriate interpolation was carried out for each element to determine its concentration in the corresponding calibration line (Table 1).

A selection of isotopes of the elements to be determined was performed, except for monoisotopic elements. The selected isotopes were those free from isobaric or important matrix-induced interferences, when possible. Otherwise, suitable elemental equations, established after preliminary tests, which are presented in Section 3, were applied to correct isobaric and matrix-induced interferences. Since Pb isotope ratios may change from sample to sample, ²⁰⁶Pb, ²⁰⁷Pb and ²⁰⁸Pb isotopes were measured. The ²⁰¹Hg and ²⁰²Hg isotopes were added in order to increase the signal intensity.

The experimental conditions used for the measurements were: dwell time = 50.0 ms; sweeps/reading = 30; reading/replicates = 1; replicates = 4; time per run = 235 s.

2.7. Semi-quantitative approach

A full mass spectrum (*m/z* = 6–240, omitting the mass ranges 16–18; 40, 41, 211–229) was obtained by full mass range scanning. A reference response table (Perkin-Elmer Total Quant III) was updated with the multielement standard solution at 10 µg l⁻¹. The software of the instrument performs automatic corrections of isobaric interferences.

The experimental conditions used for the measurements were: dwell time = 50.0 ms; sweeps/reading = 6; reading/replicates = 1; replicates = 1; time per run = 67 s.

2.8. Validation of ICP-MS procedures

The limits of detection (LOD) of the elements of interest were determined, for both calibration approaches. The LODs were calculated as being the concentrations corresponding to signals equal to three-fold the standard deviation of a blank solution signal (three replicates).

Recovery tests were carried out for a white and a red wine samples spiked with 0.2–500 µg l⁻¹ standard solution (depending of the element). Three different spikes of each element were performed and the solutions obtained were analyzed. The mean and the respective standard deviation were calculated from the three recovery values obtained for the three spikes.

The contents of Al, Cu, Cd and Pb, were determined by ETAAS [3–5], and those of Fe and Zn by FAAS. A Portuguese red wine was used in this study.

For validation of the ICP-MS results, three provisionally certified wines from a BCR intercomparison trial (BCR C white, BCR D liquor and BCR E red), for Al, Fe, Cu, Zn, Cd and Pb, were analyzed by quantitative analysis.

3. Results and discussion

As with all other atomic spectrometric analytical techniques, interferences of various types can occur during ICP-MS determinations. The most commonly observed result of matrix interference is the ion intensity if an analyte element becomes dependent upon the total composition of the sample. Several methods can be used to compensate for matrix-induced signal suppression such as matrix dilution and standard sample matrix simulation. Wine is a complex matrix that contains many inorganic and organic substances, which can affect signal intensity in ICP-MS. So, in order to reduce the matrix effect, initial studies of the method development included the establishment of the sample minimal preparation procedure. In an early step a five-fold dilution of samples was tested. Problems with signal suppression and signal instability were encountered probably due to progressive blocking of the sampling and skimmer cones. Thus a 10-fold dilution of samples was undertaken. This procedure enabled to reduce matrix effect and still yet allowed the trace elements quantification.

3.1. Cl and Ca species interference ratios and mathematical corrections

Spectral interferences are the result of other chemical species, which are present at the same atomic mass as the analyte of interest. A careful selection of isotopes was made in order to overcome isobaric overlaps. Sample constituents, usually in combination with oxygen, may generate polyatomic ions. In most cases, these interferences can be compensated by knowledge of the intensities of the oxide and the parent ion. A prior knowledge of polyatomic interferences cited in literature for a particular analyte may be helpful to the analyst for selecting reagents and conditions that would preclude or reduce [the possibility of] their formation. A good perspective of known polyatomic interferences was given by May and Wiedmeyer [32]. The fact that a spectral interference occurs at the isotope mass of an analyte does not necessarily mean that all determinations of this particular element are interfered. Whether interference occurs or not depends on the abundance of interfering species, intensities of parent ions of the interference, and on the ratio at which the interference occurs [33].

Fig. 1 shows the stability of Cl and Ca species interference ratios. $^{40}\text{Ar}^{35}\text{Cl}$ interference ratio seems to be stable all over the concentration range in study, and it was always lower than 0.0005, while $^{35}\text{Cl}^{16}\text{O}$ and $^{35}\text{Cl}^{18}\text{O}$ interference ratios increase with Cl concentration. $^{43}\text{Ca}^{16}\text{O}$ and $^{44}\text{Ca}^{16}\text{O}$ species interference ratios seem stable in the 10–1000 mg l^{-1} range of concentration. Cl level in wines is usually lower than 50 mg l^{-1}

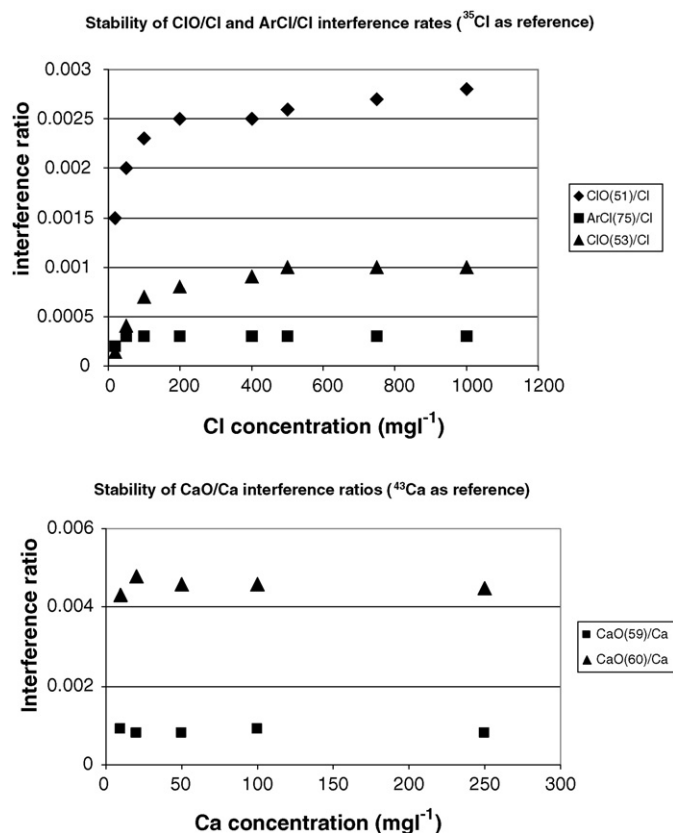


Fig. 1. Stability of Cl and Ca species interference ratios with Cl and Ca concentrations.

nevertheless in some typified and rare situations it can achieve 1 g l^{-1} . Ca level in wines is usually lower than 150 mg l^{-1} . The stability of calculated interference ratios over time were evaluated, in order to investigate their suitability to be used as interference correction factor. The interference correction factors were monitored during 3 months, showing to be fairly stable over time (20 and 10 mg l^{-1} concentrations of Cl and Ca, respectively, were used in these studies).

The interference ratios were used in elemental equations to correct for the effect of polyatomic Cl and Ca species on ^{51}V , ^{53}Cr , ^{57}Fe , ^{59}Co , ^{60}Ni , ^{75}As and ^{107}Ag isotopes. The software of the instrument performed an automatic correction for isobaric interferences. The correction equations for both types of interferences are shown in Table 2.

3.2. ICP-MS quantitative and semi-quantitative approaches figures of merit

Table 3 shows the detection limits (LODs) for both calibration approaches. The values are 10–100-fold lower than those reported by Baxter et al. [18], Pérez-Jordan et al. [19], Almeida and Vasconcelos [22], Taylor et al. [24] and Gómez et al. [26], all of them obtained using ICP-Quadropole MS apparatus. In general, major and minor elements in wine, such as Al, Cr and Fe, presents the highest LODs, nevertheless always lower than $0.1 \mu\text{g l}^{-1}$. The values are low enough to enable quality and

Table 2

Elemental equations applied for compensation of isobaric and matrix-induced polyatomic interferences in ICP-MS measurements

Analyte/isotope	Interfering species	Correction equations
⁵¹ V	³⁵ Cl ¹⁶ O	$V = I(^{51}\text{V}) - 0.0015 \times I(^{35}\text{Cl})$
⁵³ Cr	⁴⁰ Ar ¹³ C, ³⁵ Cl ¹⁸ O	$\text{Cr} = I(^{53}\text{Cr}) - 0.002 \times I(^{13}\text{C}) - 0.0015 \times I(^{35}\text{Cl})$
⁵⁷ Fe	⁴⁰ Ca ¹⁶ O ¹ H	$\text{Fe} = I(^{57}\text{Fe}) - [0.0001 \times A(^{40}\text{Ca})/A(^{43}\text{Ca}) \times I(^{43}\text{Ca})]$
⁵⁹ Co	⁴³ Ca ¹⁶ O	$\text{Co} = I(^{59}\text{Co}) - 0.0009 \times I(^{43}\text{Ca})$
⁶⁰ Ni	⁴⁴ Ca ¹⁶ O	$\text{Ni} = I(^{60}\text{Ni}) - [0.0002 \times A(^{44}\text{Ca})/A(^{43}\text{Ca}) \times I(^{43}\text{Ca})]$
⁷⁵ As	⁴⁰ Ar ³⁵ Cl	$\text{As} = I(^{75}\text{As}) - 0.0002 \times I(^{35}\text{Cl})$
⁸² Se	⁸² Kr	$\text{Se} = I(^{82}\text{Se}) - [A(^{82}\text{Kr})/A(^{83}\text{Kr}) \times I(^{83}\text{Kr})]$
¹⁰⁷ Ag	⁹¹ Zr ¹⁶ O	$\text{Ag} = I(^{107}\text{Ag}) - 0.025 \times I(^{91}\text{Zr})$
¹¹⁵ In	¹¹⁵ Sn	$\text{In} = I(^{115}\text{In}) - [A(^{115}\text{Sn})/A(^{118}\text{Sn}) \times I(^{118}\text{Sn})]$
¹³⁸ Ba	¹³⁸ Ce, ¹³⁸ La	$\text{Ba} = I(^{138}\text{Ba}) - [A(^{138}\text{La})/A(^{139}\text{La}) \times I(^{139}\text{La})] - [A(^{138}\text{Ce})/A(^{140}\text{Ce}) \times I(^{140}\text{Ce})]$
¹⁸⁷ Re	¹⁸⁷ Os	$\text{Re} = I(^{187}\text{Re}) - [A(^{187}\text{Os})/A(^{189}\text{Os}) \times I(^{189}\text{Os})]$

legal control of the contamination elements in wine. It should be referred that the International Organisation of Vine and Wine (OIV) defines maximum acceptable values for the concentration of the elements Cu, Zn, As, Cd and Pb in wine, of 1, 5, 0.2, 0.01 and 0.20 mg l⁻¹, respectively [6]. The semi-quantitative approach presents LODs, for most of the studied elements, similar or slightly higher than those obtained by the quantitative approach.

Results concerning the recovery tests are also present in Table 3. It can be seen that if Cr is excluded, recoveries between 80 and 120% were obtained for white and red wines. These recovery percentages are similar to those obtained by several authors [18,19,22] for table wines, using sample pre-preparation other than dilution or sample nebulizers other than crossflow. For Cr, an average recovery higher than 120% was obtained. This indicates that the correction of the matrix-induced polyatomic of ⁴⁰Ar¹³C and ³⁵Cl¹⁸O species was not effective for this element. Therefore, it was concluded that Cr could not be measured in the wine samples with the present methodology. The mathematical corrections used for V, Fe, Co, Ni and As interferences seem to be

appropriate. Some elements of medium mass (Ag, Cd, In) show recoveries close to 80%, despite the use of an internal standard with a similar mass (Rh). Re seems to be an appropriate internal standard for the elements with higher mass. It should be referred that in an initial step of our study, an attempt was made to use Sc as internal standard for elements of low mass, such as Li, Be and Al. However, the results were not satisfactory probably due to interferences affecting Sc. Once sample pre-treatment consists in a dilution, without destruction of the organic material, the results obtained are satisfactory. Preliminary tests in order to determine internal calibration recoveries showed similar values to those obtained by external calibration.

3.3. Comparison of analytical results obtained by ICP-MS quantitative and semi-quantitative approaches

In order to compare the results of quantitative and semi-quantitative approaches, white and red table wines were analysed, in parallel, by both modes of analysis (Cr was excluded). For each wine sample three independent replicates (external cal-

Table 3

Limits of detection (LODs)^a for both quantitative (Q) and semi-quantitative (SQ) approaches of analysis (μg l⁻¹). Recoveries percentages^b obtained using the quantitative (Q) approach

Element	LOD (Q)	LOD (SQ)	Recovery (Q)		Element	LOD (Q)	LOD (SQ)	Recovery (Q)	
			White wine	Red wine				White wine	Red wine
Li	0.003	0.003	97 ± 1	88 ± 1	Se	0.01	0.02	97.7 ± 0.9	88.9 ± 2
Be	0.001	0.003	98 ± 2	89 ± 1	Rb	0.001	0.001	96 ± 2	103 ± 3
Al	0.1	0.08	90 ± 2	99 ± 5	Sr	0.002	0.004	98 ± 18	98 ± 2
V	0.001	0.003	102 ± 1	101 ± 2	Ag	0.001	0.006	79 ± 3	81 ± 2
Cr	0.2	0.3	124 ± 4	136 ± 14	Cd	0.001	0.001	82 ± 1	83.0 ± 0.1
Mn	0.001	0.003	104 ± 4	105 ± 8	In	0.0002	0.0005	84 ± 2	83.0 ± 0.3
Fe	0.2	0.2	96 ± 4	110 ± 2	Cs	0.0003	0.0004	89 ± 1	98 ± 1
Co	0.0004	0.001	93 ± 2	90.8 ± 0.5	Ba	0.002	0.010	109 ± 2	81 ± 3
Ni	0.006	0.02	99 ± 3	102 ± 4	Hg	0.01	0.008	99 ± 3	100 ± 3
Cu	0.01	0.03	97 ± 3	91 ± 2	Tl	0.0003	0.0006	91 ± 3	93.7 ± 0.9
Zn	0.02	0.2	95 ± 3	97 ± 3	Pb	0.001	0.006	99 ± 1	92 ± 1
Ga	0.002	0.004	87.6 ± 0.6	84.8 ± 0.6	Bi	0.001	0.02	88 ± 6	93 ± 6
As	0.002	0.005	98 ± 3	95.0 ± 0.8	U	0.003	0.5	104 ± 5	106 ± 2

^a LODs were calculated as being the concentrations corresponding to signals equal to three-fold the standard deviation of a blank solution signal (three replicates).^b Mean of recovery values of three different spikes ± S.D.

Table 4

Concentrations^a of the contaminant elements obtained ($\mu\text{g l}^{-1}$), using both the quantitative (Q) and semi-quantitative methods (SQ), for wine samples

Element	White wine		Red wine	
	Quantitative mode	Semi-quantitative mode	Quantitative mode	Semi-quantitative mode
Li	4.4 (0.2)	4.3 (0.2)	7.8 (0.1)	7.55 (0.06)
Be	1.09 (0.04)	0.96 (0.06)	0.127 (0.006)	0.15 (0.07)
Al	1437 (32)	1364 (9)	671 (3)	528 (5)
V	59 (2)	58.0 (0.8)	24.2 (0.2)	23.17 (0.07)
Mn	1036 (28)	1009 (3)	879 (24)	828 (2)
Fe	2147 (21)	1168 (5)	4490 (12)	2587 (14)
Co	4.3 (0.1)	4.47 (0.09)	2.87 (0.02)	2.76 (0.03)
Ni	24.5 (0.9)	27.5 (0.8)	18.2 (0.4)	19.2 (0.5)
Cu	49 (2)	44.2 (0.7)	237 (3)	226 (4)
Zn	661 (10)	819 (6)	642 (19)	758 (6)
Ga	1.7 (0.2)	1.6 (0.1)	2.1 (0.2)	1.83 (0.07)
As	10.6 (0.6)	11.2 (0.3)	3.73 (0.09)	3.76 (0.09)
Se	0.9 (0.1)	ND ^b	0.6 (0.2)	ND ^b
Rb	453 (10)	429.9 (0.8)	674 (18)	627 (3)
Sr	243 (6)	239 (3)	326 (10)	303 (11)
Ag	ND ^b	ND ^b	ND ^b	ND ^b
Cd	0.48 (0.02)	0.28 (0.04)	0.30 (0.02)	0.21 (0.01)
In	0.12 (0.01)	0.02 (0.01)	0.005 (0.003)	ND ^b
Cs	3.6 (0.01)	3.80 (0.01)	3.59 (0.03)	3.80 (0.1)
Ba	89 (2)	89.1 (0.3)	79 (1)	77.3 (0.03)
Hg	ND ^b	ND ^b	ND ^b	ND ^b
Tl	0.24 (0.01)	0.15 (0.03)	0.147 (0.006)	0.145 (0.002)
Pb	14.43 (0.02)	14.0 (0.3)	19.2 (0.3)	19.0 (0.1)
Bi	1.3 (0.1)	1.3 (0.7)	1.2 (0.6)	1.7 (0.1)
U	0.69 (0.01)	2.04 (0.08)	0.47 (0.02)	1.67 (0.04)

^a Concentration values correspond to the mean of three independent replicates (quantitative mode) and three dependent replicates (semi-quantitative mode) corresponding standard deviation (in brackets).

^b ND – not detected.

ibration) were carried out and the mean and the respective R.S.D. (%) calculated for each element.

The values of R.S.D. (%) changed between 0.2 and 4% for most elements (Table 4), both for quantitative and semi-quantitative approach. For Ga, Se, In and Bi R.S.D. (%) values were observed in white and red wine as follows: 12 and 10; 11 and 33, 8 and 60; 8 and 50%, respectively. These results could probably be explained by their relatively low concentration in wines. Nevertheless, these precisions are slightly better than those obtained by Pérez-Jordán et al. [19] and similar to those reported by Almeida and Vasconcelos [22].

As shown in Table 4, comparable results (differences lower than 20%) were obtained for all of the elements with exception of Fe, Zn, Se, Cd, In, Tl and U (white wine) and Al, Fe, Se, Cd, In, Bi and U (red wine). In most cases the differences were lower than 6% (white wine) and 10% (red wine). For Be and Ga the differences were between 10 and 20% in both wines. Se was not detected by semiquantitative mode of analysis, neither in white nor red wine. For In and Tl the main reason for the differences in white wine, 80 and 60%, respectively, may be the fact that the respective concentrations are near the analytical limits of both modes of analysis, which precluded their accurate determination. For Cd, differences of 42 and 30% in

white and red wine were observed, probably due to their low concentration.

The differences observed for U, near to 200%, may be explained by the unsuitability of the semi-quantitative mode of analysis for such a high mass element. Differences between 42 and 45% were observed for Fe results. The results of quantitative mode were confirmed by AAS analysis, leading to the conclusion that either the automatic corrections made by the software for semi-quantitative mode were not successful or the calibration was not fit. As like for Fe, the differences observed for Al (21% in red wine) and Zn (23% in white wine and 18% in red wine) may be related to the unsuitability of the calibration used in semi-quantitative mode of analysis for major elements. In fact, it was used a standard solution with $10 \mu\text{g l}^{-1}$ of major, minor, trace and ultra-trace elements. As previously referred in Section 1, since sensitivity depends on experimental factors, it is important to update the set of response factors for each element in order to achieve accurate results. With reference to the importance of accurate results, such update should be performed with a multielement standard solution containing, for each element, the approximate concentration of those observed in wine.

In reference to the elements under legal control, it should be noted that for Zn and Cd, the comparison study revealed

large differences between the results obtained by the two calibration approaches (higher than 10%) and, hence, demonstrate the unsuitability of semi-quantitative approach in the analysis of these elements in wine. A linear least-squares adjustment of the results obtained by both modes of analysis was performed in order to evaluate the potential risk of error (the 95% confidence limits of the linear regression parameters are given). Non-comparable results (differences >20%) were not considered in the adjustment. A deviation of the slope from unity indicates proportional discrepancies between the two methods. A non-zero intercept is diagnosed as a constant discrepancy. The regression line for white wine yielded the equation: $y = (0.957 \pm 0.008)x + (1 \pm 4)$, $R = 0.9999$ ($n = 16$) (95% confidence level). For red wine the equation was $y = (0.937 \pm 0.005)x + (1 \pm 2)$, $R = 0.9999$ ($n = 15$) (95% confidence level). The regression line of the global results (including white and red wine samples) yielded the equation $y = (0.952 \pm 0.006)x \pm 2$, $R = 0.9999$ ($n = 31$) (95% confidence level). In both cases, white and red sample wines, high correlations were found, being very close to unity. However, the semi-quantitative mode analytical results are slightly lower than quantitative mode results, suggesting the existence of a slight bias. These observations are similar to those registered by Almeida and Vasconcelos [22], obtained either with table and fortified wines.

3.4. Validation of ICP-MS procedures

As referred, for validation of ICP-MS quantitative approach, six elements of a Portuguese red table wine were measured both by ICP-MS and AAS (Fig. 2). The differences between the results obtained by the two techniques were lower than 5% for all elements, with exception of Al (18%), demonstrating the accuracy of ICP-MS method. Linear least squares adjustment was applied to the six elements determined by AAS (x -axis) and ICP-MS (y -axis). The regression line of the global results yielded the equation: $y = (1.01 \pm 0.04)x + (3 \pm 40)$ (95% confidence level), with a correlation coefficient of $R = 0.9995$ ($n = 6$). Evidence of relative or fixed bias was not observed.

The results concerning the analysis of provisionally certified wines by quantitative ICP-MS approach are shown in Fig. 3.

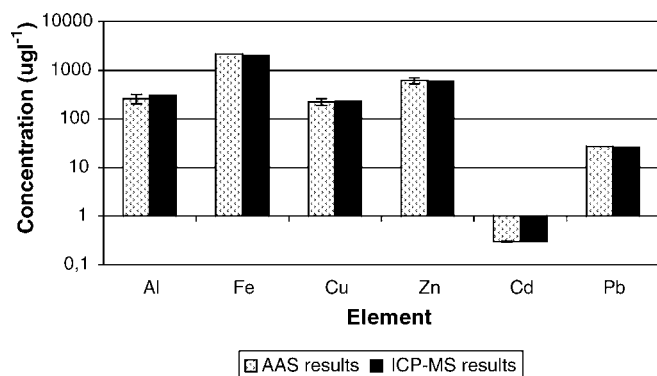


Fig. 2. Comparison between AAS and ICP-MS results ($\mu\text{g l}^{-1}$) obtained for the elements Al, Fe, Cu, Zn, Cd and Pb measured in a Portuguese red wine. The y -axis represents the logarithm of concentrations.

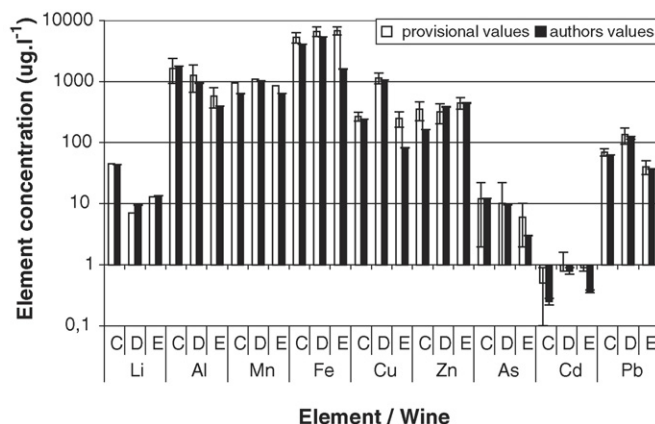


Fig. 3. Data for reference wines (BCR C, BCR D and BCR E, in the graph referred as C, D and E, respectively), showing values obtained by the authors and provisional values. The y -axis represents the logarithm of concentrations ($\mu\text{g l}^{-1}$) in order to obtain a better scatter of the data. Information about Li and Mn (provisional) concentration standard deviation of the three wines was not available.

Acceptable agreement was obtained within the tolerances supplied for all samples and analytes with the exceptions of Fe (BCR D and BCR E wines), Cd and Cu (BCR E wine). A linear least squares adjustment was applied to all elements determined, provisional data (x -axis) and our data (y -axis). For BCR C wine the equation $y = (0.79 \pm 0.09)x + (14 \pm 55)$ (95% confidence level), with a correlation coefficient of $R = 0.99$ ($n = 9$), was obtained. For BCR D wine the equation $y = (0.80 \pm 0.03)x + (40 \pm 75)$ (95% confidence level), with a correlation coefficient of $R = 0.999$ ($n = 9$), was obtained. If we exclude Fe data, for BCR E wine the equation $y = (0.8 \pm 0.2)x - (7 \pm 75)$ (95% confidence level), with a correlation coefficient of $R = 0.97$ ($n = 8$) was observed. The regression line of the global results (including the three wines), yielded the equation: $y = (0.80 \pm 0.03)x + (14 \pm 55)$ (95% confidence level), with a correlation coefficient of $R = 0.99$ ($n = 26$). The differences between our data and the provisional values indicate a systematic bias (our results were lower than the provisional values, that is, a slope significantly <1). The reason for this may related to the precipitation phenomena observed in all the wines, but more intense in red wine (BCR E wine). As previously mentioned the wines are from 1992 year and showed some precipitation material. It is known that metals interact with polyphenols and pectic polysaccharides species, which, in part, precipitate during the ageing of wines.

4. Conclusions

In the present work it was demonstrated that ICP-MS quantitative and semi-quantitative approaches offer valuable alternatives to table wine characterization and comparison purposes. However, as a tool for legal control, quantitative approach is the most suitable alternative. For some elements with legal importance, namely for Fe, Zn and Cd, the comparison study reveals large differences between the results obtained using quantitative and semi-quantitative calibration. In spite of that, analytical

results of both methods are comparable in terms of precision and accuracy (relative differences <20%) for most of the elements. In contrast with quantitative methodology, semi-quantitative methodology is faster, 67 s per sample, which allows high sample throughput. Another advantage is the lower reagent consumption.

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A 15-crown-5-functionalized carbosilane dendrimer as ionophore for ammonium selective electrodes

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Abstract

The synthesis of $\text{CH}_2=\text{CHCH}_2\text{OCH}_2[15\text{-crown-5}]$ (**III**) is achieved by the treatment of $\text{HOCH}_2-[15\text{-crown-5}]$ (**I**) with equimolar amounts of $\text{CH}_2=\text{CHCH}_2\text{Br}$ (**II**) in the presence of KOH. The hydrosilylation of **III** with $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{H})_4$ (**IV**) in the presence of the Karstedt catalyst affords the crown ether end-capped carbosilane dendrimer $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{Si-Me}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}_2[15\text{-crown-5}])_4$ (**V**). PVC-based membranes of **V** as ionophore with sodium tetraphenyl borate (NaTPB) as anion excluder and dioctyl phthalate (DOP), diphenyl ether (DPE), dibutyl amine (DBA) and dibutyl phthalate (DBP) as plasticizing solvent mediators were prepared and investigated as NH_4^+ -selective electrode. The response of the electrode was linear with a Nernstian slope of 53.3 mV/decade over an NH_4^+ ion concentration range of 7.60×10^{-6} to 1.0×10^{-1} M and a detection limit of 3.9×10^{-6} M. The response time to achieve a steady potential for NH_4^+ ions was between 6 and 10 s, and the electrode is suitable for use within the pH range of 2.2–8.5. The selectivity relative to alkali, alkaline earth, and transition heavy metal ions is good. The newly developed ionophore showed higher NH_4^+ selectivity over K^+ ($\log K_{\text{NH}_4^+/\text{K}^+}^{\text{Pot}} = -1.801$) and Na^+ ($\log K_{\text{NH}_4^+/\text{Na}^+}^{\text{Pot}} = -2.850$). The electrode could be used for at least 45 days without considerable alteration in its potential. The electrode also shows a better working concentration range and slope in comparison to other NH_4^+ -selective electrodes reported in literature.

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Keywords: Carbosilane dendrimer; Crown ether; Ammonium; Ion selective electrode; Ionophore

1. Introduction

The study of ammonium ion recognition is of considerable fundamental and practical interest because of the importance of ammonium-ion cation– π interactions which has been of great interest within the biochemical community. The availability of a highly selective ammonium ionophore is crucial for the preparation of ammonium ion chemical sensors used for the direct measurement of the ammonium ion [1–3]. Since NH_4^+ is a by-product of various reactions, much work has focussed on the fabricating of sensors for selective ammonium ion detection [4–6]. Until recently, the most common strategy involves the use of ion-selective electrodes (ISEs) employing non-actin, a natural antibiotic, as an ionophore doped into plasticized

poly(vinyl chloride) (PVC) membranes [7]. However, the NH_4^+ selectivity of non-actin over K^+ or Na^+ is not perfectly sufficient for practical use, and the development of a more highly selective ammonium ionophore as ion-sensing component for ammonium ion-selective electrodes has been desired. Consequently, significant effort is now being made to develop new synthetic ionophores with higher ammonium ion selectivity. Chin and coworkers [8] focused on the different symmetry of NH_4^+ and K^+ for the design of an ionophore and were successful in obtaining high NH_4^+ selectivity over K^+ . However, the developed ionophore showed problems such as low sensitivity and strong pH dependence. Kim et al. investigated the use of thiazole-containing benzo-crown ethers as ammonium ionophores, reported potassium selectivity comparable to non-actin, and enhances selectivity for ammonium over sodium [9]. Also, 19-crown-6 structures with decalino blocking groups were used to control selectivity, reporting increased selectivity for ammonium over both smaller and larger cations [10]. Recently,

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some efforts to find suitable NH_4^+ ionophores were successful [11–14].

In the ammonium ionophore design, two important factors are taken into consideration; the first contributes to increasing the NH_4^+ selectivity relative to smaller ions, such as Na^+ , Li^+ , or even the closest size, K^+ , and the second factor contributing to the increase in NH_4^+ selectivity over the larger ions such as Rb^+ and Cs^+ .

Our approach to design and synthesize ammonium ionophores has focused on the use of crown-ether end-capped dendrimers [15]. Dendrimers are perfect and unified macromolecules with regular and highly branched architectures that are obtained from an iterative procedure [16–20]. Due to their chemical and physical properties, dendrimers are used for many fields in material sciences [21]. Functionalized dendritic molecules with, for example, end-grafted electroactive or molecular recognition units are of interest as components in sensors, electro-devices, etc. [22–25].

A number of 1,10-phenanthroline derivatives containing crown-ether moieties were used as sensing agents for ion-selective electrodes for alkali metal ions [26]. For the purpose of extending of applicability, we have synthesized the 15-crown-5 end-capped dendrimer $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}_2[15\text{-crown-5}])_4$ and used it as ionophore for the fabrication of a ammonium selective membrane electrode. In contrast to silicon-containing ionophores, carbosilane dendrimers are more stable towards hydrolysis which makes them more suitable to be used as carriers. The number of peripheric crown ether groups attached to the carbosilane scaffold enhances the coordination of the target ion.

2. Experimental

2.1. Reagents

Ammonium thiocyanate, PVC of high relative molecular weight, dibutyl phthalate (DBP), diphenyl ether (DPE), dioctyl phthalate (DOP) and dibutyl amine (DBA) and nitrate salts of all the cations were obtained from Aldrich and used without further purification. Distilled water was used throughout to make the metal salt solutions.

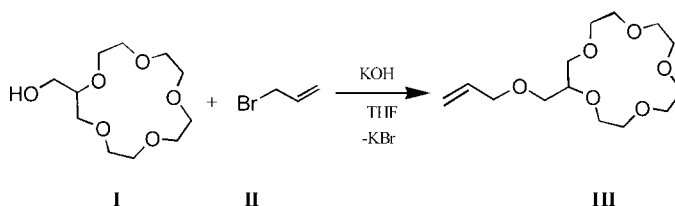
All the reactions for the synthesis of dendrimer **V** were carried out under N_2 using Schlenk techniques. Solvents and reagents were purified and dried by distillation.

2.2. Apparatus

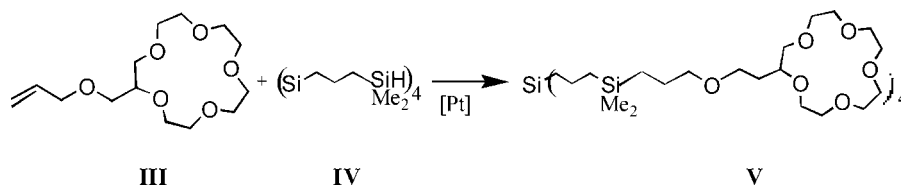
Schott Geräte Microprocessor pH/mV Meter CG840B was used for potential measurements at $27 \pm 0.1^\circ\text{C}$. IR spectra were recorded with a Perkin-Elmer FT-IR 1000 spectrometer as films between NaCl plates. ^1H , $^{13}\text{C}\{^1\text{H}\}$, and $^{29}\text{Si}\{^1\text{H}\}$ NMR spectra were recorded with a Bruker Advance 250 Spectrometer, operating in the Fourier transform mode. ^1H NMR spectra were recorded at 250.130 MHz (internal standard relative to CDCl_3 , $\delta = 7.27$ ppm); $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were recorded at 62.902 MHz (internal standard relative to CDCl_3 , $\delta = 77.0$ ppm). $^{29}\text{Si}\{^1\text{H}\}$ NMR spectra were recorded at 49.662 MHz (external standard relative to SiMe_4 , $\delta = 0.0$ ppm). Chemical shifts are reported in δ units (ppm) downfield from SiMe_4 . C, H-microanalysis was performed by the Organic Department at Chemnitz, the Technical University, using a Foss Heraeus Vario EL analyzer. The ESI-TOF mass spectra were recorded with a Mariner Biospectrometry Workstation 4.0 by Applied Biosystem.

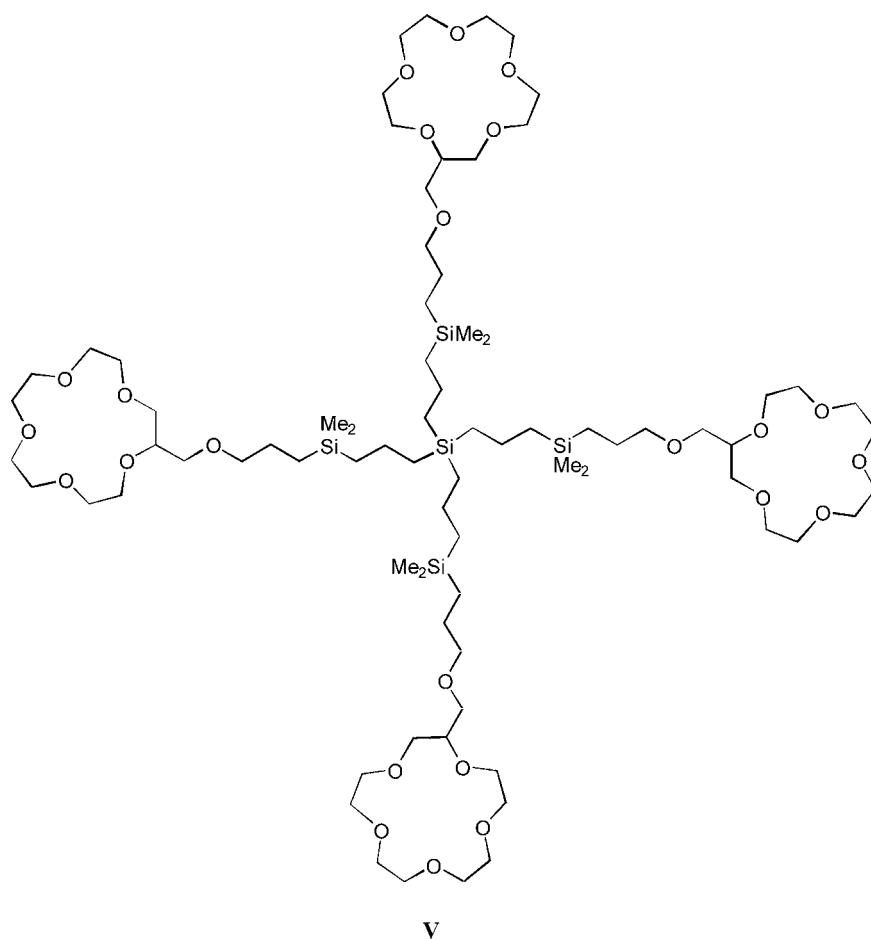
2.3. Synthesis of dendrimer **V**

The synthesis of the allyl-functionalized crown ether $\text{CH}_2=\text{CHCH}_2\text{OCH}_2[15\text{-crown-5}]$ (**III**) is achieved by treatment of $\text{HOCH}_2[15\text{-crown-5}]$ (**I**) with equimolar amounts of $\text{CH}_2=\text{CHCH}_2\text{Br}$ (**II**) in the presence of KOH.



The hydrosilylation of **III** with $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{H})_4$ (**IV**) [27,28] in presence of the Karstedt catalyst affords the crown ether end-capped carbosilane dendrimer $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}_2[15\text{-crown-5}])_4$ (**V**) in excellent yield.





0.31 g (0.716 mmol) $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{H})_4$ (**IV**) and 0.95 g (3.27 mmol) $\text{CH}_2=\text{CHCH}_2\text{OCH}_2$ [15-crown-5] (**III**) are reacted in diethyl ether (10 mL) for 16 h at 25 °C. The excess of **III** is distilled off by ball tube distillation at 200 °C in oil-pump vacuum. Yield: 1.12 g (0.702 mmol, 98% based on **IV**) of **V** as a colourless viscous oil.

IR (NaCl) ν [cm^{-1}]: 1248 (m) [$\delta_{\text{SiC-H}}$], 1130 (versus) [$\nu_{\text{C-O}}$].

^1H NMR (CDCl_3): δ -0.11 (s, 24 H, SiCH_3), 0.38 (m, 8 H, $\text{SiCH}_2\text{CH}_2\text{CH}_2\text{O}$), 0.43–0.53 (m, 16 H, $\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$), 1.23 (m, 8 H, $\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$), 1.48 (m, 8 H, $\text{SiCH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.32 (t, 8 H, $\text{SiCH}_2\text{CH}_2\text{CH}_2\text{O}$, $^3J_{\text{HH}}=7.1$ Hz), 3.40 (d, 8 H, OCH_2CH , $^3J_{\text{HH}}=5.0$ Hz), 3.50–3.81 (m, 76 H, crown ether OCHCH_2).

$^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3): δ -3.0 (SiCH_3), 11.6 ($\text{SiCH}_2\text{CH}_2\text{CH}_2\text{O}$), 17.9, 20.5 ($\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$), 18.9 ($\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$), 24.4 ($\text{SiCH}_2\text{CH}_2\text{CH}_2\text{O}$), 70.6, 70.9, 71.0, 71.2, 71.3, 71.3, 71.4, 72.0, 74.9 (OCH_2), 79.1 (OCH).

$^{29}\text{Si}\{^1\text{H}\}$ NMR (CDCl_3): δ 0.5 (Si), 2.1 (SiCH_3).

ESI-TOF-MS [m/z] (rel. int.) doped with KSCN: 933 (2) [$M+4\text{K}+2\text{SCN}$] $^{2+}$, 884 (14) [$M+3\text{K}+\text{SCN}$] $^{2+}$, 835 (63) [$M+2\text{K}$] $^{2+}$, 603 (8) [$M+4\text{K}+\text{SCN}$] $^{3+}$, 570 (100) [$M+3\text{K}$] $^{3+}$, 438 (10) [$M+4\text{K}$] $^{4+}$; doped with NaSCN: 901 (20) [$M+4\text{Na}+2\text{SCN}$] $^{2+}$, 581 (100) [$M+4\text{Na}+\text{SCN}$] $^{3+}$, 422 (10) [$M+4\text{Na}$] $^{4+}$.

Elemental analysis: $\text{C}_{76}\text{H}_{156}\text{O}_{24}\text{Si}_5$ (1594.45) calcd.: C, 57.25, H, 9.86; found: C, 57.26, H, 10.17.

2.4. Electrode preparation

The general procedure to prepare the PVC membrane was to mix thoroughly PVC, plasticizer, and anion excluder of varying amounts in a minimum quantity of THF. To this mixture was added the ionophore and the solution was mixed well. The resulting mixture was poured into acrylic rings placed on a smooth glass plate and allowed to evaporate at room temperature. After 48 h, transparent membranes of 5 mm diameter were obtained which were then cut to size and attached to a pyrex glass tube with Epoxy-bond glue. The tube was then filled with internal filling solution (1.0×10^{-3} M NH_4SCN). The electrode was finally conditioned for 48 h by soaking in a 1.0×10^{-2} M solution of NH_4SCN . The ratio of various ingredients, concentration of equilibrating solutions, and time of contact were optimized to provide membranes which result in reproducible and stable potentials.

2.5. Potential and pH measurements

All potential measurements were carried out with the following assembly: $\text{Hg}|\text{Hg}_2\text{Cl}_2(\text{s})$, $\text{KCl}|0.001\text{M}$ $\text{NH}_4^+||\text{membrane}||\text{test solution}|\text{KCl}$, $\text{Hg}_2\text{Cl}_2(\text{s})|\text{Hg}$. pH of the test solution was adjusted by using 0.1 N HCl or 0.1 N

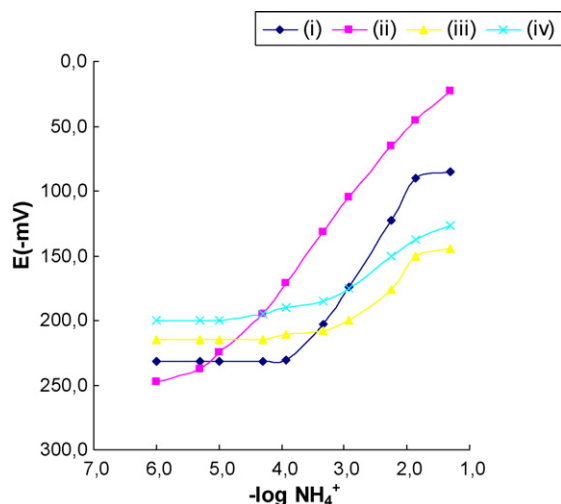


Fig. 1. Potential response of various metals: Na^+ (i), ammonium (ii), K^+ (iii), and Rb^+ (iv), ion-selective electrodes based on carbosilane dendrimer **V**.

NaOH , as per requirement, added in small increments to obtain solutions of desired pH.

3. Results and discussion

3.1. Potentiometric response

In preliminary experiments, the carbosilane dendrimer **V** was used as a neutral carrier to prepare PVC-based membrane electrodes for a variety of alkali metal ions and ammonium ion. The potential responses of the most sensitive electrodes, prepared under the same experimental conditions are shown in Fig. 1. As it can be seen, among different tested cations, NH_4^+ with the most sensitive response, seems to be suitably determined with the PVC membrane based on **V** and the potential responses obtained for all other cation-selective electrodes are much lower than that predicted by the Nernst equation. This is probably due to the selective behaviour of the ionophore against NH_4^+ in comparison to other metal ions. For NH_4^+ , the hydrogen bonding to the polyether oxygen of the crown ether ring may also be an important factor in its complexation.

3.2. Working concentration range and slope

It is well known that the sensitivity and selectivity obtained for a given ionophore depend significantly on the membrane composition and the nature of solvent mediators and additives used [29–32]. Thus, the influences of the membrane composition, nature and amount of plasticizer, amount of NaTPB as an anion excluder on the potential response of the NH_4^+ sensor were investigated. It should be noted that the presence of an anion excluder in cation-selective membrane electrodes not only diminishes the ohmic resistance [33] and enhances the response behaviour and selectivity [34], but also, in cases where the extraction capability is poor, increases the sensitivity of the membrane electrodes [35]. It is seen that, among four different plasticizers used, the use of DBP and the anion excluder

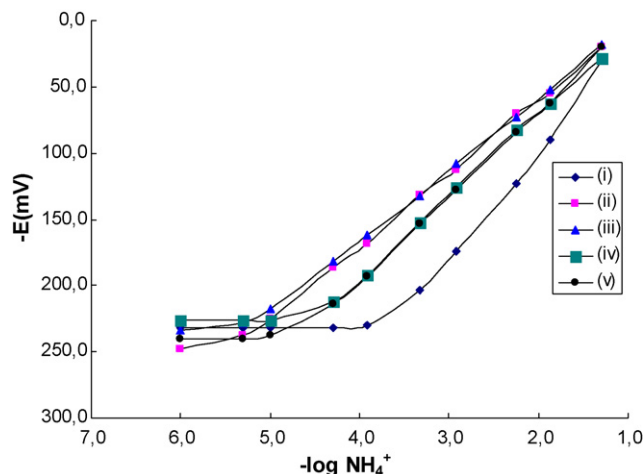


Fig. 2. Potential response of ammonium sensor based on **V** with different compositions of membrane ingredients (i–v corresponds to 1–5 of first column of Table 1).

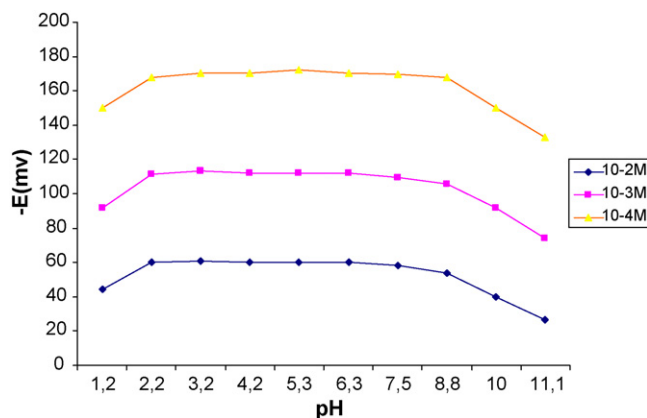


Fig. 3. Effect of pH on potential; $[\text{NH}_4^+] = 10^{-2}$, 10^{-3} , and 10^{-4} M for membrane no. 2.

exhibited a wide working concentration range of 7.60×10^{-6} to 1.0×10^{-1} M with a detection limit of 3.9×10^{-6} M and a Nernstian slope of 53.3 mV/decade (no. 2, Table 1 and Fig. 2), thereby exhibiting best sensitivity. The membrane having DPE, DOP, and DBA as plasticizer and NaTPB as anion excluder (membrane nos. 3–5) exhibited a working concentration range of 5.62×10^{-6} to 1.0×10^{-1} M, 4.46×10^{-5} to 1.0×10^{-1} M, 3.54×10^{-5} to 1.0×10^{-1} M, respectively. Thus, membrane no. 2 having composition ratio of 150:10:3:120 (mg) (DBP:V:NaTPB:PVC) was chosen for further electroanalytical studies.

3.3. pH Effect

The effect of pH of the ammonium thiocyanate test solutions (1.0×10^{-2} , 1.0×10^{-3} , and 1.0×10^{-4} M) on the sensor potential was investigated by following the potential variation over the pH range of 1–11. The influence of pH on all response of the PVC membrane electrode is shown in Fig. 3. Potentials remain constant from pH 2.2–8.5 and the same may be taken

Table 1

Composition and response characteristics of PVC-based 15-crown-5-functionalized carbosilane dendrimer **V** membrane sensor for NH_4^+ ions

Membrane no.	Components in membranes (mg)							Working concentration range (M)	Detection limit (M)	Slope (mV/decade of activity)	Response time t_{95} (s)
	V	PVC	DBP	DPE	DOP	DBA	NaTPB				
1	10	120	–	–	–	–	–	3.98×10^{-4} to 1.0×10^{-1}	2.5×10^{-4}	61.5	25
2	10	120	150	–	–	–	3	7.60×10^{-6} to 1.0×10^{-1}	3.9×10^{-6}	53.3	06
3	10	120	–	150	–	–	3	5.62×10^{-6} to 1.0×10^{-1}	3.9×10^{-6}	50.0	06
4	10	120	–	–	150	–	3	4.46×10^{-5} to 1.0×10^{-1}	3.1×10^{-5}	66.6	10
5	10	120	–	–	–	150	3	3.54×10^{-5} to 1.0×10^{-1}	1.7×10^{-5}	40.0	10

as the working pH range of the assembly. The drift in potential values at $\text{pH} > 8.5$ is attributed to the formation of ammonia.

3.4. Response and life time

The optimum conditioning time for the membrane electrode in a 10^{-2} M NH_4^+ solution was 48 h, after which it generated stable potentials on contact with Ag^+ solutions of different concentrations. The response time is calculated as the time which elapsed between the instant at which the ion-selective electrode and a reference electrode (ISE cell) are brought in contact with a sample solution (at which the activity of the ion of interest in solution is changed) and the first instant at which the emf/time ($\Delta E/\Delta t$) becomes steady. It was seen that the electrode reaches the equilibrium response in a short time of 15 s when the ammonium ion concentration was rapidly increased from 10^{-4} to 10^{-3} M. The sensing behaviour was unchanged when the potentials are recorded either from low to high concentration or vice-versa. The membrane without plasticizers and NaTPB (no. 1) showed a response time of 25 s. It could significantly be reduced by 15–20 s, when the solvent mediators were added. The best response time of 6 s was recorded for membrane having DBP and DPE as solvent mediator. The potentials generated by this membrane remained stable for more than 3 min after which it started deviating. To evaluate the reproducibility of the proposed electrode, a series of membranes (five) with similar composition (no. 2) were prepared and the responses of these electrodes to NH_4^+ ion concentration were tested. The results show the average of slopes, detection limit, and working concentration range were 53.3 ± 1 mV/decade, $3.9 (\pm 2) \times 10^{-6}$ M, and $7.6 (\pm 2) \times 10^{-6}$ to 1.0×10^{-1} M, respectively.

The lifetime of electrodes based on the ionophore in solvent polymeric membranes depends on the distribution coefficient of the ionophore and the plasticizer between the aqueous and membrane phases [36]. Hence, the lifetime of electrodes must depend on the components of the solution and the measured specimens with electrodes. The lifetime of the electrodes was determined by performing calibrations periodically with standard solutions and by calculating the response slope over the range of 1.0×10^{-6} to 1.0×10^{-1} M NH_4SCN . The electrode can be used at a stretch for 45 days, however, after this period a very slight gradual change in slope is observed. With repeated calibration over several days, the electrode can be further used for over 90 days. Subsequently, the electrochemical behaviour of the electrode gradually deteriorated which may be due to ageing of the polymer (PVC), plasticizers, and ionophore [22].

3.5. Potentiometric selectivity

The potentiometric selectivity coefficients of membranes are very important for determining their quality as ISEs. The selectivity coefficients represent their response to the species to be measured over other ions present in solution. They were evaluated by the Fixed Interference Method [37] and the Matched Potential Method [38,39]. The selectivity coefficients towards NH_4^+ were determined against the interfering cations Li^+ , Rb^+ , Ag^+ , Cs^+ , Na^+ , K^+ , Cu^{2+} , Ca^{2+} , Sr^{2+} , Ni^{2+} , Mg^{2+} , Ba^{2+} , Al^{3+} , and Fe^{3+} . The resulting values are summarized in Table 2. As can be seen, for all diverse ions used, the selectivity coefficient values are in the order of 10^{-2} or smaller, indicating they would not significantly disturb the functioning of the NH_4^+ ion-selective membrane. The electrode based on **V** exhibited good selectivity relative to K^+ and Na^+ , which are major sources of interferences in the sensing of NH_4^+ . The NH_4^+/K^+ and $\text{NH}_4^+/\text{Na}^+$ selectivity of the electrodes were -1.801 and -2.850 , respectively. Since it has been shown that FIM, based on Nicolsky–Eisenman equation, suffers some limitations in terms of the values for ions of unequal charges, a non-Nernstian behaviour of interfering ions, and the activity dependence of the values [38,40], therefore, we have also used the recommended MPM in this work, which is totally independent of the Nicolsky–Eisenman

Table 2

Selectivity coefficient values for ammonium selective membrane electrode (no. 2) for various interfering ions (B) using the Fixed Interference Method (FIM) and the Matched Potential Method (MPM)

Interfering ions (B)	Selectivity coefficient	
	FIM $\log (K_{A,B}^{\text{Pot}})$	MPM (K_{sel})
Li^+	–2.500	0.13
Ag^+	–2.752	0.12
Cs^+	–2.400	0.12
Na^+	–2.850	0.16
K^+	–2.801	0.20
Rb^+	–2.450	0.12
Cu^{2+}	–2.625	0.12
Ca^{2+}	–2.725	0.12
Sr^{2+}	–2.375	0.12
Ni^{2+}	–2.950	0.13
Mg^{2+}	–2.903	0.12
Ba^{2+}	–2.752	0.11
Al^{3+}	–2.425	0.11
Fe^{3+}	–2.701	0.11

Conditions for MPM: reference solution (10^{-6} M of NH_4^+); primary ion (10^{-4} M of NH_4^+). Conditions for FIM: interfering ion (10^{-2} M).

Table 3

Comparison of the reported electrodes with the proposed NH_4^+ assembly

S. no.	Working concentration range (M)	pH range	Slope (mV/decade)	Interferences	Ionophore	Refs.
1	10^{-5} to 10^{-1}	–	56.3	K^+	Polypyrrole	[11]
2	10^{-6} to 10^{-1}	NM	58.1	K^+	TD 19C6	[10]
3	10^{-5} to 10^{-1}	–	59.3 59.1	Na^+ Ca^+	TD 20C6 TD 21C6	[12]
4	10^{-4} to 10^{-1}	8.0	NM	Good selectivity	Pyrazole	[13]
5	10^{-5} to 10^{-1}	>2	59.0	Good selectivity	Macrocyclic polyether	[14]
6	7.60×10^{-6} to 10^{-1}	2.2–8.5	53.3	Good selectivity	15-crown-5-functionalised carbosilane dendrimer	Proposed electrode

NM: not mentioned.

equation. According to the MPM, a specified activity (concentration) of primary ions (A) was added to a reference solution and the potential was measured. In a separate experiment, interfering ions (B) were successively added to an identical reference solution, until the measured potential matched the one obtained before adding primary ions. The MPM selectivity coefficient (K_{sel}) was then given by the resulting primary ion to the interfering ion activity (concentration) ratio, $K_{\text{sel}} = a_{\text{A}}/a_{\text{B}}$. The experimental conditions employed and the resulting values are given in Table 2.

It is interesting to note that a comparison of the selectivity and working of the proposed sensor along with those reported before [10–14] (Table 3), clearly indicate a good enhancement in the behaviour of the proposed NH_4^+ electrode in terms of the working concentration range, slope, pH range, and potentiometric selectivity.

4. Conclusions

PVC membrane electrodes incorporating the crown ether end-capped carbosilane dendrimer $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{SiMe}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}_2[15\text{-crown-5}])_4$ (V) as ionophore showed high selectivity and sensitivity towards ammonium ions. The best result was obtained with the membrane containing DBP as plasticizer. The membrane exhibited a Nernstian slope to ammonium ion activity in the range of 7.60×10^{-6} to 1.0×10^{-1} M with a detection limit of 3.9×10^{-6} M. The functional pH range of the electrode is 2.2–8.5. The selectivity of the electrode towards NH_4^+ is very good over other cations and also does not show interference from other mono-, di-, and trivalent ions.

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The application of multielemental analysis in the elaboration of technology of mineral feed additives based on *Lemna minor* biomass

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Abstract

Supplementation of microelements (Mn, Zn, Cu, Cr) to livestock diet is of particular concern. There are various mineral feed additives available on the market. The most frequently used are inorganic feed additives, characterized with low bioavailability and high toxicity. Also, organic feed additives are used, including amino acids chelates, in which bioavailability was greatly improved and toxicity reduced. The problem is high price of these products. Therefore, there is the need to search for new biological mineral feed additives with designed composition, that would be characterized with high bioavailability, low toxicity, low cost and that would also possess a nutritional value. Such a possibility offers biological materials. It was found that biomaterials have metal-binding capabilities. Metal ions may be bound to biomass via either biosorption or bioaccumulation process.

When elaborating technology of the production of feed additives, containing simultaneously various elements, it is necessary to use the tool of multielemental analysis in order to simultaneously analyze the content of Mn, Zn, Cu, Cr. In the present work, ICP-OES multielemental analysis was used to investigate the process of production of mineral feed additives based on the biomass of aquatic plant *Lemna minor*. The effectiveness of the processes of biosorption and bioaccumulation of microelements by an aquatic plant *L. minor* was studied. The mechanism of the process as well as equilibrium was investigated with the use of multielemental analysis by ICP-OES Vista-MPX instrument from Varian (Australia).

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Keywords: Biosorption; Bioaccumulation; *Lemna minor*; Microelements; Mineral feed additives

1. Introduction

Supplementation of microelements to natural feeds of livestock has recently become particularly significant. Microelements are supplied in the form of mineral feed additives, the most commonly in the form of inorganic salts. This form is however characterized with low bioavailability to animals and therefore microelements are of transit character. Also, inorganic salts ingested by animals may cause adverse health effects. Therefore, there is the need to elaborate such mineral feed additives that would supply minerals in non-toxic form and the mostly bioavailable to animals. This can be achieved when the minerals are supplied in the form possibly similar to the natural. It would be also advantageous if these products would have a nutritional

value. Recently, amino acid chelates have been used in supplementation of livestock diet. The problem of bioavailability and toxicity has been greatly reduced, however these products are still expensive (the cost is 10 times higher than inorganic salts). The problem of high cost could be overcome by binding microelements to readily available biomass of, i.e. aquatic plants [1]. It is well known that various types of biomaterials possess metal-binding properties. This topic has been widely discussed in the literature [2–5]. These capabilities however are employed in industrial wastewater treatment processes in which biomass is mainly used to remove heavy metal ions from solutions. In bioremoval methods, two distinctive processes can be distinguished: biosorption (the process performed by not-living biomass) and bioaccumulation (the process carried out by living cells). The same processes, although at different process conditions may be used to bind metals. The aim would be not to remove cations, but to incorporate them into biological material. Therefore, in bioremoval processes the aim would be to

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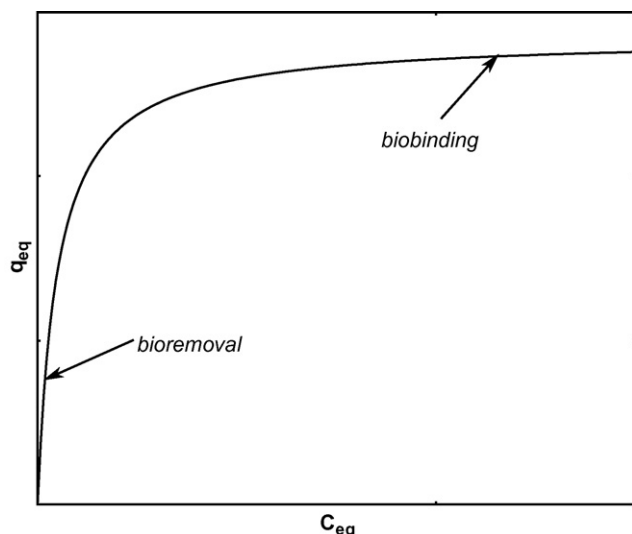


Fig. 1. Differences in bioremoval and biobinding equilibrium.

reach possibly low equilibrium metal ions concentration and in 'biobinding' processes the aim would be to reach possibly high equilibrium metal-binding capacity (Fig. 1).

In the present work, microelements, important in livestock feeding were bound to the biomass of commonly worldwide available freshwater floating aquatic plant *Lemna minor* (duckweed) [6]. *L. minor* was found to be cold tolerant, it grows rapidly and is easy to harvest. The plant is used as a valuable feed [7]. Since *L. minor* is an organism that can grow in highly polluted environments, it was applied in wastewater treatment processes to remove either nutrients from municipal wastewaters (so-called constructed wetlands) [8–11] or to bind heavy metals from industrial effluents, since it possesses a capability to concentrate metal ions from solutions, several fold (1000–100,000 times) [12–16]. The produced biomass was used either as feed for poultry [8,17,18], fish [19] and cattle (as soybean substitute) [6] or as fertilizer [20,21]. It was found that the supplementation of *L. minor* to hens diet resulted in increased yolk pigmentation [17,22]. Also, *L. minor* can be considered as rich in protein (30 mass%), minerals and vitamins animals feed [8,17] and poor in fiber that was considered advantageous [9].

The processes of biobinding in real systems occur in the presence of various ions. Also, in the case of technology of biological mineral feed additives, binding of cations of feeding significance (Mn, Cu, Zn, Cr) could be considered as multi-ion biosorption system. To study this process, simultaneous multi-elemental analysis is indispensable. Such a possibility offer ICP techniques, commonly used in biosorption experiments, in particular in studies on multi-metal systems [23–27].

The aim of the present work was to investigate the applicability of an aquatic plant *L. minor* in the production of mineral feed additives in the processes of biosorption and bioaccumulation. The experiments were performed in a multi-metal system, in the presence of minerals of feeding significance: Mn, Cu, Zn, Cr. Inductively coupled plasma optical emission spectroscopy (instrument Vista-MPX, Varian, Australia) was used in this study.

2. Materials and methods

2.1. Characterization of an organism

L. minor (duckweed) was used as research material in the experiments on biosorption and bioaccumulation of microelements from aqueous solutions. *L. minor* was obtained from a drainage ditch in Wrocław (Poland). The plant was washed and rinsed with demineralized water three times for 10 min.

The multi-elemental analysis of biomass was performed with the use of ICP-OES technique. Before the analysis, the samples were digested in the microwave system. The biomass underwent also macroelemental analysis (CHNS) with the use of Eurovector EA 3000 (Milan, Italy) instrument. Cation-exchange capacity was determined with potentiometric titration according to the procedure described previously [28].

2.2. Biosorption experiments

In biosorption experiments, dry biomass was used to assure that cells were not alive. The biomass was dried at 60 °C for 5 days and exposed to microelements solution for 2 h (kinetic experiments showed that the equilibrium was reached after few minutes) at various initial concentrations of microelements in the ratio (mm⁻¹) Cr:Mn:Zn:Cu (1:1:1:0.1). The following inorganic salts were used to prepare the solutions: Cr(NO₃)₃·9H₂O, MnCl₂·4H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O (from POCH, Gliwice). The proportions and the salts used, were in conformance with animal feeding standards [29,30]. Biosorption tests were performed in water bath shaker at ambient temperature and pH 5. The initial concentration of microelements solution (C_0) was determined by ICP-OES technique. After biosorption was finished, the suspension was filtered through no. 2 paper filters and was analyzed for the content of microelements with ICP-OES (C_{eq}).

2.3. Bioaccumulation experiments

In bioaccumulation experiments living cells were used. Fresh *L. minor* was used in these tests. Two types of bioaccumulation experiments were performed: with the use of non-growing cells (in the solution of microelements in demineralized water) and with the use of growing cells (in the solution of microelements in boiled tap water). The contact time was also 2 h. The aim was to compare the efficiency of three processes: biosorption, bioaccumulation without and with growth. Bioaccumulation tests were performed in water bath shaker at ambient temperature, with natural lighting (light intensity ca. 400 lx). The solution (before and after bioaccumulation) was analyzed for the content of microelements with ICP-OES method.

2.4. Analytical methods

Multi-elemental analysis was performed with the use of ICP-OES measurements by Vista MPX instrument with charge coupled device (CCD) simultaneous detection systems (Varian Inc., Victoria, Australia). Demineralized water was prepared using

an Elga water purification system (Aquadem, Nice, France). A microwave-assisted acid digestion Milestone MLS-1200 MEGA apparatus was used for biomass sample dissolution. Reagent used for digestion of samples was nitric acid, 65% trace-pure grade Merck (Darmstadt, Germany). Plant biomass (1 g) was digested with 5 ml of nitric acid and after digestion filled with demineralized water to the volume 50 ml.

Aliquots of an ICP multielement standard solution (100 mg l^{-1} Astasol® Mix Calibration Standard Solution from Analytika Ltd. (Czech Republic)) containing the analyzed elements, was used in the preparation of all calibration solutions. Calibration solutions ranged from 0.1 to 100 mg l^{-1} and were prepared every day by adequate dilution of the stock standard solution.

ICP-OES parameters (RF power, nebulizer pressure, auxiliary gas flow rate sample uptake and rinse time) were optimized in order to reach high signal to blank ratios at emission lines of least interference (Table 1). Also, the mostly suitable spectral wavelength was selected (Table 1). Analytical process was con-

trolled by the use of matrix standard Certified Reference Material Hard Drinking Water (UK)—metals from LGC (LGC6010) for analysis of solutions and Polish Certified Reference Material for multielement trace analysis Oriental Tobacco Leaves (CTA-OTL-1) from Institute of Nuclear Chemistry and Technology (Poland) for analysis of digested samples of the biomass. The comparison of obtained results and certified concentrations is shown in Table 2.

3. Results and discussion

Literature reports that an aquatic plant *L. minor* can be used to remove metal cations from effluents, since it possesses high metal ions binding capacity. The studies however focus on metal ions removal, not binding. Also, the results of the studies on the mechanism of metal ions binding by *L. minor* were not reported in the literature.

In the present study, similarly as in the literature [12,16], it was found that kinetics of biosorption by *L. minor* was rapid and the equilibrium was reached after few minutes. For this reason, it was supposed that the mechanism of metals ions binding would be rather chemical than physical adsorption. This theory was confirmed by potentiometric titration that enabled to evaluate cation-exchange properties. Unexpectedly, it was found that the studied biomass possessed high ion-exchange capacity, when compared with other sorbents. The experimental results of potentiometric titration were described with the mathematical model proposed previously ($R^2 = 0.997$) [28]. Three functional groups similarly as in the case of other biomasses [28,31] capable of cation-exchange were identified on the surface of cells (Table 3): carboxyl, phosphoryl and hydroxyl and the total ion-exchange capacity was evaluated as $26.2 \text{ mequiv. g}^{-1}$ and thus theoretical maximum biosorption capacity of Cr(III) ions would be 454 mg g^{-1} . Many authors report [27,28,31,32] that the most important role in biosorption is played by carboxyl groups, being deprotonated in the wide pH range (in biosorption experiments in the present study pH was adjusted to 5.0), yielding maximum sorption capacity of Cr(III) at pH 5: $15.8 \text{ mequiv. g}^{-1}$ (that corresponds to 274 mg g^{-1} of Cr(III) ions).

Biosorption and bioaccumulation (by non-living and living cells) of Cr(III) ions was performed in a single-metal system. The obtained results are presented in Fig. 2. The experimental results were described with Langmuir equation that yielded higher conformity with the experimental results than Freundlich equation. Also, Langmuir equation is used more frequently to describe sorption equilibrium if the mechanism was identified as chemisorption. For this reason, the experimental results were described with Langmuir equation. The model parameters are shown in Table 4. Unexpectedly, it was found that the highest performance was reached in biosorption ($q_{\text{max}} = 9.24 \text{ mequiv. g}^{-1}$), afterwards in bioaccumulation by growing ($7.41 \text{ mequiv. g}^{-1}$) and by not-growing cells ($5.95 \text{ mequiv. g}^{-1}$). The affinity of the biomass to Cr(III) in each process was very similar.

It is known that different ions possess different metal-binding affinity to the biomass. Also, metal-binding capacity should depend on whether we work in a single- or multi-ion system,

Table 1
The operating parameters of determination of elements by ICP-OES

Method parameters	Value
RF power (kW)	0.95
Plasma flow (l min^{-1})	15.0
Nebulizer flow (l min^{-1})	0.69
Viewing height (mm)	12
Pump rate (rpm)	15
Rinse time (s)	10
Auxiliary flow (l min^{-1})	1.5
Replicates	3
Replicate read time (s)	10
Instrument stabilization (s)	15
Sample uptake delay (s)	30
Element	Emission line (nm)
Ag	328.068
Al	396.152
As	188.980
B	249.772
Bi	223.061
Ca	396.847
Cd	214.439
Co	238.892
Cr	206.158
Cu	324.754
Fe	259.940
K	766.491
Li	670.783
Mg	279.553
Mn	257.610
Mo	202.032
Na	589.592
Ni	231.604
Pb	220.353
Sb	206.834
Sr	407.771
Ti	336.122
Tl	190.794
V	292.401
Zn	213.857

Table 2

The comparison of results obtained for Certified Reference Materials (certified concentrations)

Element	Unit	Certified concentration		Obtained result		
		Mean	Uncertainty	Mean	Uncertainty	Recovery (%)
Certified Reference Material LGC6010						
Ag	μg l ⁻¹	6.2	1.1	6.1	0.4	98
Al	μg l ⁻¹	208	19	200	14	96
Cr	μg l ⁻¹	48	3	47	3	98
Fe	μg l ⁻¹	236	6	238	17	101
Mn	μg l ⁻¹	48	2	47	3	98
Pb	μg l ⁻¹	95	3	93	6	98
Sb	μg l ⁻¹	11.9	1.2	12.0	1	101
Zn	μg l ⁻¹	542	16	526	37	97
Certified Reference Material CTA-OTL-1						
Al	μg g ⁻¹	1740	290	1723	172	99
Ca	mg g ⁻¹	31.7	1.2	32.0	4.8	101
Cd	μg g ⁻¹	1.12	0.12	1.10	0.22	98
Co	μg g ⁻¹	0.879	0.039	0.92	0.14	105
Cr	μg g ⁻¹	2.59	0.32	2.67	0.40	103
Cu	μg g ⁻¹	14.1	0.5	14.2	2.1	101
K	mg g ⁻¹	15.6	0.5	15.0	2.2	96
Li	μg g ⁻¹	23.0	1.8	22.1	3.3	96
Mg	μg g ⁻¹	4470	210	4336	650	97
Mn	μg g ⁻¹	412	14	404	60	98
Ni	μg g ⁻¹	6.32	0.65	6.04	1.21	96
Pb	μg g ⁻¹	4.91	0.80	5.01	1.0	102
Sr	μg g ⁻¹	201	20	201	30	100
V	μg g ⁻¹	3.08	0.42	3.01	0.45	98
Zn	μg g ⁻¹	49.9	2.4	49.1	7.4	98

in which the competition between ions for metal-binding sites is observed. Biosorption and bioaccumulation was also performed in multi-metal systems in the simultaneous presence of Cr(III), Cu(II), Mn(II), Zn(II) cations. Biosorption was carried out with the use of dry biomass and bioaccumulation with fresh plant, the concentration of which was the same as in the case of biosorption (the determined moisture content of fresh biomass was 94.9%). The results are presented in Figs. 3–5. The experimental data in multi-ions system were also described with Langmuir equation. The model parameters are shown in Table 4.

The highest total maximum binding capacity showed the biomass in the biosorption process ($3.02 \text{ mequiv. g}^{-1}$), afterwards in bioaccumulation by living cells. The total maximum metal-binding capacity obtained in a single-metal system (expressed as mequiv. g^{-1}) was the same as in multi-metal system. This confirms the theory, that there is a finite number of metal-binding sites present on the cellular surface that possess cation-exchange properties. It was found that in multi-ion system, the affinity of sorbent to sorbate was significantly higher. This could be explained by the competition between the cations and could be the reason that the sorbent becomes saturated at

lower equilibrium concentration of metal ions when compared with a single-metal system. In biosorption, as well as in bioaccumulation experiments the cells showed the highest sorption capacity for Cr(III) ions, afterwards (in decreasing order) for: Cu, Zn and Mn. The total sorption capacity was the highest in the case of biosorption (the total maximum sorption capacity was $2.23 \text{ mequiv. g}^{-1}$).

Reassuring, it is possible to produce mineral feed additives with the use of *L. minor* biomass that contain up to 7.45% (m/m) minerals via biosorption, 3.39% (m/m) via bioaccumulation by

Table 3

The concentration of cation-exchange functional groups on *Lemma minor* cells

Functional group	pK _a	Cellular concentration (mequiv. g^{-1})
Carboxyl	3.02	12.7
Phosphoryl	6.78	7.86
Hydroxyl	11.0	8.99

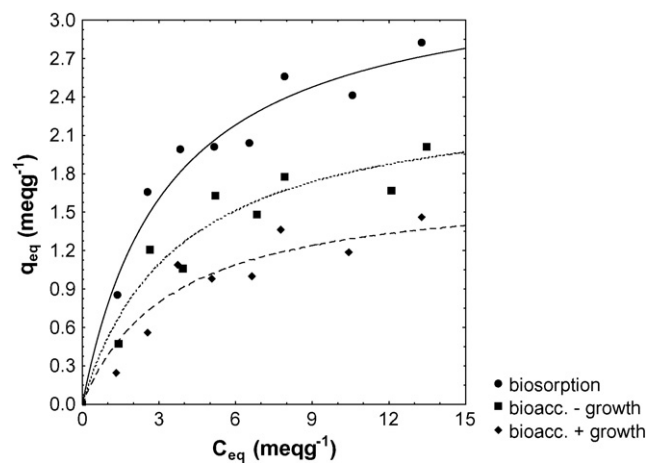


Fig. 2. Biosorption, bioaccumulation without cellular growth (bioacc. – growth) and bioaccumulation with cellular growth (bioacc. + growth) of Cr(III) ions.

Table 4
Langmuir parameters for biosorption and bioaccumulation by both non-growing and growing cells of *L. minor*

	Single-ion system	Multi-ion system			
	Cr	Cr	Cu	Mn	Zn
Biosorption					
$q_{\max}$ (mequiv. g ⁻¹)	3.40	1.40	0.738	0.364	0.514
b (l mequiv. ⁻¹)	0.298	4.98	1.33	3.2	2.66
R^2	0.983	0.977	0.964	0.991	0.992
Bioaccumulation by non-growing cells					
$q_{\max}$ (mequiv. g ⁻¹)	2.46	0.773	0.0869	0.287	0.301
b (l mequiv. ⁻¹)	0.267	5.11	37.6	2.53	6.55
R^2	0.932	0.977	0.948	0.964	0.972
Bioaccumulation by growing cells					
$q_{\max}$ (mequiv. g ⁻¹)	1.72	1.26	0.116	0.321	0.326
b (l mequiv. ⁻¹)	0.287	1.84	26.9	0.605	2.22
R^2	0.937	0.952	0.958	0.967	0.982

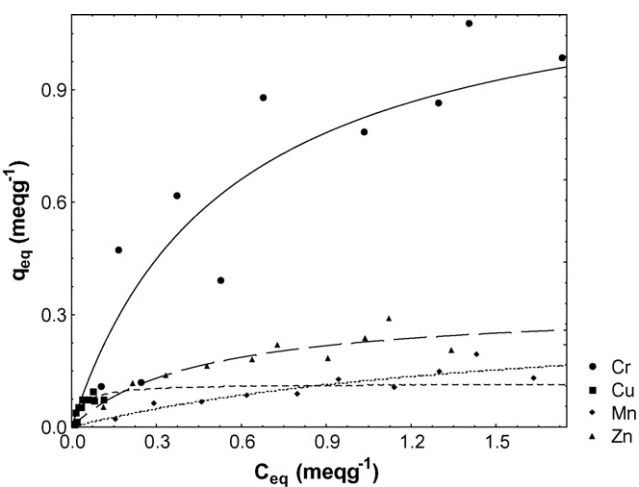


Fig. 5. Bioaccumulation of Cr, Cu, Mn, Zn ions by living *L. minor*.

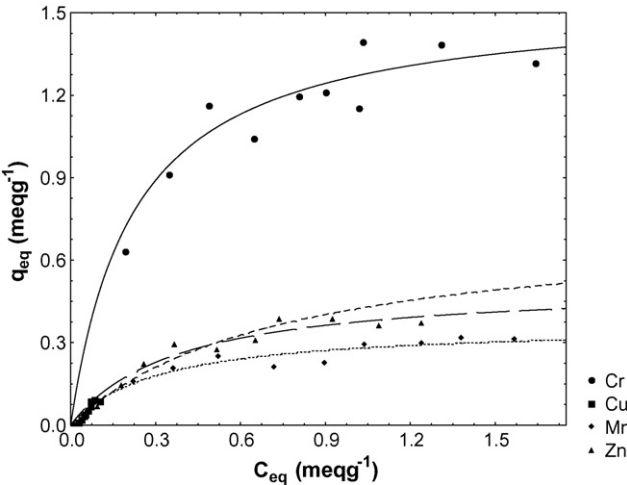


Fig. 3. Biosorption of Cr, Cu, Mn, Zn ions by non-living *Lemma minor*.

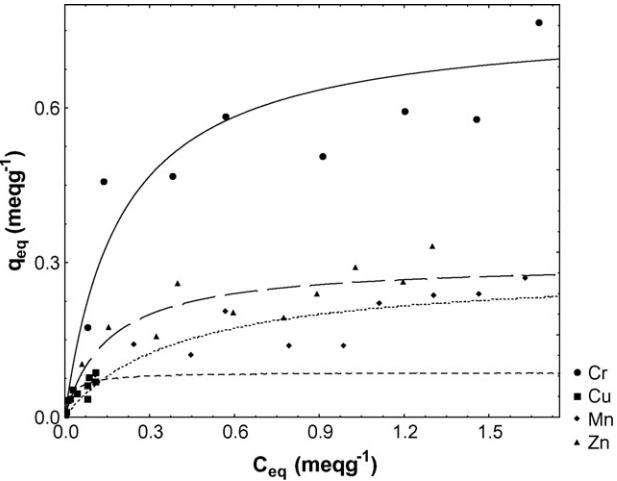


Fig. 4. Bioaccumulation of Cr, Cu, Mn, Zn ions by non-living *L. minor*.

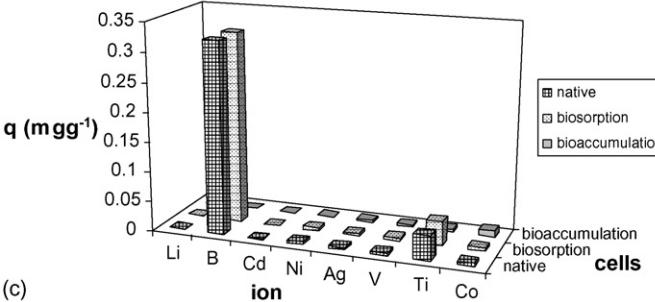
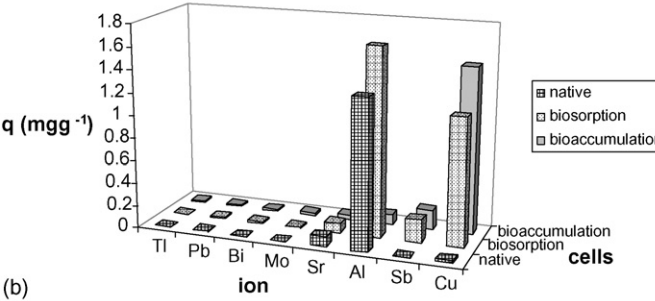
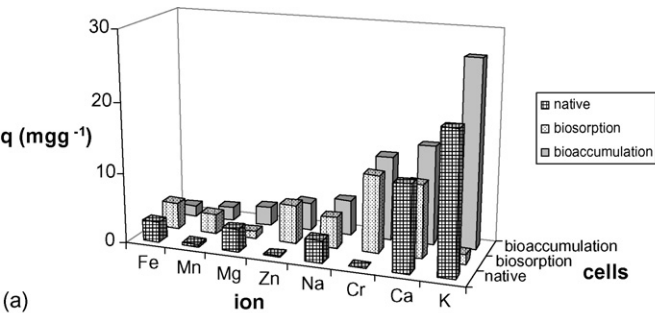


Fig. 6. The elemental composition of native biomass, cells after the processes of biosorption and bioaccumulation with growth.

non-growing cells and 4.50% (m/m) via bioaccumulation by growing cells. The mostly advantageous in terms of efficiency seem to be biosorption process. Since the lowest minerals binding capacity showed cells in the process of bioaccumulation without growth, this process was assumed not to be advantageous technique of the production of mineral feed additives and for this reason the other two processes were used in the further experiments.

In order to investigate the nutritional value of *L. minor*, the native biomass (that did not undergo the process of biobinding) was analyzed for the content of macroelements, microelements and trace elements. *L. minor* contains 32.1% carbon, 5.86% hydrogen and 3.48% N that corresponds to the protein content 19–22%, which was found to be comparable with soybean [6]. The mineral composition of the biomass native and after the process of biosorption and bioaccumulation by growing cells is presented in Fig. 6. The native cells contain mainly the following minerals: (in decreasing order): K (20.0 mg g^{-1}), Ca (12.2 mg g^{-1}), Mg (3.3 mg g^{-1}), Na (3.14 mg g^{-1}), Fe (2.9 mg g^{-1}), biomass after biosorption contains: Cr (11.0 mg g^{-1}), Ca (10.3 mg g^{-1}), Zn (5.47 mg g^{-1}), Na (4.40 mg g^{-1}), Fe (2.8 mg g^{-1}), Mn (2.75 mg g^{-1}), Cu (1.12 mg g^{-1}), living biomass after bioaccumulation: K (26.6 mg g^{-1}), Ca (14.0 mg g^{-1}), Cr (11.9 mg g^{-1}), Na (4.97 mg g^{-1}), Zn (3.97 mg g^{-1}), Mg (2.62 mg g^{-1}), Mn (1.96 mg g^{-1}), Cu (1.47 mg g^{-1}). Totally, native biomass contains 44.2 mg g^{-1} minerals, after biosorption: 43.6 mg g^{-1} and after bioaccumulation 69.4 mg g^{-1} . This showed that probably the only mechanism of metal ions binding in the process of

biosorption was ion-exchange. In bioaccumulation, additional mechanism must occur, including the transport of metal ions into the cellular interior. It was observed that the minerals present in the native biomass (that were exchanged in biosorption process: K, Ca, Na) were not released in the process of bioaccumulation. This could explain why the total content of minerals in the biomass after the process of bioaccumulation with growth was the highest. Also, the multielemental analysis of the solution before biosorption and after the process (Fig. 7) showed that the following ions were bound by the biomass: Cr (18.2 mg g^{-1}), Zn (15.6 mg g^{-1}), Mn (10.7 mg g^{-1}), Cu (3.11 mg g^{-1}), Na, Tl, Sb, Ag and the following were released by the cells: K (30.8 mg g^{-1}), Ca (19.1 mg g^{-1}), Mg (7.52 mg g^{-1}), Sr, Al, Ti, Mo, Co. Totally 49.2 mg g^{-1} ions were bound by the biomass and 57.7 mg g^{-1} ions were released.

4. Conclusions

In the present study, it was found that the biomass of an aquatic plant *L. minor* has the potential to be used as mineral feed additive in livestock nutrition. The investigation could have been performed thanks to the powerful tool of simultaneous multielemental analysis provided by ICP-OES technique. The results showed that the efficiency of so-called ‘biobinding’ depended on the process that was employed (either biosorption or bioaccumulation by non-living or living cells) and it was found that the mostly efficient process of binding was biosorption, afterwards bioaccumulation by growing cells. The experiments were performed in a single- and multi-metal system. It was found that the maximum sorption capacity in a single-ion system (Cr(III) ions) was the same as in multi-ion system (simultaneous presence of Cr(III), Mn(II), Zn(II), Cu(II) ions). The experiments showed that the biomass possessed different efficiency in cations binding that was (in decreasing order): Cr, Zn, Mn, Cu. Totally up to 7.45% of minerals can be bound by the biomass in biosorption process. The efficiency of ‘biobinding’ process was described with Langmuir equation that enabled evaluation of maximum biosorption capacities and affinities of sorbent to sorbate. Also, the mechanism of ‘biobinding’ was investigated. The experimental results showed that ion-exchange was the only mechanism of cations binding in the process of biosorption. Microelements were exchanged mainly with K, Ca and Mg ions. The total content of minerals in the native and biosorption biomass was very similar. In bioaccumulation process, additional mechanisms occurred. The ions were transported into the cellular interior. The minerals were bound by the biomass, but K, Ca and Mg ions were not released by the biomass.

In the present study, chemical experiments were performed to investigate the efficiency of ‘biobinding’ process, to select the process in which metal ions would be bound to the biomass with the highest efficiency. However, it is necessary to perform further studies in order to investigate in which form microelements would be the mostly bioavailable to livestock.

In the paper, the impact was stressed on the applicative part of the analytical method and the interpretation of the results obtained with this technique. ICP-OES Varian Vista-MPX is a relatively simple and reliable instrument, the cost of analysis

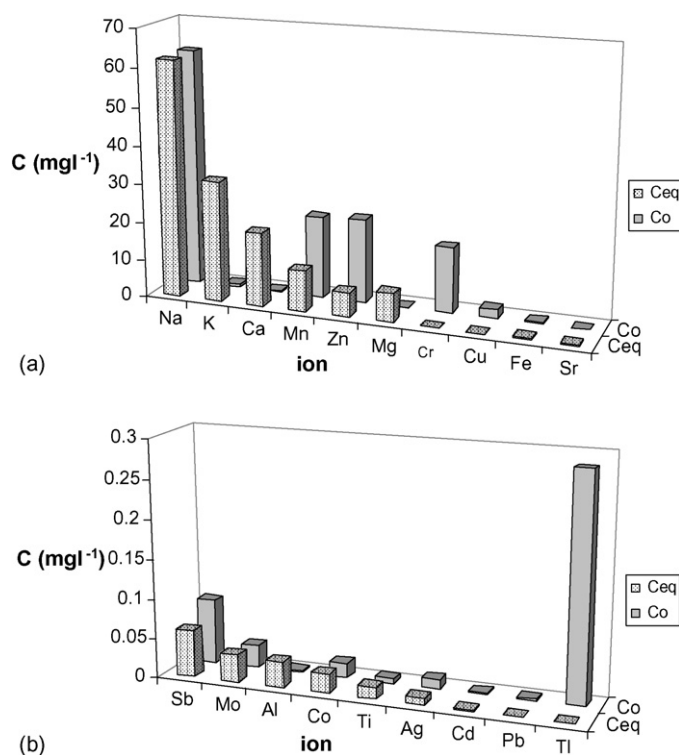


Fig. 7. The elemental composition of solution before biosorption and after the process.

is low when comparing with other ICP techniques and therefore can find an application as a powerful tool in the elaboration of technologies of the production of biological mineral supplements in both, research and development studies, as well as in the routine quality control in an industrial process.

Acknowledgement

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Chemical analysis of polycyclic aromatic hydrocarbons by surface-enhanced Raman spectroscopy

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Abstract

The use of surface-enhanced Raman spectroscopy (SERS) for trace determination of polycyclic aromatic hydrocarbons (PAHs) is described. This paper focuses on the development of SERS-active substrates that are specific for the characterization and spectroscopic study of PAHs. The SERS-active substrates are based on thin gold films evaporated on a glass surface previously treated with a mercaptoalkylsilane. SERS of PAHs was investigated over uncoated gold island films and over such films coated with a self-assembled monolayer (SAM) of 1-propanethiol. Adsorption of PAHs on a plain SERS-active Au-film led to a surface-induced decomposition of PAHs, due to catalytic properties of nanostructured gold. Thus, the functionalization of the SERS-active substrates by means of SAM was done aiming at a specific chemical interaction toward PAHs. Thus, in addition to preventing decomposition of the PAHs, the coating also concentrates the hydrophobic PAHs close enough to the SERS-active interface. Results show that high sensitivity, SERS-active nanostructured gold substrates that show selectivity towards PAHs were obtained, with the following properties: strong intensification of the Raman signal, reproducibility, and stability over time. The employed methodology enables the observation of excellent Raman spectra of PAHs in aqueous environment at ppm levels.

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Keywords: Surface-enhanced Raman scattering; SERS; Polycyclic aromatic hydrocarbons; Pyrene; Benzo(a)pyrene; PAHs

1. Introduction

The applications of vibrational spectroscopic techniques for investigating different chemical species of environmental relevance have grown significantly in the last years. There are many reasons for this: the availability of more sensitive and less expensive instruments; the development of high-sensitivity enhanced spectroscopic techniques, capable of detecting even a single individual molecule; the needs associated to the study of problems with increasing complexity, that require the combination of different techniques. Raman spectroscopy has a combination of characteristics that make it convenient to the study of environmentally relevant samples: it requires little sample preparation, allows for the study of aqueous solutions, allows for the identification and quantification of chemical species, and has high specificity and sensitivity.

Modern spectroscopic techniques include a powerful set of enhanced spectroscopic techniques, which allow the observa-

tion of molecules adsorbed on surfaces (normally metallic) in concentrations lower than a monolayer. The enhancement of the Raman signal of molecules by their interaction with nanostructured metallic surfaces is the basis of the surface-enhanced Raman spectroscopy (SERS) [1]. It is generally accepted that the surface enhancement results from two major contributions: the electromagnetic mechanism is related to a plasmonic excitation of the metallic substrate, and does not depend on the nature of the adsorbed species; the chemical mechanism, on the other hand, relies on an electronic interaction between adsorbed species and metallic surface, and is thus less general. The SERS effect is recognized nowadays as an important analytical surface technique for the characterization of adsorbates and of the chemical transformations that occur on metallic surfaces. It allows the use of Raman spectroscopy in a much broader way, and opened the possibility for the application of vibrational spectroscopy for the analysis at the pico and eventually femtogram regime. Historically, the analytical applications of Raman spectroscopy were limited by its low sensitivity, as compared to fluorescence techniques, for instance. Recent developments had a particularly important impact, in the sense of changing this tendency: huge enhancement factors were observed in specially prepared

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SERS active surfaces, making it possible to observe the Raman spectrum of even a single molecule [2–4]. So, the technique is already sensitive enough to compete with fluorescence-based techniques. However, considering the wealth of information contained in a vibrational spectrum as compared to a fluorescence spectrum, it is expected that the SERS technique becomes increasingly important in the area of chemical analysis. Thus, from the point of view of chemical analysis, the most remarkable aspects of SERS are its tremendous enhancement of analytical response and the large amount of available structural information. However, SERS has not yet been established as a routine analytical tool for the detection of organic compounds owing to some limitations, such as reproducibility and the need to prepare specific substrates to each desired compound, or class of compounds. In other words, there is no universal enhancing substrate. In the case of polycyclic aromatic hydrocarbons (PAHs), which are the object of study of this paper, there are additional problems. First, although coupling of π -electrons of PAH with the metal substrate should occur, this class of compounds does not present a specific functional group (such as sulfur or nitrogen) that could act as a strong anchor to the surface of the substrate that would intensify the SERS effect via a significant chemical mechanism. Moreover, in the presence of the substrates traditionally used in SERS analysis, the PAHs were observed to decompose. So, as in other cases, substrates have to be tailored for this specific class of substances.

In order to use SERS effect in practical applications, studies need to be conducted to develop long-lasting, substrates that are low-cost, and reproducibly prepared. There are a great variety of SERS active substrates. Researchers have selected architectures for substrates accordingly to each experimental need. Though the choice of the working metal, use of protective layers, the SERS-active substrate may be developed in order to selectively adsorb selected compounds with similar chemical structures. An important aspect on the practical use of a metallic nanostructure in chemical analysis is its fabrication in a reproducible manner. Many interesting applications of SERS exist in the environmental area [5–8]. To determine compound in ppb concentrations in complex environmental samples, the sensors must have enough sensitivity to differentiate compounds and identify specific functional groups. In many situations, the combination of different techniques is necessary for identification and quantification. The characteristics of Raman spectroscopy allow it to be used for identifications and quantitative analysis without the need of previous chromatographic separation.

It is notable that relatively few studies reporting the SERS study of PAHs [9–11] which are known hazardous pollutants [12]. In this work, SERS effect of PAHs and their nitro derivatives is investigated. PAHs are composed of fused aromatic rings, and are products of incomplete combustion in relatively low temperatures. They are present in the atmosphere as volatile and semivolatile pollutants, as well as particulate matter. These compounds are object of special interest and concern due to their mutagenic properties and carcinogenic effects. PAHs biochemical persistence arises from dense clouds of π -electrons on both sides of the aromatic structures, making them resistant to nucleophilic attack [13]. The study of their properties may clar-

ify their behavior in the environment, and thus help to a better assessment of the risks involved.

The composition and the complexity of the PAHs mixtures depend on the emission sources. Mixtures are generally very complexes, with a large variety of PAHs in different levels of concentrations [12]. More than 150 PAHs, with structures having between two and seven rings, were identified in smoke. Although PAHs present high molecular fluorescence, the use of conventional fluorescence spectroscopy is limited by the broadening of emission bands caused by the multiplicity of vibrational and rotational states. In addition, this technique has low specificity, and does not distinguish similar molecules. Raman spectroscopy is a vibrational spectroscopic method that has the inherent ability to distinguish between molecules with great similarity [14]. This can be very useful in the PAHs analysis. Another characteristic makes SERS very convenient to be used in this field: it is normally accompanied by fluorescence suppression, characteristic of many PAHs, for instance.

2. Experimental

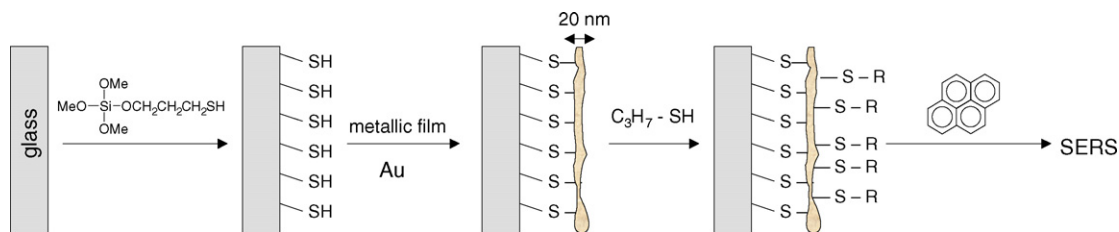
2.1. Materials and instrumentation

UV–vis–NIR spectra were obtained with a Shimadzu UVPC-3101 scanning spectrophotometer. Raman spectra were acquired on a Renishaw Raman System 3000 equipped with a CCD detector and coupled to an Olympus microscope (BTH2) that allows a rapid accumulation of Raman spectra with a spatial resolution of about 1 μm (micro-Raman technique). The laser beam was focused on the sample by a $\times 50$ lens. Laser power was always kept below 0.7 mW at the sample. The experiments were performed at ambient conditions using a back-scattering geometry. The samples were irradiated with the 632.8 nm line of a He–Ne laser (Spectra Physics). FT-Raman spectra of solid samples were recorded at room temperature in a RFS 100 FT-Raman Bruker spectrometer using the 1064.0 nm radiation from a Nd:YAG laser. A Digital Instruments Scanning Probe Microscope model NanoScope IIIA was used for obtaining the AFM image. The AFM was operated in the intermittent contact mode, and measurements were performed under ambient conditions. The image was manipulated by WS $\times$ M free software and are presented without further corrections.

Chemicals and solvents were supplied by Aldrich and Merck. All chemicals were analytical grade and were used as received without any further purification. The organic solvents were spectroscopic grade. The working solutions were prepared using analytical grade chemicals and deionized water. The gold targets used for the preparation of thin films, were 99.99% pure polycrystalline materials.

2.2. SERS substrate preparation

In order to improve the substrate stability, the thin gold film was evaporated onto immobilized alkoxysilane having pendant a functional group with high affinity for gold. The surface derivatization was accomplished by the following procedure: [15] clean glass substrates were immersed in methano-



Scheme 1. Schematic representation of SERS active substrate preparation.

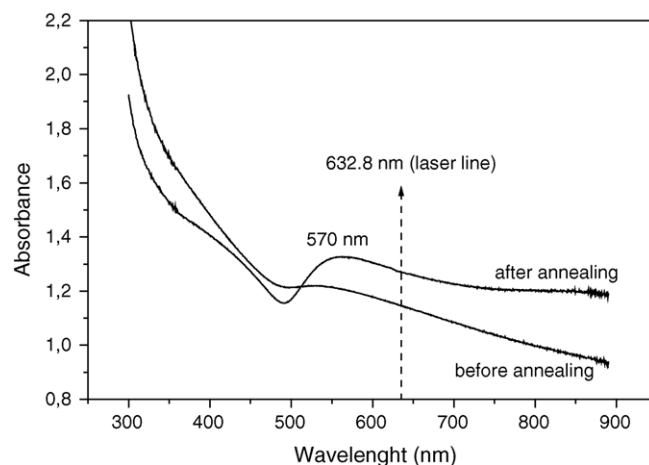
lic solution (25 vol%) of (3-mercaptopropyl)trimethoxysilane (MPTMS) for 24 h. The substrates were then removed and rinsed with methanol. Twenty nanometer (mass thickness) of gold was evaporated onto the silanized substrate. Gold thin films were grown by argon plasma sputtering using an Edwards High Vacuum system, model E096-02-000. Metal deposition occurred at a pressure of 2×10^{-1} Torr, at a rate of 0.04 nm s^{-1} . The average mass thickness of the film was measured with a quartz crystal microbalance from Edwards High Vacuum, model FTM6, and confirmed by AFM measurements. The gold thin films were annealed to 160°C under nitrogen atmosphere by 1 h to the formation of gold island structures. Coating of the gold layer was done by immersion of the substrate in a 1-propanethiol methanolic solution (10 vol%) for 24 h (see Scheme 1). The SERS-active substrates were immersed in an aqueous solution of the PAH for 30 min. The substrates were removed, rinsed profusely with water and dried over nitrogen. The SER spectra were then obtained.

3. Results and discussion

3.1. Substrate characterization

Fig. 1 shows the extinction spectrum of the SERS-active gold film deposited over glass. This spectrum is characterized by a broad absorption band centered at ca. 570 nm that corresponds to the plasmon excitation of the gold particles shown in the AFM image in Fig. 2.

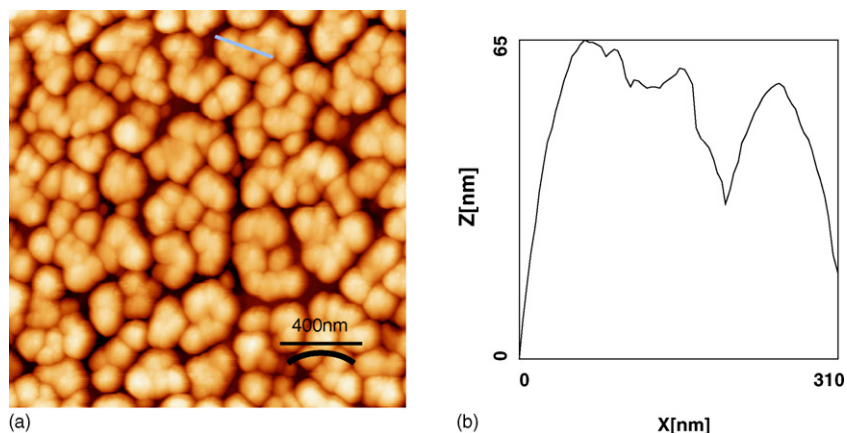
The basic electromagnetic enhancement of the analytical signal in SERS is dependent upon the optical properties of the metal substrate, the shape of the metal particles, the dimensions of the

Fig. 1. Extinction spectra of SERS-active 10 nm gold films deposited over silanized glass before and after annealing procedure at 160°C for 1 h.

metal roughness or metal particles and the packing of the metal particles. The image profile shows that the metal islands have (60 ± 20) nm in height and lateral dimension. Such gold island films were optimized to obtain the best SERS enhancement.

3.2. SERS of PAHs over gold island films

Fig. 3 shows SER spectra of gold substrates immersed in aqueous pyrene and benzo(a)pyrene solutions. In both cases, the most prominent band is observed at 2133 cm^{-1} . This vibrational region is typical of $\text{C}\equiv\text{O}$ stretching mode, indicating the presence of carbon monoxide. Thus, it can be concluded that the adsorption of the PAH molecules on the nanostructures gold

Fig. 2. (a) AFM image of gold film annealed at 160°C for 1 h; (b) AFM height profile of the gold nanoparticles along the line indicated in the image.

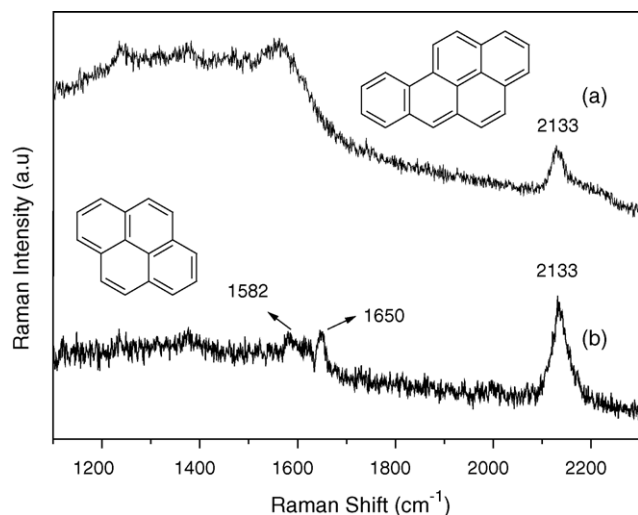


Fig. 3. SER spectra of (a) benzo(a)pyrene and (b) pyrene adsorbed over gold surfaces ($\lambda_{\text{exc.}} = 632.8 \text{ nm}$).

layer significantly enhances their chemical reactivity towards oxidation processes. This precludes the study of PAHs through SER spectroscopy on thin films. Infact such catalytic activity of gold islands films has already been reported for other chemical species [16]. It is important to mention that such behavior is typical of every PAH studied. In order to adequate the SERS active substrate to the study of PAHs, it is necessary to chemically modify the gold surface. The chosen approach consisted on the chemical modification through a self-assembled monolayers (SAM), so that the PAH would adsorb over the SAM and not directly over the gold nanoparticles. Molecules with thiol groups present a high affinity for gold or silver, and promptly react with these surfaces originating SAMs [17]. It has been verified that SERS-active substrates modified with different lengths alkanethiols are very efficient in the detection of chlorated hydrocarbons and aromatic compounds [18]. The chosen compound was 1-propanethiol [19]. In addition to protecting

the PAH from direct contact to the metal, the surface modification makes the surface hydrophobic, favoring the adsorption of non-polar compounds.

Fig. 4 shows the SER spectra of three different PAHs adsorbed on chemically modified gold substrate. For comparison purposes, normal Raman spectra of the solid-state samples at two different laser excitation wavelengths is also presented. The metallic surfaces have the positive effect of quenching PAH fluorescence that interferes with the normal Raman spectrum when obtained with visible exciting radiation. The normal Raman and SER spectra of fluoranthene are very similar. Changes in the relative intensities cannot be observed and small frequency shifts, around $2\text{--}3 \text{ cm}^{-1}$, indicates a weak interaction with the gold surface. In the case of benzo(a)pyrene, the bands observed in the normal FT-Raman spectrum at 1237, 1387, 1584 and 1634 cm^{-1} undergo wavenumber shifts in the SER spectrum, being observed at 1233, 1385, 1581 and 1631 cm^{-1} , respectively. Changes in the relative intensities also occur: the bands at 333, 1621, and 1631 cm^{-1} are enhanced while the bands at 1233, 1385 and 1581 cm^{-1} show lower relative intensity in SERS spectrum. In the same way, the SER spectrum of pyrene undergo small wavenumber shifts (ca. 4 cm^{-1}) as compared to the normal Raman spectrum. Changes in relative intensity can also be identified. Such behavior indicates weak interaction between the PAH and the surface. Nevertheless, the differences observed can be associated with changes in the electronic structure of the adsorbed molecules due to the proximity with the metallic surface.

The influence of the nitro group on the SERS effect of PAHs was also investigated. The normal Raman spectra of anthracene and 9-nitroanthracene at two different exciting radiation and their respective SER spectra are shown in Fig. 5. As the others PAHs, the SER spectrum of anthracene shows small differences as compared to the solid state, indicating weak interaction with the surface. On the other hand, the SER spectrum of 9-nitroanthracene presents larger frequency shifts and changes in relative intensities (the bands at 402, 775, 1264 and

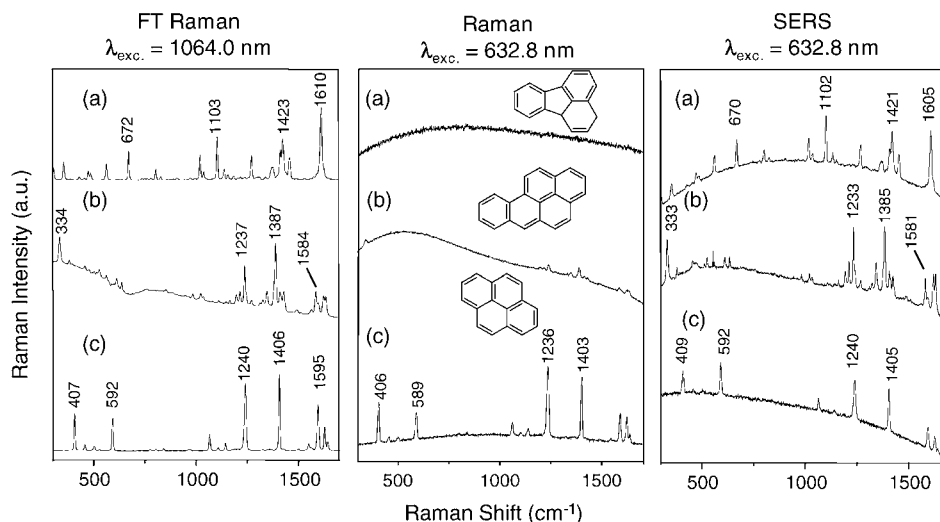


Fig. 4. Solid-state Raman and SER spectra over 1-propanethiol coated gold surface of (a) fluoranthene; (b) benzo(a)pyrene; and (c) pyrene at the indicated laser excitation wavelengths.

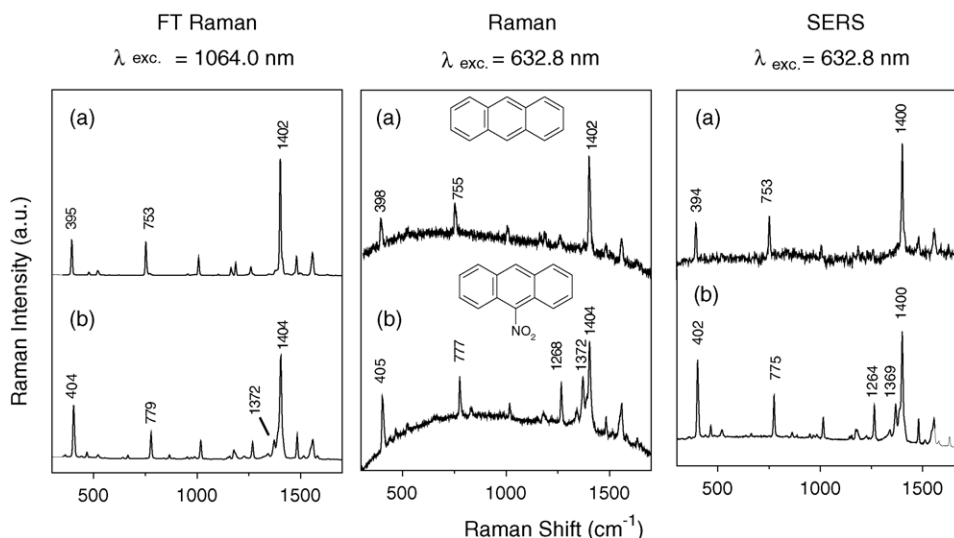


Fig. 5. Solid-state Raman and SER spectra over 1-propanethiol coated gold surface of (a) anthracene and (b) 9-nitroanthracene at the indicated laser excitation wavelengths.

1631 cm^{-1} are enhanced in SER spectrum). According to the surface selection rule [20] an increase in the relative intensities of the bands related to vibrational modes that give rise to induced dipole moments perpendicular to the surface are expected. For 9-nitroanthracene, the bands that undergo frequency shift and enhancement are assigned to the in-plane normal modes, suggesting that this molecule is adsorbed in a vertical geometry.

The bands at 1372 and 1515 cm^{-1} , observed in the spectrum of the solid, can be assigned to the NO_2 symmetric and asymmetric stretching modes, respectively. These bands shift to 1369 and 1512 cm^{-1} , suggesting that the nitro group is a possible adsorption site of the molecule. Although the surface was covered with a SAM, a chemical interaction of the nitro groups with the gold surface could be observed. Fig. 6 highlights the weaker features in Raman and SER spectra of anthracene and 9-nitroanthracene. The bands at 667 and 866 cm^{-1} , observed in the spectra of the 9-nitroanthracene are absent in the spectrum of the anthracene, can be assigned to the out of plane normal modes [21]. Such bands are less intense in the SER spectrum (Fig. 6c), corroborating the hypothesis of vertical adsorption.

3.3. The chemical selectivity of the coated substrate

In addition to prevent the degradation of the PAH, the surface modification enhances the chemical affinity towards the compounds of interest, and makes them more selective. This aspect is illustrated in Fig. 7, that shows the spectra of SERS substrate with and without the 1-propanethiol SAM exposed to crystal violet (CV), a polar molecule. It is worth mentioning that CV is an organic dye that gives rise to very strong SERS signal [22]. While the SERS signal of CV is clearly seen in the non-protected substrate, the chemically modified surface, treated in the same conditions, does not show the Raman signal of this dye molecule. This result demonstrates the importance of the

chemical affinity between the adsorbed monolayer and the target compound: while high affinity may be expected between 1-propanethiol and hydrophobic non-polar PAH molecules, the CV, a cationic hydrophilic molecule does not show affinity for

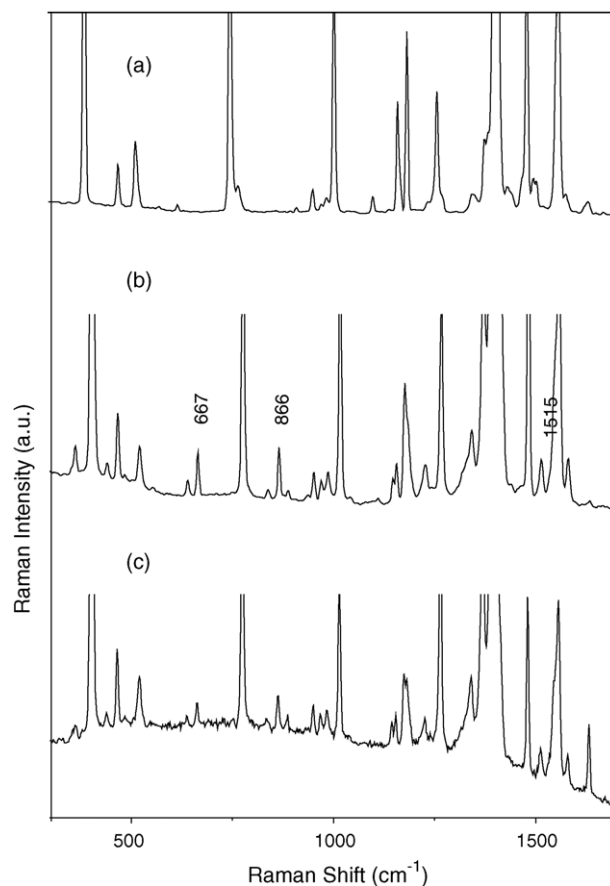


Fig. 6. Raman spectra of (a) anthracene; (b) 9-nitroanthracene ($\lambda_{\text{exc.}} = 1064\text{ nm}$, solid state); and (c) SER spectrum of 9-nitroanthracene over 1-propanethiol coated gold surface ($\lambda_{\text{exc.}} = 632.8\text{ nm}$).

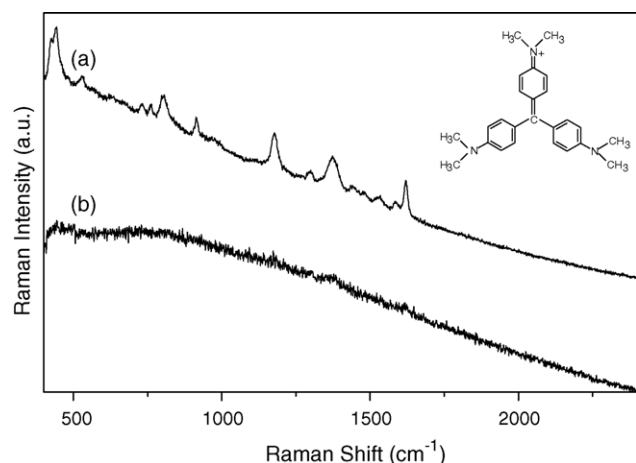


Fig. 7. SERS spectra of crystal violet over gold surface (a) without and (b) with 1-propanethiol coating ($\lambda_{\text{exc.}} = 632.8 \text{ nm}$).

the 1-propanethiol layer. So, the SAM works as a solid extraction phase associated with the SERS platform. This approach can be used to enhance the sensitivity and selectivity of SERS technique and avoid interference from other species in solution for PAH analysis through SERS technique.

4. Conclusions

In this work, we employed metal nanomaterials in the development of sensitive chemical methods of detection and identification of PAHs through Raman spectroscopy. Study of PAHs over traditionally prepared Au-films was not possible because of surface-induced decomposition of PAHs due to catalytic properties of nanostructures gold. Decomposition of the adsorbate is avoided by the use of 1-propanethiol SAM. Chemical modification was very useful for controlling the physical and chemical properties of SERS active substrate allowing the choice of a more or less hydrophobic SERS substrate in function of the properties of the investigated adsorbate. Adsorption of the PAHs onto self-assembled 1-propanethiol surface is a very favorable process. Such strategy led to the interaction of the PAHs with the surface by weak Van der Waals forces. Small differences in the normal Raman and SER spectra of such compounds indicate that the metallic surface led to their physisorption with π redistribution in the electronic structure of the adsorbed molecules. The affinity of the nitro group with gold atoms allows chemisorption of 9-nitroanthracene although a 1-propanethiol SAM had previously been adsorbed on gold substrate.

The utility of the coated Au film demonstrated as a robust SERS substrate for detection and identification of PAHs and nitro-PAHs. The strategy employed here does not require previous extraction methods (the extraction phase is in the SERS

platform) or separation, does not require the use of organic solvents, is capable of detecting and identify simultaneously many compounds, allowing the analysis of trace concentration aqueous solutions.

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Kinetics of oil saponification by lead salts in ancient preparations of pharmaceutical lead plasters and painting lead mediums

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Abstract

Lead soaps can be found in archaeological cosmetics as well as in oil paintings, as product of interactions of lead salts with oil. In this context, a better understanding of the formation of lead soaps allows a follow-up of the historical evolution of preparation recipes and provides new insights into conservation conditions. First, ancient recipes of both pharmaceutical lead plasters and painting lead mediums, mixtures of oil and lead salts, were reconstructed. The ester saponification by lead salts is determined by the preparation parameters which were quantified by FT-IR spectrometry. In particular, ATR/FT-IR spectrometer was calibrated by the *standard addition method* to quantitatively follow the kinetics of this reaction. The influence of different parameters such as temperature, presence of water and choice of lead salts was assessed: the saponification is clearly accelerated by water and heating. This analysis provides chemical explanations to the historical evolution of cosmetic and painting preparation recipes.

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Keywords: FT-IR; Saponification; Lead soap; Plaster; Oil; Painting

1. Introduction

Reading ancient pharmaceutical recipes, one is amazed by the intensive use of metallic salts, like lead-based, arsenic-based, mercury-based, each of them being considered, nowadays, as potentially toxic. The presence of lead in ancient cosmetics and pharmaceutical products discovered in burials from Ancient Egypt attests the importance of these metal salts in medicine [1,2]. Lead salts were employed a solid powder, or in a mixture with fat. In some ancient cosmetics containing fat and lead salts, lead soaps can be identified [3]. A remaining question is to know how these lead soaps were prepared: could have they been intentionally synthesized more than 30 centuries ago, or are they the result of long-term degradation? Making soap enhances the consistency of fat. Hence, it can justify the need to saponify a part

of fat. In an attempt to understand how and why lead soaps have been synthesized for galenic purposes, we have studied ancient pharmaceutical recipes, mentioning preparation of fat and lead salts, such as lead plasters. These pastes were used very often during the Antiquity and up to the 20th century, to heal up ulcers or to treat burns.

Surprisingly, similar processes involving the hot mixture of oil and lead salts occur in the painting field. The first lead medium is attributed to Antonello da Messina (1430–1479) and was prepared with one part of litharge (PbO) combined by cooking with three to four parts of linseed oil. This process enabled to thicken the oil and get a more ductile paste and appeared as a *radical change* in oil painting history [4]. From this point, the preparation of lead medium evolved, and became a usual process of oil, still employed by some artists today [5,6]. For instance, lead was used as a siccative in at least 102 of the 155 colored grounds from French paintings of the 17th and 18th centuries studied by Duval [7]. Famous painters, such as Leonardo da Vinci, Giorgione, Titian, Tintoretto,

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Rubens used such preparations, with personal adaptations [4].

This work deals with the chemical analysis of fat saponification involved in the preparation of both lead plasters, in pharmacology, and lead mediums, in painting. It aims at identifying experimental parameters that could influence the saponification processes, in two different contexts: first, from an historical point of view, for the study of processing evolutions along centuries and, second, from a conservation point of view, for a better understanding of the formation of lead soaps by interaction of oil with lead salts. It is noteworthy that aggregates of lead soaps, called protrusions, can appear in oil paintings [8–13], and such lumps are puzzling alterations for conservators. Recently, an intense scientific research arose to investigate the apparition and evolution of lead soaps in painting, and also to help conservators in choosing the best treatments with regards to this phenomenon [14]. Lead introduced as pigment is often considered as responsible for such protrusions. Yet, it seems very relevant to shed light on the role of lead introduced as siccative or drier, i.e. mixed with oil, in cold or hot conditions, to accelerate the drying process.

Lots of parameters can influence the saponification kinetics. Here we present, as an example, the dependency on temperature, addition of water or nature of lead salts. Although this study is not exhaustive in terms of controlled parameters, it aims at providing a simple and generic method to follow and quantify the oil saponification. In this context, FT-IR spectroscopy offers an easy and rapid quantification method. Unlike chromatography or X-ray diffraction-based methods, this analytical technique enables simultaneous analyses of both organic and mineral phases. Furthermore, it is rather handy since it does not require any chemical preparation. FT-IR spectroscopy has already been successfully used to reveal the presence of lead soaps in ancient cosmetics [3] and remains a common way to identify lead soaps in paintings [8–10,15]. Here this technique is applied here to follow the transformation of fat during the preparation of reconstructed recipes. Methodological processes and analytical techniques are developed in the next section. Data and interpretations on ancient lead plasters and lead mediums reconstruction are given later. In the conclusion, outlook on the study of cultural heritage related objects is tackled.

2. Experimental

2.1. Operating protocol reproducing ancient pharmaceutical recipes

One of the most ancient text describing the preparation of a lead plaster is a recipe written on papyrus and dating from 1500 B.C. It originates from a collection of pharmaceutical texts related to the treatment of skin and eye diseases. In this text, it can be understood: “*make-up to be prepared during summer, winter and flooding season: mineral-mesdemet*. It will be ground in goose-terep fat, in the morning, without permitting it to drop on fire. Make-up for the night” [16]. The significance of the word *mesdemet* is the natural mineral galena (PbS) or a mixture of lead salts used for cosmetic purposes

[17]. This recipe is interesting not only because it is one of the oldest, but also because it mentions two important features: the choice of lead salts and the use of fire. We have searched how these recipes, made of fat and lead salts, have evolved through History from the Antiquity up to the 20th century. Despite some modifications in the operating methods, the main acts are commonly reported [18]. The recipe consists in mixing olive oil, water and lead oxide, in the mass concentration ratio 2–2–1, and heating it to get the consistency of a plaster [19]. Gradually, and more precisely from the 18th century, these starting steps were considered as a basic recipe, called by the pharmacists “simple plaster”. Then various ingredients were added later on to this excipient giving rise to different plasters. Reconstructions of lead plasters were performed by following and adapting this most common and famous recipe.

To synthesize the plasters, we have used triolein (SIGMA, St. Louis, MO, USA) as a model of olive oil. It is a well known triester made by the association of 1 molecule of glycerol with 3 molecules of oleic acid. To evaluate the effect of lead salts, we have studied, for comparison, the saponification kinetics in the presence of PbO, PbCO₃, Pb₃O₄ (VWR, Fontenay S/Bois, France), PbS (natural, from USA), and two synthetic lead salts, identified in some ancient cosmetics from Egypt: laurionite (PbOHCl) and phosgenite (Pb₂CO₃Cl₂). These salts were prepared in aqueous solutions, following ancient recipes reported by classical authors [20].

For each experimental condition, 2 parts of triolein and 1 part of a lead salt are introduced into a beaker, with or without water, mixed with a propeller (60 rpm) and heated up at a controlled temperature. At regular time intervals, a compound collection is done, and three infrared spectra of this sample are acquired for homogeneity and reproducibility purposes. The methods used to quantify the soap amount are detailed below.

2.2. Preparation of a painting lead medium

Lead medium reconstruction was done according to a recipe given by various authors, among them J. Maroger, who attributes the discovery of this technique to Leonardo da Vinci [4], and T. Turquet de Mayerne. The later gave a detailed description of the process [21]. The preparation of painting lead medium differs from that of pharmaceutical lead plaster by the choice of oil – non-siccative olive oil in pharmacy versus siccative linseed or walnut oil in painting and by the proportion lead salt/oil – around 1/2 in pharmacy versus around 1/3 to 1/4 in painting. The introduction of water in lead medium is not systematic, and indeed, it is rather marginal. Yet, it presents some technical advantages in terms of consistency and color [4]. This is the reason why we chose to incorporate water in our protocol. The experimental reconstruction was based on the *Formula for the second lead medium, probable technique of Leonardo da Vinci*, reported by J. Maroger [4], as well as on the Folio 28 of T. Turquet de Mayerne’s treatise [pp. 43–44 of 21b]. We used a home-made walnut oil, water and PbO, in the mass proportions 4–4–1. PbO was first mixed with oil, for 40 min at 60 °C. Then, water was added and temperature

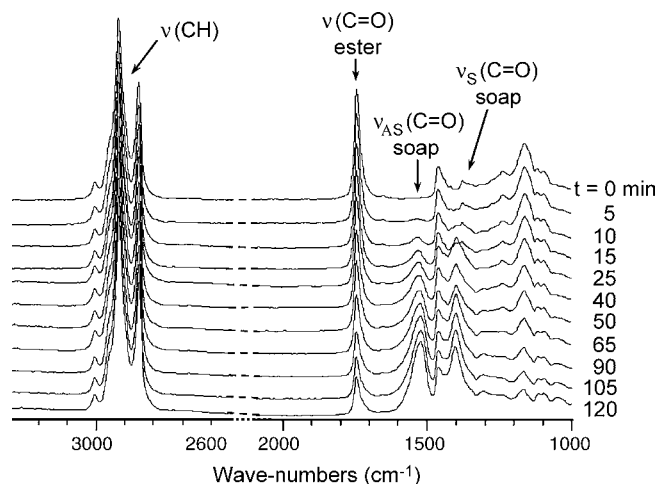


Fig. 1. Evolution of the FT-IR spectra acquired during the saponification of triolein by PbO, with water, at 100 °C, from 3300 to 2600 cm⁻¹ and from 2000 to 1000 cm⁻¹ (50 scans, resolution 8 cm⁻¹).

increased up to 100 °C. The reaction was followed during 3 h.

2.3. ATR/FT-IR measurements

ATR/FT-IR spectroscopy was chosen because of its appropriateness with the paste analysis, the adhesive properties of the samples precluding the use of KBr pellets. Besides, it does not require any chemical preparation. However, the absorbance value does not have a quantitative significance, especially during ATR measurement, where intimate contact between the ATR crystal and the sample is required, which is hardly achieved in a reproducible manner. Accordingly, an indirect calibration is required, the ultimate goal being to determine the fraction of saponified ester in a mixture of ester and soap.

The spectrometer used is an Impact 420 (Nicolet), with an ATR accessory in diamond. Acquisitions were performed at room temperature. Each spectrum results in an accumulation of 50 scans, with a spectral resolution of 8 cm⁻¹. For each sample, a minimum of three spectra was recorded, in order to estimate the sample's homogeneity and measure's reproducibility.

Fig. 1 illustrates the time evolution of the spectra during the preparation of a lead plaster. The intensity of the C=O ester stretching band (ν(CO)), at ~1745 cm⁻¹, decreases, whereas the two stretching bands of lead soap – asymmetric (ν_{AS}(CO)) at ~1520 cm⁻¹ and symmetric (ν_S(CO)) at ~1400 cm⁻¹ – show up and grow. It is not straightforward to quantify this change, without a detailed knowledge of the dynamic dipole moment strength of the respective vibrational motions. To do so, the so called *standard addition approach* was used.

2.4. Standard addition calibration

This quantification was performed on both triolein and walnut oil. First, known masses of oils (m_0) and lead oxide have been added up in beakers, then mixed and annealed at 140 °C. It

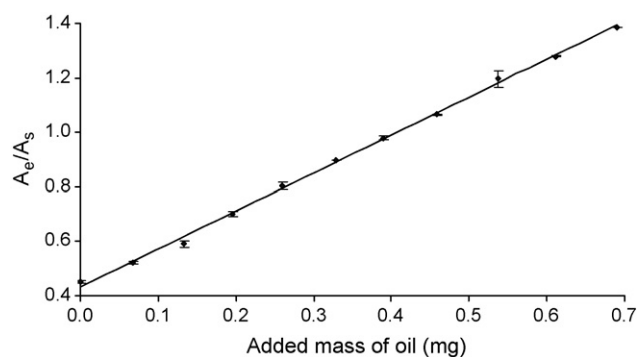


Fig. 2. Calibration by the *standard addition method*: evolution of the ratio area ester/soap vs. addition of walnut oil.

resulted in the synthesis of an unknown fraction of soap, the original esters being either unsaponified (m_E) or saponified (m_S).¹ Subsequently, infrared spectra have been recorded on this initial mixture. Second, a known mass of pure triolein ($m_A^{(1)}$) has been added to the above prepared compounds. It is worth mentioning that the reaction of saponification is stopped at room temperature. Hence, the additional triolein does not affect the soap content. After mixing again, infrared spectra have been collected. Comparison of infrared spectra shows an increase of the relative absorbance of ester-related versus soap-related vibrational bands rises, due to the additional triolein. The lead soap can be considered as an internal reference. Ten successive additions were done, with the acquisition of at least three spectra for each mixture.

After each addition of triolein and following the Beer–Lambert law, the increase of the relative absorbance can be expressed as:

$$\frac{A_E^{(n)}}{A_S^{(n)}} = k \times \frac{m_E + m_A^{(n)}}{m_S} = k \times \frac{m_E + m_A^{(n)}}{m_0 - m_E}$$

where $m_A^{(n)}$ is the total mass of triolein added after the n th addition, $A_E^{(n)}$ the integrated absorbance of the ν(CO) ester band (in the frequency domain 1759–1728 cm⁻¹) and $A_S^{(n)}$ the ν_{AS}(CO) band of soap (in the frequency domain 1577–1493 cm⁻¹). k is the absorptivity ratio of ester versus soap, independent of the mixture.

The relationship between $A_E^{(n)}/A_S^{(n)}$ and $m_A^{(n)}$ is linear ($y = ax + b$) (Fig. 2), of slope $a = k/(m_0 - m_E)$ and origin value $b = k \times (m_E/(m_0 - m_E))$. With the spectral regions considered, a fit of the experimental values gives $a = 3.26 \pm 0.02$ g⁻¹ and $b = 0.25 \pm 0.06$ ($R^2 = 0.9995$) for triolein, $a = 1.39 \pm 0.02$ g⁻¹ and $b = 0.428 \pm 0.007$ ($R^2 = 0.9984$) for walnut oil.

Determination of a and b allows m_E and then k to be calculated. The results give $k = 0.84 \pm 0.03$ for triolein and 0.47 ± 0.03 for walnut oil. The difference between the two oils

¹ It must be outlined that m_S is the mass of saponified esters and not the mass of lead soap. The latter could be calculated by taking into account the molecular masses of the different compounds. All the following reasoning is led with the mass of esters as reference. According to the law of conservation of matter, $m_0 = m_E + m_S$.

may be due to different chemical composition. The soap percentage, τ_S , in all the mixtures ester–soap, can be derived from the following equation:

$$\tau_S = 100 \times \frac{m_S}{m_S + m_E} = \frac{100}{1 + m_E/m_S} = \frac{100}{1 + A_E/k \times A_S} \quad (1)$$

2.5. Calibration in the presence of a FT-IR signal interfering with the carboxylate band

It is of great interest to study the influence of the lead salts on the saponification rate, since various lead salts (oxide, carbonate, sulphide, ...) are reported in ancient recipes or have been identified in archaeological cosmetics and in oil paintings [1–3,20]. Some lead salts, like galena (PbS) or lead oxide (PbO) are totally transparent in the 4000–700 cm^{-1} domain. This is different for phogesnite ($\text{Pb}_2\text{CO}_3\text{Cl}_2$), which exhibits a strong absorption band around 1535 cm^{-1} [3]. This band overlaps with that of the $\nu_{\text{AS}}(\text{C}=\text{O})$ band of the soap, making the determination of the lead soap content ambiguous (Fig. 1S). To get rid of this band overlapping effect, a specific calibration method was optimized. It avoided measuring directly the absorbance of the $\nu_{\text{AS}}(\text{CO})$ band of soap and rather considered the following spectral range: $\nu(\text{CO})$ band of ester (A_E , area under the peak extending from 1759 to 1728 cm^{-1}) and the $\nu(\text{CH})$ band of the alkyl chain (A_{al} , area from 2950 to 2880 cm^{-1}). The absorptivity of the alkyl chain depends upon the nature of the head group, either ester or soap [22]. It is the reason why alkyl chain signal cannot be considered as a direct internal reference [23]. Conversely, assuming a linear law, A_{al} , the area of $\nu(\text{CH})$ band of the alkyl chain can be expressed as:

$$A_{\text{al}} = \varepsilon_{\text{al}}^{\text{E}} \times m_E + \varepsilon_{\text{al}}^{\text{S}} \times m_S$$

where $\varepsilon_{\text{al}}^{\text{E}}$ and $\varepsilon_{\text{al}}^{\text{S}}$ are the mass absorptivity of the alkyl band (al), ending with an ester (E) or with a soap (S), and where m_E and m_S are the masses of ester unsaponified and saponified, respectively.

Considering the $\nu(\text{CO})$ band of ester, the area, A_E , is proportional to m_E : $A_E = \varepsilon_E \times m_E$, with ε_E the mass absorptivity of ester groups.

Finally, we define the fraction of ester in percentage, τ_E , as $\tau_E = 100 \times m_E/(m_E + m_S)$.

The combination of these three relations gives:

$$\frac{A_{\text{al}}}{A_E} = \frac{(\varepsilon_{\text{al}}^{\text{E}} - \varepsilon_{\text{al}}^{\text{S}})}{\varepsilon_E} + \frac{\varepsilon_{\text{al}}^{\text{S}}}{\varepsilon_E} \times \frac{100}{\tau_E}$$

The plot A_{al}/A_E versus $1/\tau_E$ has been generated, using 150 spectra acquired on triolein–PbO mixtures. For this plot, τ_E is calculated using Eq. (1). The slope of the linear relationship is 1.630 ± 0.007 , with a zero-abscissa value of 0.0125 ± 0.0003 ($R^2 = 0.997$) (Fig. 2S).

These values were used to calculate the soap amount, τ_S (%), in the presence of phogesnite with triolein, following:

$$\tau_S = 100 - \tau_E = 100 - \frac{1.630}{A_{\text{al}}/A_E - 0.0125} \quad (2)$$

3. Results and discussion

3.1. Saponification kinetics in pharmaceutical lead plasters

The classical preparation of a ‘simple plaster’ consists in mixing the same weight of olive oil and water, and half the lead oxide. In these conditions, lead oxide is added in excess (the stoichiometric proportion PbO/oil would be 0.38). The plaster consistency is obtained after 2 hours. One can notice, in Fig. 3(A), that it does not correspond to a complete saponification. Around 10% of unsaponified ester remains. This fact is important to understand the sample’s consistency. Considering its application on skin, pure oil would not be applicable, since it would flow along the skin. Conversely, pure lead oleate, which is solid at room temperature, would not be soft enough to make a paste. An intermediate saponification rate leads to a mixture of liquid oil and solid soap, which results in optimized mechanical properties for application on skin. The paste is supple enough to be applied on skin and remains stuck in this tissue.

3.1.1. Effect of water

In the Antiquity, the addition of water was not systematic while preparing a plaster. Gradually, people discovered that adding water could permit a control of the temperature below 100 °C, thanks to the bain-marie principle. It also avoids burning fat which occurs when heating at higher temperatures. Fig. 3(A and B) presents two kinetics, performed at 100 °C, with and without water. Clearly, water is not only a thermostat but also a catalyst. It enables to reach an amount of soap of ~90% after 2 h, instead of ~10% in the absence of water. Therefore, it appears essential to add water to oil and lead salts if one wants to make a white plaster in a relatively short-time-scale.

3.1.2. Effect of temperature

Conversely, a dry process can be performed at higher temperatures. Fig. 4 illustrates the saponification kinetics for a PbO–triolein mixture, heated in the absence of water, at different temperatures, from 100 to 180 °C. Saponification rates

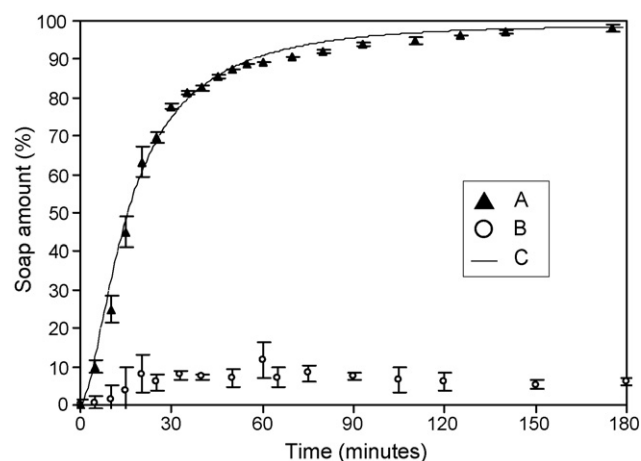


Fig. 3. Kinetics of the saponification of triolein by PbO, at 100 °C; A: with water; B: without water; C: modelization of (A). The error bars indicate the standard deviation with a minimum of three acquisitions per point.

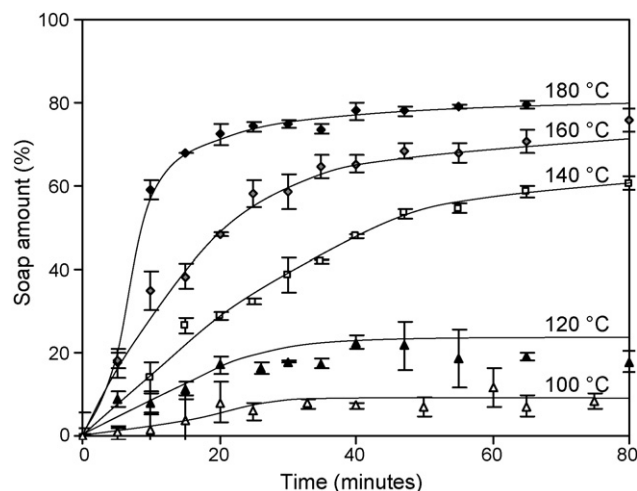


Fig. 4. Kinetics of saponification of triolein by PbO, without water, at temperatures ranging from 100 to 180 °C. The error bars indicate the standard deviation with a minimum of three acquisitions per point.

of 80% can be reached after only 30 min, at 180 °C. In return, such a high heating temperature leads to a beginning of darkening, due to the fat degradation. The color of the products varies from beige to black for a temperature range from 120 to 180 °C. Indeed, in the ancient texts, recipes of lead plasters were divided into two different classes: the burnt and the non-burnt plasters, which corresponds to syntheses made without and with water, respectively. The former will be brown or black, the latter white [24].

3.1.3. Effect of lead salt

The effect of lead salt was evaluated by following the saponification kinetics of triolein in the presence of water, at 100 °C, by different lead salts. The amount of soap was calculated using Eq. (1) for PbO, Pb₃O₄, PbS, PbCO₃ and PbOHCl and Eq. (2) for Pb₂CO₃Cl₂. Results are plotted in Fig. 5. Among the different lead salts studied, only PbO and Pb₃O₄ exhibit a significant saponification. With the other lead salts, no soap is detected after

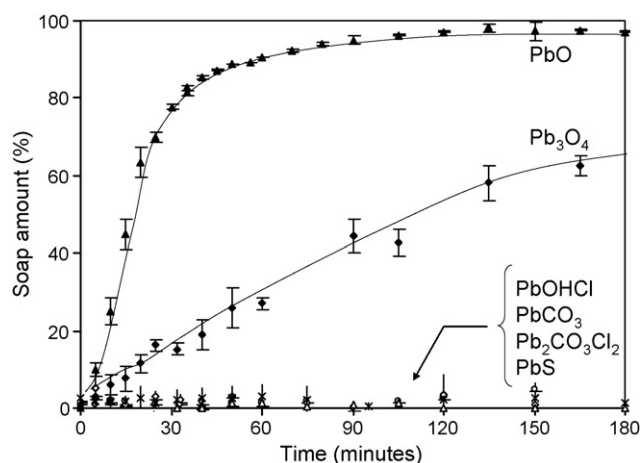


Fig. 5. Kinetics of saponification of triolein, with water, at 100 °C, by different lead salts. The error bars indicate the standard deviation with a minimum of three acquisitions per point.

Table 1

Saponification of triolein by different lead salts, at 100 °C, in presence of water

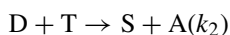
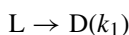
Lead salts	PbO	Pb ₃ O ₄	PbOHCl	PbCO ₃	Pb ₂ CO ₃ Cl ₂	PbS
Soap amount (%)	97 ± 3	63 ± 3	NS	NS	NS	NS
pH	10	8	7	6	6	6

NS = non-significant, <3%.

3 h (Table 1). A survey of about 100 recipes from 15th B.C. to 20th A.D. shows that the lead salt most commonly employed in lead plasters was PbO. This trend may indicate a clear intention to favor oil saponification.

3.1.4. Kinetic modelizations

Fig. 3 shows the influence of water on the chemical reaction mechanism between triolein and PbO. Water clearly acts in the saponification process. Besides, the kinetic behavior, with water, is convex at the beginning of the reaction (from 0 to 20 min). These two observations lead to the hypothesis of a two-step mechanism: first, the slow dissolution of the lead salt (L) in water, second, the reaction between dissolved species (D) and the triolein (T), ending in the formation of soap (S) and alcohol (A).



where k_1 and k_2 are the kinetic constants of the reaction steps. Considering that each molecule of triolein contains three ester groups and each PbO two basic functions, we can calculate the initial molar ratio PbO versus triolein, r_0 :

$$r_0 = \frac{2}{3} \times \frac{m_{\text{PbO}}^0 / M_{\text{PbO}}}{m_{\text{triolein}}^0 / M_{\text{triolein}}}$$

where m^0 are the initial masses of PbO and triolein, and M the molar masses. With the classical mass proportions PbO/triolein 1:2, $r_0 = 1.3$ and so, PbO is in excess. Assuming a reaction order of 1 for each reagent, kinetic equations can be written:

$$\frac{d\tau_s}{dt} = k_2 \times [r_0(1 - \exp(-k_1 t)) - \tau_s] \times (1 - \tau_s)$$

A numeric solution to this equation is plotted in Fig. 3(C). The convex shape at the beginning of the reaction is well fitted. The discrepancy between experiment and fit after 1 h could be explained by a physical limitation of the chemical interaction: the formation of lead increases the viscosity hence gradually restrains interfaces and interactions salt–water–oil.

A second experimental observation reinforces this model. It relies on the comparison between the pH of the aqueous solution at the beginning of the reaction and the saponification rate after 3 h at 100 °C, for the different lead salts (Table 1). Not surprisingly, it appears that the solution must be basic to generate soaps. The different pH must be related to both a different basicity and solubility of the lead salts.

Third, calculations of the partial charges of oxygen in the dry and wet environment give a value of −0.36 for the oxide PbO and −0.46 for the hydroxide Pb(OH)₂ [25]. The higher nucleophilicity

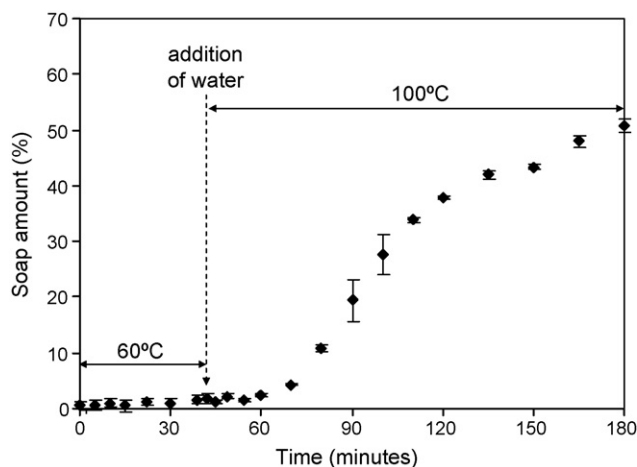


Fig. 6. Kinetics of saponification of walnut oil by PbO. Temperature ramp and water addition are indicated. The error bars indicate the standard deviation with a minimum of three acquisitions per point.

of hydroxides versus oxides is a supplementary explanation for the catalytic action of water in the saponification of triolein.

3.2. Saponification kinetics in painting lead medium

The kinetic saponification of walnut oil by PbO is plotted in Fig. 6. During the first 40 min at 60 °C, no soaps are formed. Conversely, as soon as water is added and temperature is increased, soaps are detected. As for pharmaceutical synthesis, addition of water and increase of heating accelerate the oil saponification.

After 3 h, the soap amount is $51 \pm 2\%$. The main explanation for this low proportion in comparison with the similar preparation with triolein (Fig. 1) involves the mass proportion PbO/oil (1/4 in lead medium versus 1/2 in lead plaster). The intentions of pharmacists and painters are different: the former tend to obtain a thick paste, whereas the latter only require a light thickening of oil. Lead plasters would be too thick to impregnate canvas and lead medium too liquid to be applied on skin. In addition to the appropriate consistency, the color of the product is of prime importance in painting. The addition of water was a crucial evolution to avoid the darkening of oil and get a light paste. The story goes that da Vinci was the first to add water to thermostat the mixture at 100 °C and to avoid the darkening of oil [4].

4. Conclusions

The initial aim of this study was the reproduction of ancient pharmaceutical and painting recipes, made of fat and lead salts. ATR–FT-IR was successfully used as an easy and rapid method to estimate the saponification rate during the different experiments. In some cases, lead soaps can be obtained, which results in oil thickening, giving lead plasters thick and soft enough to remain in the skin, and lead medium more ductile for the applications of canvas. The amount of lead soaps will depend on the duration of preparation, the heating temperature, the presence of water, the choice of lead salts, . . . Saponification kinetics are noticeably accelerated by both adding water and heating the mixture. The usefulness of water addition is clearly demonstrated

since it offers the best trade-off between consistency, color and preparation duration. These chemical observations are in agreement with historical evolutions of recipes.

Although, lead plaster recipes have existed since at least 35 centuries, it is difficult to determine whether the lead soaps found in archaeological cosmetics were willingly prepared by the Egyptians, or if they are resulting from a slow degradation of the original material. As previously established, both the addition of water and preparation temperature have dramatic effects on the saponification kinetics, and we do not know if the archaeological cosmetics were prepared via cool/hot dry/wet processes. Furthermore some uncertainty remains about the ingredients known and used by Egyptians. Lead oxide, for example, seems to be ignored during the Pharaonic period in Egypt [2]. Its use seemed to be linked to the development of the extraction of silver from lead minerals in Laurium, near Athens. These techniques have produced large amounts of slags mainly constituted of lead oxide. Yet, during the Greco-Roman Antiquity, this chemical was often used, particularly in medicine.

Concerning oil painting, lead soaps currently observed in ancient paintings could have been intentionally prepared by the painters, as well as they could have resulted from a long-term interaction of oil with lead salts. From the conservation point of view, it is shown that cold and dry conditions are more adapted to reduce oil saponification by lead salts.

Further experiments are required to better understand reactions that may occur in the long-term and identify the effects of other parameters such as the choice of the oil, the presence of other pigments. Kinetic evolutions over time-scale ranging from a few days to several months at ambient temperature are ongoing. This problem is obviously crucial for the improvements of the conservation techniques of cultural heritage materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2006.03.007.

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Infrared study of chlorophenols and products of their photodegradation

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Abstract

The presents study of the 3-chlorophenol, 2,4-dichlorophenol, 2,3,4,5-tetrachlorophenol, pentachlorophenol and products of their photodegradation using FT-IR spectroscopy and GC/MS. Spectra of pure chlorophenols with the spectra of their solutions after photodegradation were compared. FT-IR spectra of pure chlorophenols investigated in the region of 3700–3000 cm⁻¹ show that in particular cases the position and shape of bands corresponding to stretching vibration of hydroxyl groups are different. In all cases, the differences between spectra of pure chlorophenols and irradiated solutions were observed. It was confirmed that different distribution of types of hydrogen bonds appearing in particular chlorophenols has strong influence on the process of irradiation reaction and final products.

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Keywords: Photodegradation; Chlorophenols; FT-IR; Hydrogen bond; GC/MS

1. Introduction

Chlorophenols are synthetic organic compounds, obtained on large, industrial and commercial scales by chlorinating of phenol or hydrolysing of chlorobenzenes. Chlorophenols (CP) also form as intermediate products at some stages of 2,3-dichlorophenoxyacetate acid production. They may also be formed as by-products during disinfection of drinking water by chlorinating, production of paper, coking process, or distillation of wood [1–4].

In the natural environment, chlorophenols decay—they are biodegraded, degraded in photochemical reactions, and physically transformed by evaporating or adsorbing [5,6]. These processes are often accompanied by oxidation and hydrolysis. In the aquatic environment, chlorophenols exist as dissociated or non-dissociated. The forms of chlorophenols depend on pH of the environment, as well as on physical and chemical properties of the particular compounds. In most cases the dissociated species are more stable than the undissociated.

Hydrogen bonding refers to a stronger-than-usual dipole attraction between molecules in which hydrogen is bonded to a highly electronegative atom. Typically, this occurs when the

partially positively charged hydrogen atom lies between partially negatively charged oxygen and nitrogen atoms, but it can also be found elsewhere, e.g., between fluorine atoms in HF₂⁻ and between water and smaller halide ions F⁻, Cl⁻ and Br⁻ (e.g., HO–H···Cl⁻). It is well-known that some of the chlorophenols occur as two geometric conformers resulting from two orientations of hydroxyl hydrogen for each of the asymmetric chlorine substitution. Fig. 1 shows the structure of the *syn* and *anti* conformers for 2,4-DCP. In the case of *syn* conformers of chlorophenols the hydroxyl group and the *ortho*-substituted chlorine may interact and lend to intramolecular hydrogen bonding in this conformers. Such hydrogen bonding does not exist in the *anti* conformers, except for those with chlorine substitution at both *ortho* positions. The hydrogen bond energies of chlorophenols are typically 3.0 kcal mol⁻¹ [7].

Chlorophenols and polar solvent molecule (e.g., water, alcohol) could act as proton donors as well as proton acceptors and two types of hydrogen bonds are possible. When chlorophenol is the proton donor, electronic charge is transferred from the solvent to the phenolic oxygen and in consequence intermolecular hydrogen bonds may be formed [12–14]. The influence of hydrogen bonding on rate of reaction, especially radicals reaction, is discussed in various papers [8–11].

At infrared spectrum, hydrogen bonds cause broadening of the bands which are characteristic for stretching vibrations of hydroxyl group and their shift to lower wavenumber. It is the

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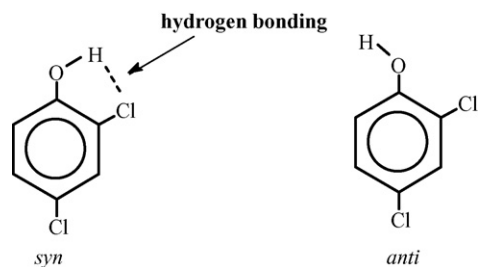


Fig. 1. *Syn* and *anti* conformers of 2,4-dichlorophenol.

result of changes in the length of O–H bonds. Distributions of hydrogen bonds of particular type are different even in the case of polymers depending on their low molecular weight models [15,16]. The study of the dynamics of the system with hydrogen bonding is interesting in itself, since bonds play a decisive role in kinetics of a number of processes, in particular, in the processes of proton transfer and proton exchange. Bureiko and Denisom [17] has shown that formation of an intramolecular hydrogen bonding by the proton acceptor group is accompanied by a considerable decrease in the rate of H-exchange, and the values of activation energy are close to the estimates of the intramolecular hydrogen bonding energies for the molecule under investigation. Their results confirmed the conclusion that the rate of the molecular H-exchange processes of the considered type is determined by the same peculiarities of electronic structure, which control the hydrogen bonding ability of the functional groups of the molecules and the reaction takes place formation of via cyclic intermediate complex which formed by hydrogen bonding between the functional groups of the molecules—partners.

The photochemical processes comprise, among other reactions, also photodissociation, photosubstitution, photooxidation, and photoreduction [18–20]. Usually, photodegradation of substances in the environment is resultant of all the listed above processes, and its rate and efficiency depend on many factors. The factors of the greatest importance to the photochemical processes are: maximum light absorption of a compound undergoing the process, wavelength of the radiation, time of exposure, and the state of the reaction environment.

In this work, study of the 3-chlorophenol, 2,4-dichlorophenol, 2,3,4,5-tetrachlorophenol, pentachlorophenol and products of their photodegradation using FT-IR spectroscopy is presented. Spectra of pure chlorophenols were compared with the spectra of products of their photoreaction in water solutions and methylene chloride extracts from irradiated solutions. The results obtained were correlated with GC/MS method.

2. Experimental

2.1. Reagents and chemicals

All the used solvents, i.e., methylene chloride, methanol, carbon tetrachloride, were of the analytical grade. The 3-chlorophenol (3-CP), 2,4-dichlorophenol (2,4-DCP), 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP), pentachlorophenol (PCP) (Supelco) were used without purification. Triply distilled water was used for all experiments.

2.2. Photolysis experiments

Water solutions of chlorophenols were placed in quartz tubes and exposed to radiation of the wavelength of 254 nm using low-pressure 6 W mercury lamp. The total experimental time was 120 min. Experiments were carried out at 25 °C. The concentration of chlorophenols in water was 0.2 g l⁻¹.

2.3. Analytical methods

2.3.1. FT-IR method

Infrared spectra of pure chlorophenols and products of their photoreaction were measured with FT-IR spectrophotometer (Digilab FTS-40A) in the range of 4000–400 cm⁻¹. The spectral resolution was 2 cm⁻¹, and accumulation was 32 scans.

The investigated samples were in a form of films obtained after evaporation in room temperature from solutions in methanol onto potassium bromide and water under vacuum onto KRS plates.

To study the formation of hydrogen bonding in chlorophenols, FT-IR spectra of 0.1 mol l⁻¹ solutions in carbon tetrachloride placed in a 0.67 mm potassium bromide cells were recorded.

In order to determine the quantitative relations among the bands, which were detected in the regions characteristic for stretching vibrations of hydroxyl groups, a WIN-IR curve fitting program was used. The initial parameters, i.e., number of peaks, centres, heights and widths of particular peaks, were obtained from the second derivative spectra. An interactive procedure and Gaussian–Lorentzian fitting of curves were chosen to correct these parameters.

Assignments of the bands in the infrared spectra of the investigated pure chlorophenols are shown in the Table 1.

2.3.2. GC/MS method

Irradiated solutions were extracted with methylene chloride. The extraction was repeated three times. The methylene chloride extracts were mixed together, dehydrated, and evaporated under stream of nitrogen to volume of 0.5 ml. Qualitative and quantitative analyses, without derivatization, were performed using Perkin-Elmer Clarus 500 gas chromatograph (GC) equipped with a quadrupole mass spectrometry (MS) detector, and a 30 m × 0.25 mm i.d. DB-5MS column with a film thickness of 0.25 μm. Quantitative analysis was performed using selected ion monitoring method (SIM), choosing two or three typical for each compound ions.

3. Results and discussion

3.1. Investigation of pure chlorophenols

Due to two types of polar group, in chlorophenols two types of intermolecular hydrogen bonds, between two chlorophenol molecules, i.e., HO···HO, and OH···Cl can be formed. The interactions between hydrogen and chlorine atoms are weaker than those between hydrogen and oxygen atoms, therefore strong shifts of these bands can be observed in the case of HO···HO type of hydrogen bonds.

Table 1
Frequencies (cm^{-1}) and tentative assignments of the bands in the IR spectra of some chlorophenols

Compounds				Assignment
3-CP	2,4-DCP	2,3,4,5-TeCP	PCP	
3550	3524	3518		$\nu\text{OH free}$
3352	3460	3465	3417	$\nu\text{OH ass.}$
			3394	$\nu\text{OH ass.}$
	3096			νCH
3064	3070	3076		νCH
1603		1582		νPh
1590	1584	1552	1547	νPh
1488				νPh
1474	1477			νPh
1444	1407	1422	1420	$\nu\text{Ph} + \delta\text{OH}$
		1394	1382	νPh
1318	1330		1311	νPh
1298	1279	1286	1295	δCH
1244	1254	1271		δCH
1214	1239		1222	νCO
		1196	1196	
1158	1188	1149		δCH
1089	1095	1091		δCH
1070	1053			δCH
998	1004		987	νPh
	948	976		γCH
886	866			γCH
849	855	855	862	νCCl
	815	841		νCCl
770	724	729	722	$\nu\text{CCl} + \gamma\text{Ph}$
			708	νCCl
			718	
678	651	592	649	
530	556	576		Torsion OH
	514		516	
442	441			

Besides 3-chlorophenol, also intramolecular $\text{OH} \cdots \text{Cl}$ hydrogen bonds can be formed in investigated chlorophenols. The FT-IR spectra of CPs are presented in Figs. 2–5.

As it is shown in the FT-IR spectra of chlorophenols investigated in the region of $3700\text{--}3000\text{ cm}^{-1}$, the position and shape of bands corresponding to stretching vibrations of hydroxyl groups in particular cases are different. It can be a result of influence of substituents and it is related to hydrogen bonds forming.

Detailed investigations to define quantitative relations between different types of hydrogen bonding were made by curve fitting calculations in that region.

As indicated in the Table 2 for all the investigated chlorophenols several peaks in both regions were obtained. Each peak corresponds to the O–H bond in hydroxyl group of definite bonds length.

In the case of 3-chlorophenol, the highest area band appeared at 3341 cm^{-1} which can be ascribed as vibrations of hydroxyl group in a $\text{HO} \cdots \text{HO}$ type of hydrogen bonds. Since 3-CP has a chlorine atom at the *meta* position, three candidates for the dimer structure are considered: *cis-cis*, *trans-trans* and *cis-trans*. Nakagawa et al. [25] suggested that one isomer may be dominant, presumably the *cis-cis* isomer (the *cis* conformer should be more stable than the *trans* one). The bands of the proton donor and proton acceptor are arising. The authors also indicated that the hydrogen bonding of the 3-CP dimer is weaker than the phenol dimer. This phenomenon may result from the charge distribution on the oxygen atom, affected by the substituted Cl atom at the *meta* position. The initial geometries of the dimer represented sandwich-type structure and the V-shaped hydrogen bonding phenol dimer structure, in which phenyl planes are perpendicular. The optimized structures for the 3-CP dimer in the ground state is shown in Fig. 6. The relatively smaller area of the bands at 3450 cm^{-1} can result from $\text{OH} \cdots \text{Cl}$ interactions. In the region characteristic for vibrations of free hydroxyl groups also two bands at 3567 and 3532 cm^{-1} have been obtained. The presence of two peaks in this region can be explained by interactions in crystal lattice.

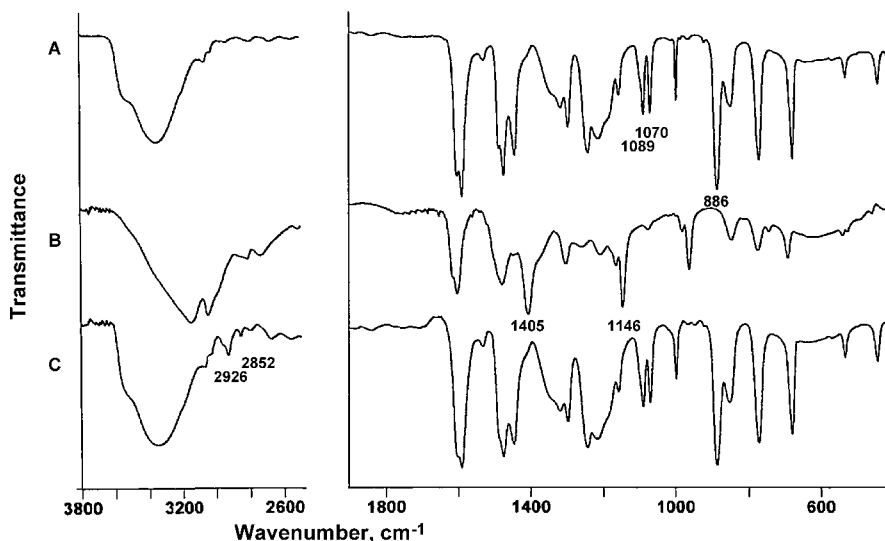


Fig. 2. FT-IR spectrum of 3-CP (A) pure, (B) irradiated water solution and (C) methylene extract from irradiation solution.

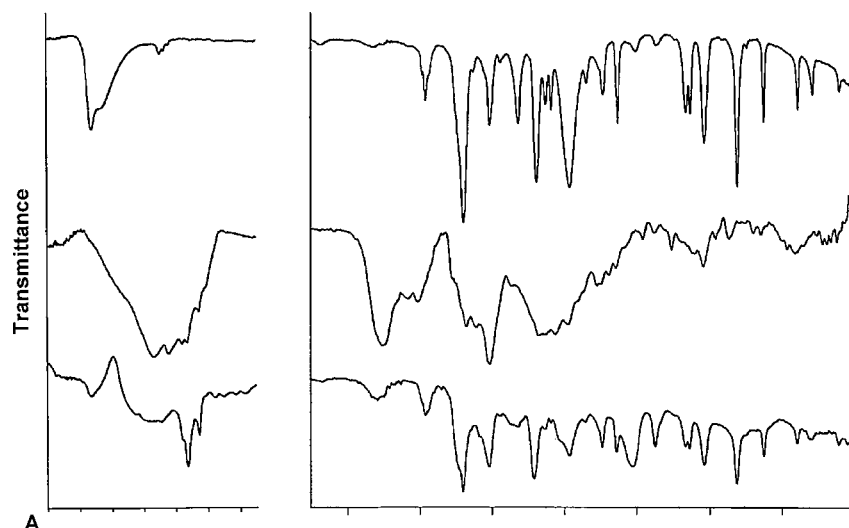


Fig. 3. FT-IR spectrum of 2,4-DCP (A) pure, (B) irradiated water solution and (C) methylene extract from irradiation solution.

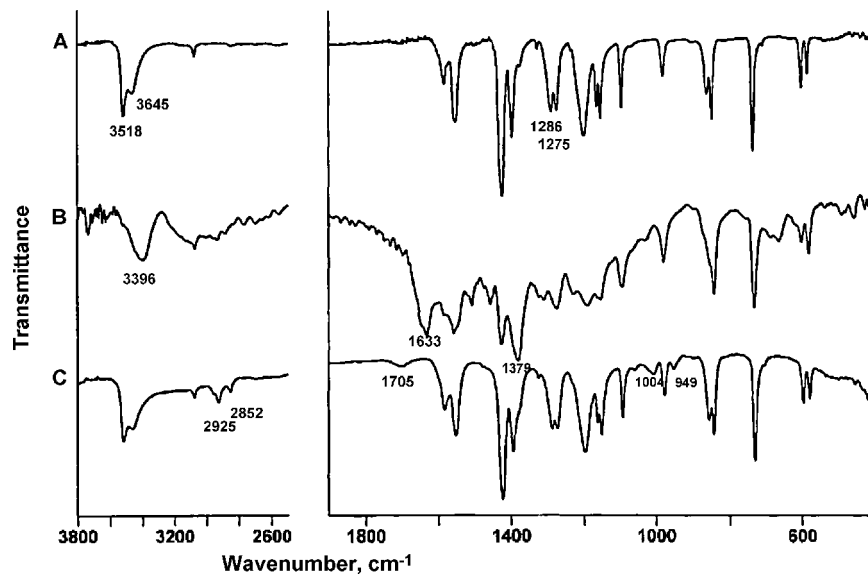


Fig. 4. FT-IR spectrum of 2,3,4,5-TeCP. (A) pure, (B) irradiated water solution and (C) methylene extract from irradiation solution.

In 2,4-dichlorophenol spectrum almost the same area bands at 3473 and 3390 cm^{-1} , which correspond to stretching vibrations of hydroxyl group in the case of $\text{OH}\cdots\text{Cl}$ and $\text{HO}\cdots\text{HO}$ interactions, respectively, were calculated. In the range of free hydroxyl group vibrations two bands at 3536 and 3521 cm^{-1} were obtained. The relations between areas of those bands are similar as for 3-chlorophenol. By analogy with spectral analysis

of 3-CP, the $\text{HO}\cdots\text{HO}$ interactions may result from formation of dimer.

Relatively high area band at 3465 cm^{-1} , attributed to $\text{OH}\cdots\text{Cl}$ interactions was calculated in 2,3,4,5-tetrachlorophenol. Two bands ascribed to vibrations of free hydroxyl groups at 3521 and 3511 cm^{-1} are significantly smaller than in the case of the bonded one.

Table 2
Curve fitting results in the region characteristic of OH stretching vibrations region

Compound	ν_1	Area	ν_2	Area	ν_3	Area	ν_4	Area	ν_5	Area	ν_6	Area	ν_7	Area	ν_8	Area	ν_9	Area
3-CP	3567	2.465	3532	5.496							3450	11.804					3341	65.947
2,4-DCP	3536	2.162	3521	6.119							3473	15.207			3390	14.077		
2,3,4,5-TeCP			3521	1.873	3511	3.101					3465	15.723						
PCP											3443	5.028	3419	6.556	3394	11.491	3367	36.201

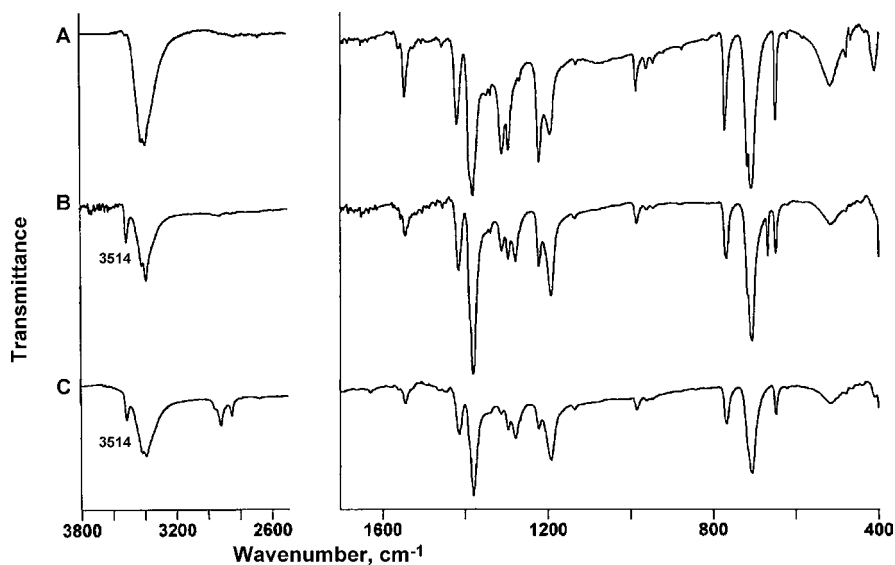


Fig. 5. FT-IR spectrum of PCP: (A) pure, (B) irradiated water solution and (C) methylene extract from irradiation solution.

For pentachlorophenol four bands at 3443, 3419, 3394 cm^{-1} and the highest area band at 3367 cm^{-1} were obtained. As it can be seen in this case free hydroxyl groups are not detected.

As it can be observed from FT-IR spectra, which were obtained for 0.1 mol^{-1} solutions of chlorophenols investigated, only one, strongly shifted to higher wavenumbers band was recorded. The highest shift to 3607 cm^{-1} is observed for 3-chlorophenol. We can conclude that the investigated chlorophenols only intermolecular hydrogen bonds appeared. Therefore, it was confirmed that only intermolecular hydrogen bonds are formed in the investigated chlorophenols.

It is not possible to conduct similar investigations in water solutions using infrared spectroscopy because in spectrum of water, a broad, high intensity band also appears in this region. The distribution of free and bonded hydroxyl groups in water solutions will be different due to the interactions of chlorine atoms and hydroxyl groups from chlorophenols with hydroxyl

groups from water which certainly influence the process of photoreaction.

3.2. Investigations of photoreaction products

The photodecomposition of CPs in aqueous solution irradiated at 254 nm may include various reactions, such as direct photolysis, and secondary reactions [26–28]. The chlorophenols are mainly converted into hydroxybenzenes and less-chlorinated phenols through photolytical dechlorination [29,30] and dimerization. The photodegradation rate of chlorophenols depends on the form in which the compound occurs (as non-dissociated or as anionic phenolates), pH of the reaction environment, and structure of the compound (especially of a position of the chlorine atom in relation to the hydroxyl group). Cl^- ions are formed in all cases of CP photodegradation. Degradation of chlorophenols is presented in Table 3.

FT-IR spectra of methylene chloride extract of irradiated solution and pure chlorophenols were very similar in the range 2600–3800 cm^{-1} . Distribution of free and bonded hydroxyl groups are the same as for 3-chlorophenol before photoreaction. That suggested formation of chlorophenol dimer in methylene extracts as in the case of pure chlorophenols. In the presented study, the differences between FT-IR spectra of irradiated water solutions of chlorophenols and their methylene chloride extracts in range 2600–3800 cm^{-1} were observed. This difference may be explained by the formation of chlorophenols complexes with water or/and HCl. As for cluster formation, water clusters have

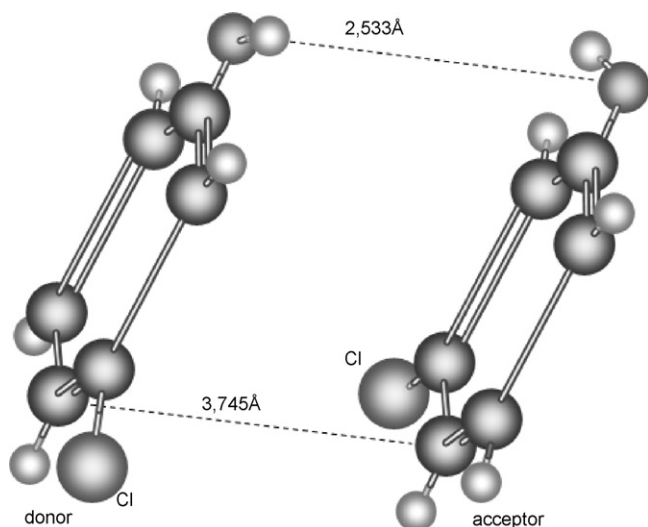


Fig. 6. The structure of the dimer of 3-CP in the ground state [26].

Table 3
Conversion of chlorophenols after 120 min irradiation

Compound	Conversion (%)
3-Chlorophenol	40
2,4-Dichlorophenol	44
2,3,4,5-Tetrachlorophenol	47
Pentachlorophenol	51

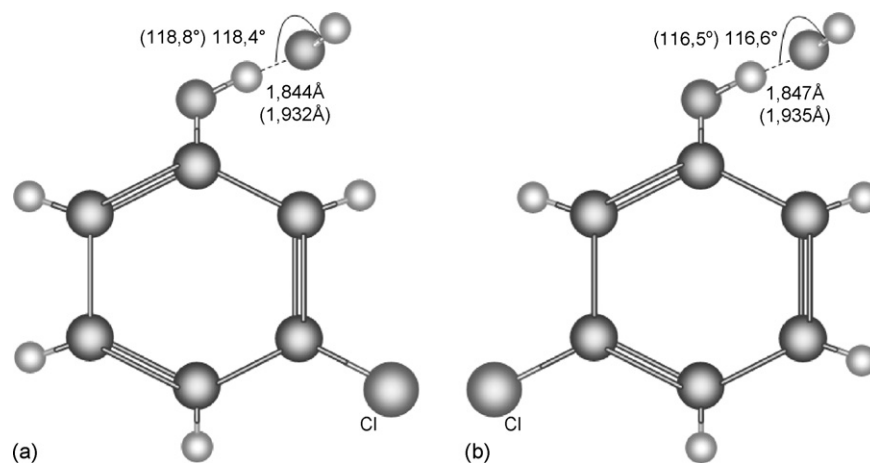


Fig. 7. The structures of (a) *cis*-3-CP–water cluster and (b) *trans*-3-CP–water cluster [26].

been observed for some phenol derivatives [17,21–24]. Nakagawa et al. [25] and Bazyle et al. [31] have shown the formation of chlorophenol–water clusters. Fig. 7 shows the molecular structures of 3-chlorophenol and their complexes with water.

The changes of spectra in the range $2600\text{--}3800\text{ cm}^{-1}$ for water solutions of chlorophenols may be explained by correlation between pK_a and number of Cl atoms in molecule. It is well-known that under the near-neutral pH conditions the chlorophenols are deprotonated to various degrees depending on number of Cl substitutions. The increased number of substituted Cl atoms leads to decrease of the negative log of the acidity constant (pK_a). For chlorophenols with the same number of Cl substitutions, pK_a increases with increasing proximity of the substituted Cl atom to the OH group.

3.2.1. 3-Chlorophenol

In FT-IR spectrum of the soluble in water 3-chlorophenol photoreaction product, significant changes are noticed (Fig. 2). Intensity of the bands arising from stretching and deformation vibrations in the region of $3600\text{--}3000$ and $1350\text{--}1250\text{ cm}^{-1}$, respectively, diminished notably. Simultaneously, the bands at 886 cm^{-1} , which is characteristic for C–Cl, and at 1070 and 1089 cm^{-1} — ascribed to deformation vibrations of C–H in aromatic ring, disappeared. Therefore, it can be seen as a proof that during photoreaction some hydroxyl groups reacted and some chlorine atoms disconnected from aromatic ring. GC/MS analy-

ses of irradiated solutions show that the main photodegradation product is resorcinol (the chromatogram of methylene chloride extract of irradiated solution is given in Fig. 8). The mechanism of the resorcinol formation is explained by polarization of the C–Cl bond and heterolytic scission of a molecule of water with the concerted formation of Cl^- and resorcinol. The obtained results confirm the mechanism of photodegradation of 3-CP as presented in Fig. 9.

These changes are accompanied by new strong bands appearance at 1405 , 1146 and 861 cm^{-1} . The band at 1146 cm^{-1} can correspond to aromatic ether groups, but also can be the result of changes in the ring substitution.

In the spectrum, obtained for methylene chloride extract from irradiated solution, only additional bands at 2926 and 2852 cm^{-1} , which due to vibrations of hydrogen chloride and at 946 cm^{-1} , arising probably from changing the ring substitution, are observed (Fig. 2).

3.2.2. 2,4-Dichlorophenol

After photoreaction of 2,4-dichlorophenol quite different spectrum was obtained for products soluble in water (Fig. 3). In the range characteristic for stretching vibrations of hydroxyl group broad band with maximum at 3137 cm^{-1} was present. Such a broad band in this region can suggest the presence of acid hydroxyl groups. It can be confirmed by the strong band at 1704 cm^{-1} ascribed to stretching vibrations of carbonyl group.

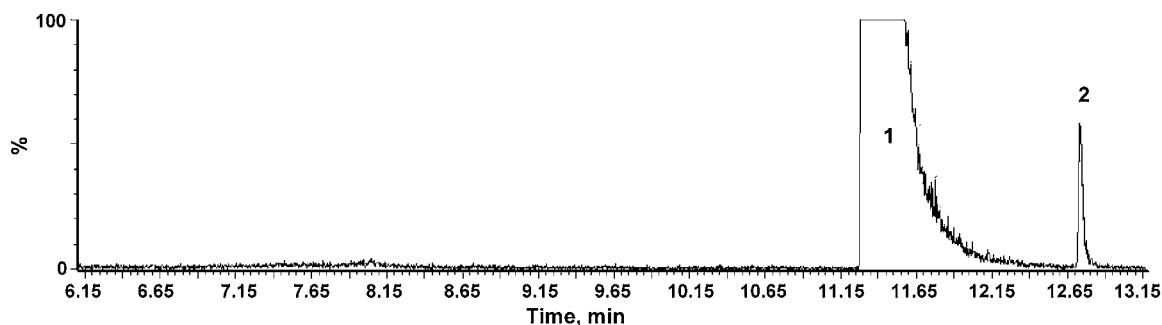


Fig. 8. The chromatogram of methylene chloride extract from irradiated solution of 3-CP: (1) 3-CP and (2) resorcinol.

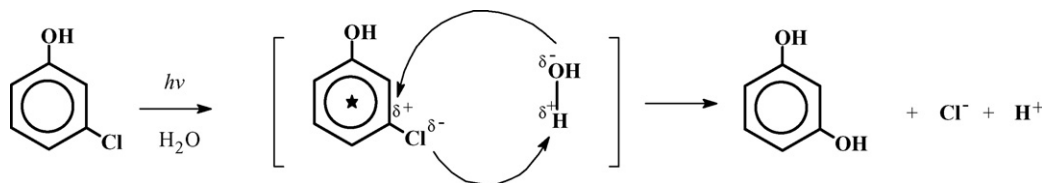


Fig. 9. Mechanism reaction formation of resorcinol during photoreaction of 3-CP [7].

In the spectrum appeared also the bands at 2930 and 2855 cm^{-1} , due to hydrogen chloride, and the band at 1633 cm^{-1} attributed to ketone or C=O group.

The photodegradation of anionic forms of 2,4-DCP leads to the formation of dimers of chlorocyclopentadienic carboxyl acid according to the Diels–Alder reaction [19]. GC/MS analysis showed that photodegradation process leads to the formation of compounds of molecular mass about 144 m/z , probably chlorocyclopentadienic acid, and small amounts of 2-chlorophenol, 4-chlorophenol and dihydroxychlorobenzenes. Due to lack of analytical standard of chlorocyclopentadienic carboxy acid it is impossible to confirm the presence of these compounds using typical GC/MS parameters, e.g., retention time and mass spec-

trum. Presence of the bands at 1704 and 1633 cm^{-1} may be indirectly confirmed by the presence of this compound in irradiated solutions. Monochlorophenols are the result of dechlorination of the parentheses compound. Lack of the band in the region of 1000–600 cm^{-1} in the FT-IR spectrum of irradiated solutions indicates that the change in the ring substitution is probably the result of small yield of dechlorination reaction. Photodegradation pathway of 2,4-DCP is presented in Fig. 10.

Just as for 3-chlorophenol, in the spectrum of the soluble in methylene chloride fraction only additional bands due to hydrogen chloride vibrations are present. The presence of these bands indicated possibility of formation of hydrogen bonding between molecules of 2,4-DCP in extracts of methylene chloride.

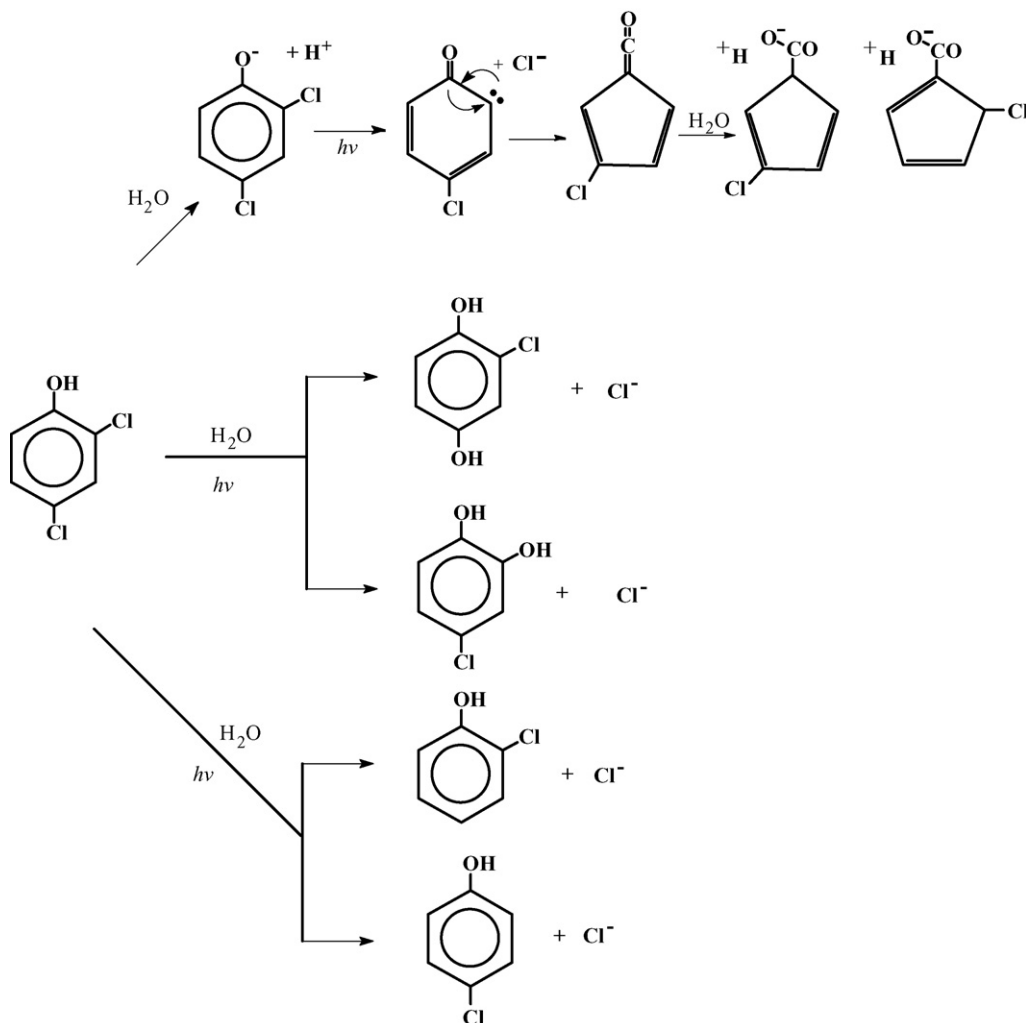


Fig. 10. Photodegradation pathway of 2,4-DCP.

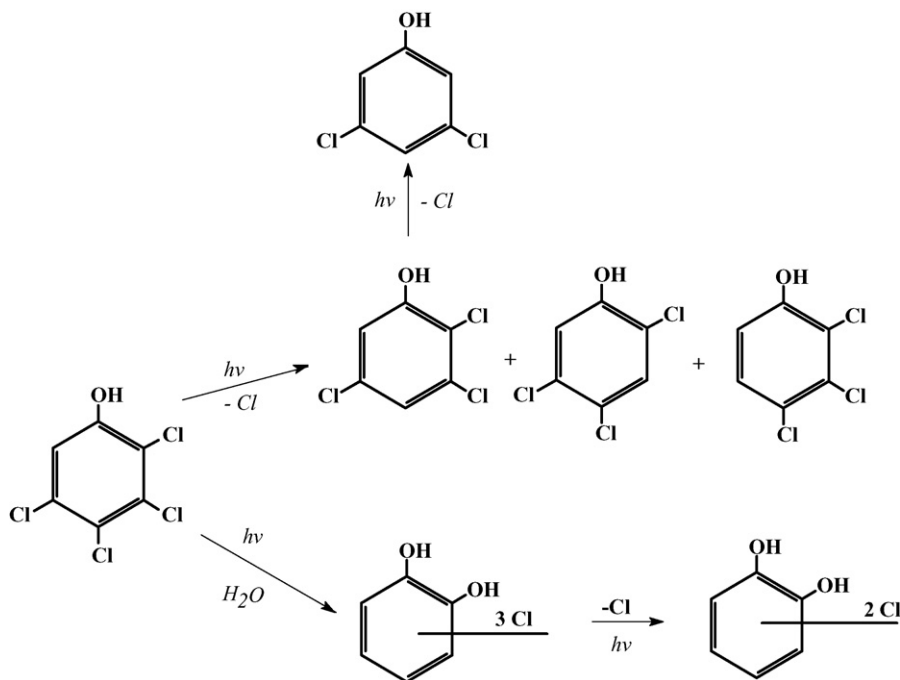


Fig. 11. Photodegradation pathway of 2,3,4,5-TeCP.

3.2.3. 2,3,4,5-Tetrachlorophenol

Spectrum of the water-soluble products of photoreaction of the 2,3,4,5-tetrachlorophenol shows a weak band with a maximum at 3396 cm^{-1} instead of two characteristic bands with maximum at 3518 and 3465 cm^{-1} which can be attributed to the stretching vibrations of hydroxyl group. Simultaneously, the band appearing in pure 2,3,4,5-tetrachlorophenol at 1286 and 1275 cm^{-1} and ascribed to deformation of vibrations of hydroxyl group has disappeared. This shows that some O–H bonds were broken during the reaction.

Additionally, relatively strong bands at 1633 and 1379 cm^{-1} were observed. The first band can be interpreted as the stretching vibration of a carbonyl group and the other one as a result of a change in aromatic ring substitution. The other bands were slightly moved and their intensities were slightly changed in relation to the pure 2,3,4,5-tetrachlorophenol spectrum. The spectrum of the methylene chloride extract photodegradation product

shows, besides the bands attributed to vibrations of hydrogen chloride at 2925 and 2852 cm^{-1} , the bands at 1705 , 1004 and 949 cm^{-1} . The first one can be interpreted as stretching vibrations of carbonyl group while the other as the result of changes in aromatic ring substitution.

GC/MS data show that irradiation of 2,3,4,5-TeCP yielded the following products: trichlorophenols, especially 2,3,5-TCP, dichlorophenols, dihydroxytrichlorobenzenes and dihydroxydichlorobenzenes. The photodegradation pathway of 2,3,4,5-TeCP is presented in Fig. 11. Results of photodegradation of 2,3,4,5-TeCP presented by Czaplicka, Czaplicki [30] showed untypical correlation of photodegradation rate of this compound versus irradiation time (Fig. 12). The observed anomaly may be explained by the formation of intermolecular hydrogen bonds between substrate and one of the phototransformation products and/or hydrogen bonds which have arisen between products of these reactions (the shift of the band to 3396 cm^{-1} , which

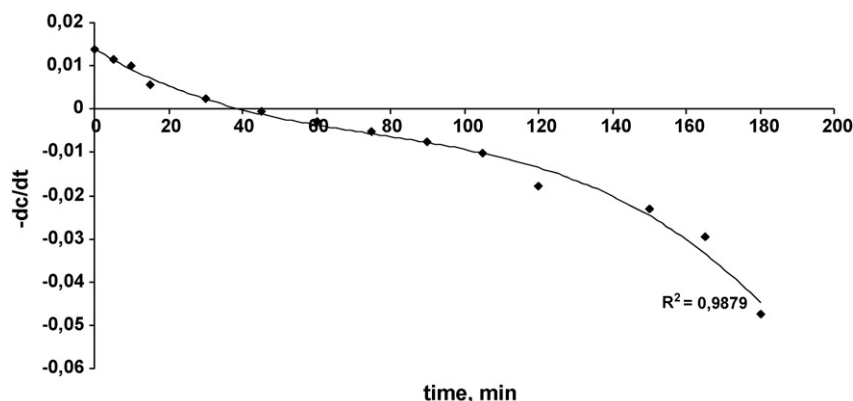


Fig. 12. Relationship reaction rate of 2,3,4,5-TeCP vs. photodegradation time [25].

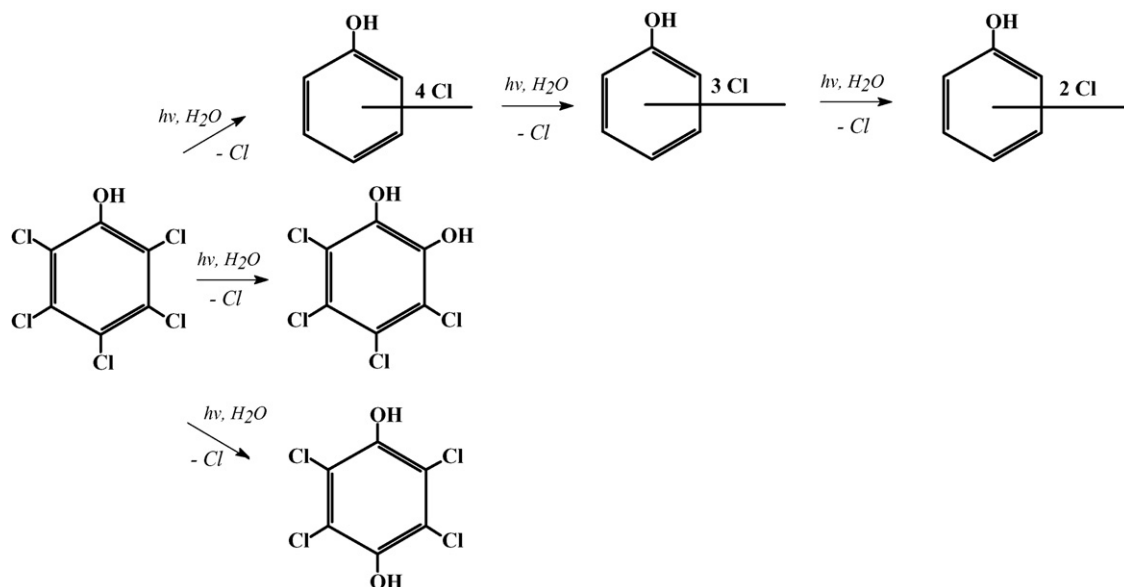


Fig. 13. Photodegradation pathway of PCP.

responded to the stretching vibrations of hydroxyl group). Fein et al. [31] and Daughney et al. [32] have shown that the deprotonated species of chlorophenol can form aqueous complexes with free cations in solution.

3.2.4. Pentachlorophenol

The PCP was mostly dechlorinated while maintaining the mono phenol structure, which produced 2,3,5-TeCP and 2,3,5,6-TeCP. After re-chlorination, they produced DCP via the trichlorophenols. The tetrachlorohydroquinone and tetrachlorocatechol were also formed [33,34]. The photodegradation pathway of PCP is presented in Fig. 13. The mass spectrometry reveals formation of many photoproducts in the irradiation of pentachlorophenol solution. In FT-IR spectra of pentachlorophenol products of photoreaction the changes are observed in the range of stretching and deformation vibrations of hydroxyl groups only (Fig. 5). Additional band appeared at 3514 cm^{-1} corresponding to vibrations of free hydroxyl groups. The changing of spectrum at about 3500 cm^{-1} between initial and irradiated solutions can be explained by formation of chlorohydroxybenzene, especially tetrachlorohydroquinone and tetrachlorocatechol.

The band arising from vibrations of hydrogen chloride additionally appeared in the spectrum recorded for products soluble in methylene chloride.

4. Conclusion

FT-IR study of pure chlorophenols indicated the possibility of formation of hydrogen bonding of the 3-CP dimer. We observed strong differences between infrared spectra of pure chlorophenols and their photodegradation products (water-soluble and methylene chloride extracts). The obtained results indicated that formation of complexes of these compounds with water in irradiated water solutions of chlorophenols is possible.

FT-IR study of irradiated solution of 3-CP indicated a change in the aromatic ring substitution. The results were confirmed by GC/MS method. In the case of 2,4-DCP, bands representing of stretching vibration of hydroxyl group (3137 cm^{-1}) and carbonyl group (1704 cm^{-1}) were observed. It indicates the possibility for formation of chlorocyclopentadienic carboxylic acid. Formations of intra- and intermolecular hydrogen bonding between substrate and photoproducts in irradiated solutions of 2,3,4,5-TeCP was observed.

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Off-line coupling of pressurized liquid extraction and LC/ED for the determination of retinyl acetate and tocopherols in infant formulas

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Abstract

An analytical methodology including pressurized liquid extraction (PLE) as sample treatment to isolate retinyl acetate and tocopherols from infant formulas has been developed. The milk extracts were kept at -18°C for 30 min and after filtration could be injected directly into the chromatographic system. Thus, a rapid and simple routine control method of these products is possible.

The parameters affecting both the extraction process and the liquid chromatography (LC) system were optimized. PLE was performed using one cycle of extraction during a static time of 5 min. Methanol was chosen as the extraction solvent for a temperature of 50°C . Chromatographic separation was accomplished using a RP-18 column; the mobile phase used was methanol–water (94:6, v/v) containing 2.5 mM acetic acid/sodium acetate buffer. Electrochemical detection in amperometric mode with a glassy carbon electrode at +1100 mV was applied. The proposed methodology was successfully used for the determination of retinyl acetate, δ -tocopherol, ($\beta + \gamma$)-tocopherol and α -tocopherol in different infant formulas. The analytes were evaluated in the same chemical form present in the samples. Recoveries were between 92 and 106%. A certified reference material of milk powder was also analyzed.

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Keywords: Food analysis; Infant formula; Retinyl acetate; Tocopherols; Pressurized liquid extraction; LC; Amperometric detection

1. Introduction

Food samples are always complex and when the determination of substances of interest is required sufficiently selective and sensitive analytical methods must be used. Sample treatment, including the isolation and preconcentration steps, is generally an important part of chromatographic determinations, but it is also one of the most tedious and time-consuming aspects of the overall analytical process. Another important feature in sample preparation, which is not always taken into account, is its effect on error propagation. Automation of this stage may reduce analysis time to an important extent, considerably simplifying the analytical process, and reducing the errors inherent to the analytical method.

Pressurized liquid extraction (PLE) is a recently introduced extraction technique that uses an organic solvent or a combination of solvents at high pressure and a temperature above the boiling point of the solvent to extract substances from a solid matrix [1–3]. PLE offers several advantages over other extraction techniques such as the possibility of automation, low solvent volumes and reduced extraction time. PLE can be accomplished in the static mode, the dynamic mode or a combination of both. In the static mode, sample and solvent are maintained for a user-specified time at constant pressure and temperature, whereas in the dynamic mode the solvent flows through the sample in a continuous manner.

PLE has been widely used for the extraction of environmental pollutants present in soil matrices, sediments, etc., and more recently in the pharmaceutical and food industries. In the field of foods, PLE has been successfully used in the analysis of contaminants such as polychlorinated biphenyls (PCBs) and pesticides. Müller et al. [4] have used PLE for the determination of PCBs in feeding stuffs and food matrices. The authors achieved the on-line extraction and clean-up of fat-

Abbreviations: ASE, accelerated solvent extraction; LC/ED, liquid chromatography with electrochemical detection; PCBs, polychlorinated biphenyls; PLE, pressurized liquid extraction; SPE, solid-phase extraction

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containing matrices using sulphuric acid impregnated with silica gel. Sporring and Björklund [5] studied the effect of different parameters, such as cell dimensions, solvent type, temperature and flush volume, in the determination of PCBs from fat-containing food and feed matrices using PLE. Suchan et al. [6] also investigated the effect of several operational parameters on the efficiency of PLE in polychlorinated biphenyls and organochlorine pesticides in fish samples. Lehotay and Lee [7] determined 58 pesticides in tomatoes using PLE as the extraction technique prior to gas chromatography with ion-trap detection.

Naturally occurring substances have been also analyzed. Papagiannopoulos et al. developed a new instrumental setup for automated extraction of solid samples by online coupling of PLE, automated solid-phase extraction (SPE) and liquid chromatography (LC). They successfully applied this device in the determination of proanthocyanidins in malt samples [8] and polyphenols in hops [9]. Catechins have been extracted from tea leaves and grape seeds [10], and also procianidins, dihydrochalcones, flavonols and anthocyanidins from apples [11] using this technique before LC analysis. Schäfer [12] used PLE to extract fatty acids from several biological samples such as cereals and animal tissues; the composition of the lipids extracts was determined by gas chromatography. Toschi et al. [13] studied the effect of different binary extraction mixtures on the extraction of total lipids in poultry meat. The composition of the extracts was determined by thin-layer chromatography and the fatty acid composition was carried out by capillary gas chromatography. Dunford and Zhang [14] studied the effect of temperature, extraction time, sample size and solvent type on the extraction efficiency and oil quality of wheat germ oil. Carotenoids, used as food colouring additives in liquid and solid samples such as beverages, pudding mixes, cereals and cookies, have been isolated using PLE [15]. Recent applications have demonstrated the possibility of using this technique for the extraction of vitamins. Analysis of Vitamin E isomers in seeds and nuts has been accomplished using PLE prior to analytical determination by liquid chromatography with coulometric detection [16]. In medical foods, Vitamin K₁ has been determined using PLE in conjunction with matrix solid-phase dispersion before determination by LC with fluorescence detection [17]. Carotene, tocopherols and tocotrienols have been determined in residue oil from palm pressed fiber using PLE followed by normal phase liquid chromatography [18].

The most commonly used methods of sample preparation for the analysis of liposoluble vitamins in foods include sample saponification and later extraction of the vitamins from the unsaponifiable matter [19,20] or direct solvent extraction of the vitamins from the sample [21,22]. A cleaning step may be necessary before injection into the chromatographic system.

The aim of the present work was to develop a simple sample treatment including PLE methodology for the extraction of retinyl acetate and tocopherols from infant formulas without additional cleaning steps before injection of the extracts into the chromatographic system. Detection was carried out using an

amperometric detector. The proposed methodology is simple, rapid and can be automated, enabling its use in routine analysis of vitamins in this kind of sample.

2. Experimental

2.1. Apparatus

2.1.1. Chromatographic system

Isocratic LC determination was carried out with a Spectra-Physics (San José, CA, USA) SP8800 ternary pump, equipped with a Rheodyne (Berkeley, CA, USA) valve with an injection loop of 20 μ L and a XTerraTM RP-18 (4.6 mm $\times$ 250 mm, 5 μ m) Waters column (Barcelona, Spain). An EG & G PAR (Princeton, NJ, USA) Model 400 electrochemical detector equipped with a working glassy carbon electrode, Ag/AgCl reference and steel counter electrodes was used. Data were processed using a Shimadzu (Duisburg, Germany) CBM 10-A communication module and Class-LC10 Software (Shimadzu).

2.1.2. PLE system

A Dionex (Germany) model ASE 100 automated extraction system for accelerated solvent extraction was used. Extraction cells of 34 mL, with cellulose filters and Dionex vials of 250 mL for extract collection, were used.

Water was purified in an ElgaStat water purification system (Elga, High Wycombe, UK). A Büchi (Flawil, Switzerland) RE 121 rotavapor with a Büchi 461 water-bath was used.

2.2. Reagents

The following compounds and chemicals were used: retinol (>97%, Fluka, Madrid, Spain), retinyl acetate (>95%, Fluka), α -tocopherol (>98%, Sigma, Madrid, Spain), γ -tocopherol (Sigma), δ -tocopherol (90% Sigma), β -tocopherol (Merck, Darmstadt, Germany), gradient grade for liquid chromatography acetonitrile (Merck), gradient grade for liquid chromatography methanol (Merck), special LC quality *n*-hexane (Merck), R.A. grade glacial acetic acid (Scharlau, Barcelona, Spain), R.A. grade sodium acetate (Panreac, Barcelona, Spain), R.A. grade potassium hydroxide (Scharlau), R.A. grade ascorbic acid (Panreac), R.A. grade ethanol (Scharlau) and Hydro-matrix Celite (Varian, Scharlau). The reference material was CRM 421 milk powder, supplied by the Community Bureau of Reference-BCR (Institute for Reference Materials and Measurements, European Commission-DG JRC, Geel, Belgium).

All samples of milk powder analyzed were from commercial sources.

The mobile phase used was a solution of 2.5 mM acetic acid/sodium acetate in MeOH/H₂O (94:6, v/v). Flow rate was set at 1.0 mL/min. The solutions were filtered through Millipore (Madrid, Spain) nylon membranes with a pore size of 0.22 μ m and a diameter of 45 mm, and were degassed with helium (99.999%, Air liquide, Valladolid, Spain).

2.3. Sample treatment

2.3.1. Sample treatment using PLE

The accelerated solvent extraction (ASE) system consisted of a stainless steel extraction cell with electronically controlled heaters and pumps to maintain the extraction parameters following an established program.

The general procedure for the analysis of retinyl acetate and tocopherols in milk powder was as follows: the sample (approximately 2 g) was mixed homogeneously with the drying agent (Hydromatrix Celite) in the extraction cell, containing a cellulose filter at the bottom. After placing the cell in the ASE system, the optimized program was applied. This included a temperature of 50 °C and a static time of 5 min in one cycle, at a pressure of 1600 psi, using methanol as solvent. The extracts were diluted with methanol to 50.0 mL and kept in a refrigerator at –18 °C for at least 30 min. The extracts showed a cloudy aspect, possibly due to the proteins extracted in the process. Refrigeration produces the coagulation and precipitation of proteins and hence permits clarification of the extracts. This clarified extract was filtered through 0.22 µm nylon membranes and injected (20 µL) into the chromatographic system.

2.3.2. Sample treatment with saponification

The procedure including saponification of samples was as follows: the sample (approximately 2 g) was mixed with hydrolysis reagent, whose composition was 50 mL of ethanol, 5 mL of aqueous 10% ascorbic acid, 10 mL of aqueous 80% KOH and 25 mL of water. The mixture was stirred over 2 h at room temperature protected from light. After this time, the analytes were extracted with hexane (2 × 25 mL) and the extracts were washed with water (2 × 10 mL). The organic phase was then evaporated to dryness in a rotavapor at 50 °C, and the residue was dissolved

in methanol (50 mL), filtered through 0.22 µm nylon membranes and injected into the chromatographic system.

3. Results and discussion

3.1. Optimization of chromatographic parameters

Different methanol–water solutions as mobile phases have been used in previous works [16,23]. In the present case, optimum results for the separation of analytes were obtained with methanol–water (96:4, v/v), containing 2.5 mM acetic acid/sodium acetate; the flow rate of the mobile phase was set at 1.0 mL/min. Under these conditions, analyte elution was completed in 12 min. To obtain the best analytical signal, the potential applied to the glassy carbon electrode was optimized. The hydrodynamic voltammograms revealed that for an applied potential of 1100 mV (versus Ag/AgCl) all vitamins reached the highest values. Fig. 1 shows the chromatograms obtained on applying the method to a standard solution and to several milk samples.

3.2. Optimization of PLE parameters

Choice of the extraction solvent aims at the selective extraction of analytes, with minimum extraction of interferences. A wide range of solvents can be used in PLE, except those with an autoignition temperature from 40 to 200 °C. The polarity of the solvent should be close to that of the analyte. In addition, it is also important to take into account the compatibility with the later treatment step, in this case the mobile phase of the chromatographic system. After assays using methanol, ethanol and acetonitrile as solvents, methanol was chosen because it achieved the best extraction yields.

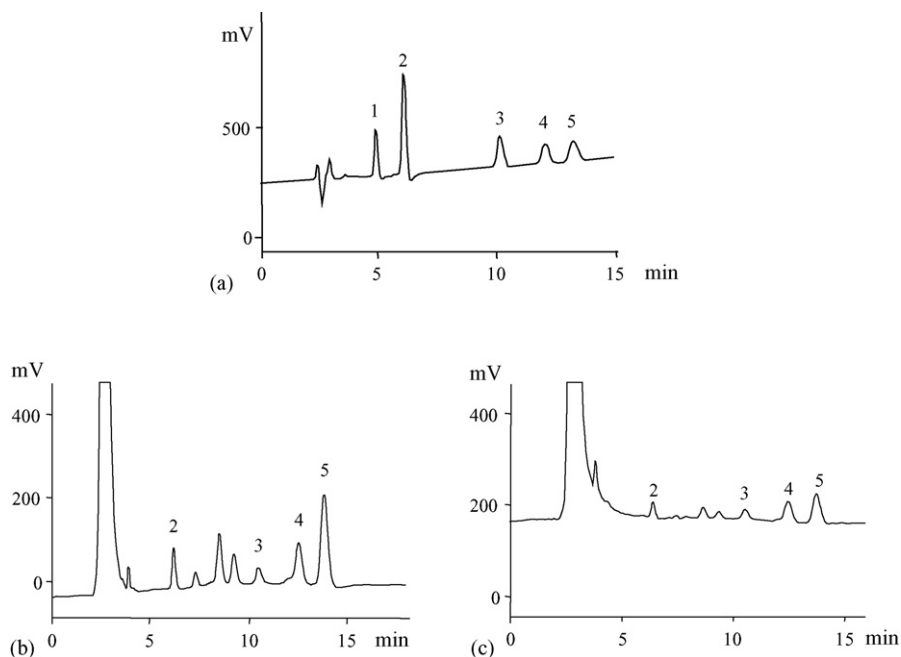


Fig. 1. Chromatograms obtained after application of the proposed method to a solution of standards (a) and two different kinds of infant formulas (b and c). Peaks: (1) retinol, (2) retinyl acetate, (3) δ -tocopherol, (4) $(\beta + \gamma)$ -tocopherol and (5) α -tocopherol.

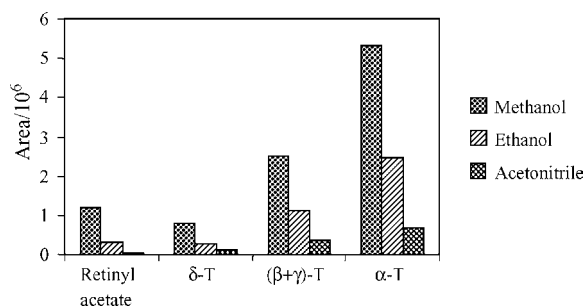


Fig. 2. Influence of solvent on PLE.

Fig. 2 shows the effects of different solvents on the extraction yield.

The extraction cell in the PLE system contained the sample mixed with a drying agent in order to avoid the generation of a dead volume in the extraction cell and to allow the best contact between the sample and the solvent. Optimization of the sample amount is important in order to achieve the best phase equilibrium between sample and solvent in the extraction cell. To obtain the best analyte extraction conditions, amounts of sample ranging from 1 to 5 g were weighed and mixed with the drying agent (Hydromatrix Celite) until the extraction cell was full. For sample amounts above 4 g, the signal tended towards a constant value asymptotically, as shown in Fig. 3. Because the relationship between the amount of sample and the analytical signal must be linear, it was necessary to work in the ascending zone of the curve, such that amounts of 2 g were used.

High temperature is one of the most important parameters in PLE extraction because it increases the solubility of the analytes and the extraction rate, and it decreases the viscosity and surface tension of the solvents. This allows better penetration of the solvent among the matrix particles, which increases mass transfer. The use of high pressure permits the solvent to be kept in the liquid state at elevated temperatures and, in addition, the pressure forces the solvent into the pores, which improves extraction efficiency [3]. ASE 100 only allows operation at 1600 psi (110 atm), and hence only temperature could be optimized to increase the efficiency of the extraction process. Temperature values ranging from 40 to 80 °C were tested, without obtaining significant differences among the analytical signals. Higher values were not used because vitamins are thermally unstable. However, some authors [2] have reported that this extraction system does not produce degradation, even for thermally unstable substances.

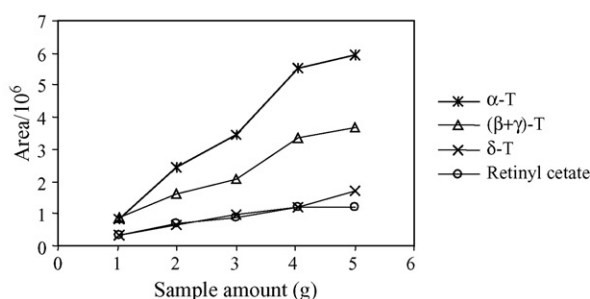


Fig. 3. Influence of sample amount on PLE.

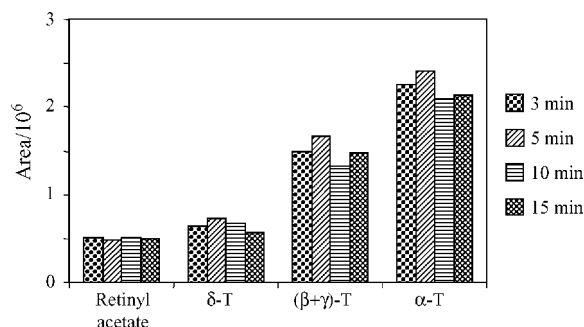


Fig. 4. Influence of static time on PLE.

In the static mode, the matrix is in contact with the solvent for a period of equilibrium at constant pressure and temperature. During this static time, the analytes are extracted from the sample. This period affects analyte recovery and hence to study this, different extractions were carried out at 3, 5, 10 and 15 min, respectively, obtaining the signals shown in Fig. 4. The main extraction occurred during the first 5 min; longer times increased the cycle time without improving extraction. Therefore, a static time of 5 min was chosen. The static process can be repeated several times if low recoveries are obtained in a single step. In this case, no significant differences were observed when the number of cycles was increased. Furthermore, the use of two or more cycles increased analysis time; as a consequence, one extraction cycle was used.

3.3. Analytical characteristics

The analytical characteristics of the proposed methodology, including pressurized liquid extraction of vitamins, separation of analytes by liquid chromatography and amperometric detection, were obtained.

Analyte quantification was performed using the external standard method. The relationships between chromatographic peak areas and vitamin concentrations were linear in the 0.1–4 mg/L range. The equations are shown in Table 1. The table also includes the detection limits, the inter-day and intra-day precisions and the recoveries. To study the recovery of the method, extraction and determination were carried out with spiked and unspiked samples. The spiked amount of each analyte was close to that of the original sample.

Using the proposed methodology, a sample of milk powder with a certified α-tocopherol content of 99 mg/kg was analyzed (CRM 421). The chromatogram obtained, Fig. 5a revealed the peak of γ-tocopherol but not that of α-tocopherol. In contrast, when alkaline hydrolysis was carried out before chromatographic determination (Fig. 5b), α-tocopherol eluted in about 12 min. All procedures used in the analysis of the certified sample included alkaline hydrolysis, as can be seen in the BCR information. It was possible to conclude that the Vitamin E in the certified sample was present as α-tocopheryl acetate.

The oxidation of α-tocopheryl acetate on a glassy carbon electrode requires higher potentials than α-tocopherol. Whereas the alcohol is oxidized at 300–400 mV, the ester requires a potential of 1300 mV and it elutes 2 min later in the chromatographic

Table 1
Analytical characteristics of the proposed method

	Intercept $\pm$ S.D. (UA)	Slope $\pm$ S.D. (UA) (mg/L)	r^2 ^a	DL (3S/b) (mg/L)	Inter-day precision ^b (%)	Intra-day precision ^b (%)	Recovery (%)
Retinyl acetate	$(1 \pm 3) \times 10^4$	$(1.95 \pm 0.05) \times 10^6$	0.998	0.014	8.9	7.7	95
δ -Tocopherol	$(1 \pm 3) \times 10^4$	$(8.2 \pm 0.1) \times 10^5$	0.9991	0.034	9.2	6.2	92
γ -Tocopherol	$(2 \pm 2) \times 10^4$	$(7.8 \pm 0.1) \times 10^5$	0.998	0.036	8.7	4.2	106
α -Tocopherol	$(-3 \pm 1) \times 10^4$	$(6.37 \pm 0.05) \times 10^5$	0.9992	0.044	6.4	3.3	95

^a Nine standards of calibration.

^b Relative standard deviation for 10 replicates of a milk sample.

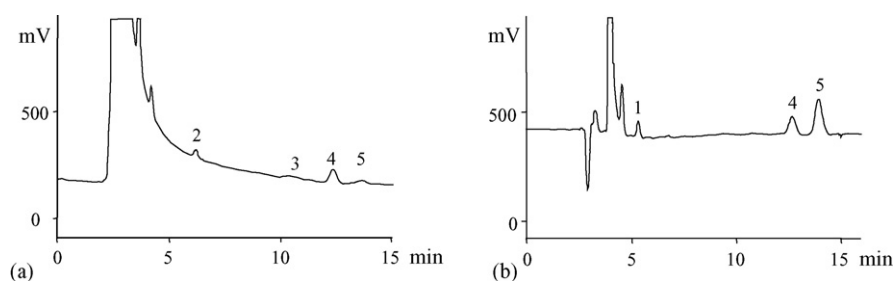


Fig. 5. Chromatograms obtained after application of the proposed method to a milk sample with certified content (a), and when classical sample treatment including alkaline hydrolysis was used (b). Peaks: see Fig. 1.

Table 2
Analysis of retinyl acetate and tocopherols in different infant formulas using PLE and LC with amperometric detection (results from three replicate analyses)

Infant formula	Retinyl acetate (mg/100 g)		δ -Tocopherol (mg/100 g)	$(\beta + \gamma)$ -Tocopherol (mg/100 g)	α -Tocopherol (mg/100 g)	
	Found	Label ^a	Found	Found	Found	Label ^a
Starter hypoallergic	0.74 ± 0.05	0.62	–	1.33 ± 0.08	5.30 ± 0.08	6.1
Starter adapted proteins and without lactose	0.59 ± 0.05	0.52	1.1 ± 0.1	3.4 ± 0.1	10.1 ± 0.1	11
Follow-up	0.60 ± 0.06	0.57	1.27 ± 0.05	3.28 ± 0.06	6.78 ± 0.05	7.4
Follow-up with <i>bifidobacterium</i>	0.67 ± 0.07	0.57	1.93 ± 0.04	5.00 ± 0.03	7.45 ± 0.04	7.4

^a Only the amounts of Vitamin A and α -tocopherol are labelled.

system. Oxidation at 1300 mV produces a high background noise in the chromatographic system and under these conditions it was not possible to evaluate α -tocopheryl acetate. The BCR information about this sample included a γ -tocopherol content of 4.74 mg/100 g. This analyte was evaluated and the content obtained was 4.5 ± 0.2 mg/100 g. This is in agreement with that indicated for the sample. Consequently, the accuracy is acceptable.

Infant formulas may be fortified by the addition of α -tocopherol or α -tocopheryl acetate during their manufacture. The addition of α -tocopheryl acetate ensures the Vitamin E content, but, regarding its stability, it is doubtful whether it can act as an antioxidant during the shelf-life of the product. Nevertheless, α -tocopherol plays an antioxidant role, contributing to the stability of the lipid fraction in infant formulas [24].

3.4. Determination of Vitamins A and E in infant formulas

Analysis of different kinds of milk powder samples was carried out applying the proposed procedure. Fig. 1 shows the chromatograms obtained for two of these samples.

The samples were analyzed in triplicate and the results are shown in Table 2. The retinyl acetate and α -tocopherol contents were in agreement with those indicated by the manufacturer. Infant formulas are fortified with retinyl acetate, α -tocopherol or α -tocopheryl acetate. In addition, different tocopherols are also present in these samples coming from the vegetable oils used in their manufacture. In a previous work, the tocopherol contents of different kinds of oils were evaluated [23] and, taking into account these data and the oil content in these formulas (24–30 g/100 g), the milk powder samples could have contents in the ranges 0.25–2.5 and 0.25–12.5 mg/100 g for δ -tocopherol and $(\beta + \gamma)$ -tocopherol, respectively. As shown in Table 2, the amounts calculated are within these ranges.

4. Conclusions

Pressurized liquid extraction is appropriate for the isolation of retinyl acetate and tocopherols in infant formulas, with the advantages inherent to this technique such as the low consumption of solvent used, automation and speed. After extraction it is only necessary to refrigerate the samples and no other cleaning

step is required. The reduction in sample manipulation, implying a decrease in error propagation, is of great interest. Additionally, pressurized liquid extraction permits chromatographic evaluation of the analytes in the same form as they are present in the samples. The method can be recommended for routine analysis.

Acknowledgements

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Development and validation of an improved liquid chromatographic method for the analysis of dirithromycin

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Abstract

The official method for the determination of dirithromycin and related substances in the European Pharmacopoeia (Ph. Eur.) and in the United States Pharmacopoeia (USP) is an isocratic liquid chromatographic (LC) method using an ODS column. With this method, the separation of the main component dirithromycin from its epimer is not complete. Moreover, this method suffers sometimes from drift of the baseline and from subsequent quantitation problems. The required resolution is not easy to obtain.

Using an adapted method derived from the one prescribed in the pharmacopoeias, the selectivity of a set of more than 40 reversed-phase columns towards dirithromycin components was investigated. The selection of the most suitable column was achieved by the chromatographic response function (CRF) approach. Several changes were introduced to the method in order to improve the separation and to overcome the baseline drift problem. The resulting method uses a Zorbax Extend column maintained at 30 °C and a mobile phase containing acetonitrile, methanol, 2-propanol, water and a phosphate buffer at pH 7.5. The method allows a good separation of dirithromycin components, which is much better than that obtained with the existing methods. Several impurities of unknown identity are also separated. The method shows good repeatability, linearity and sensitivity, and it is robust. In addition, it proved to be applicable to a wide number of C18 reversed-phase columns.

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Keywords: Validation; LC; Dirithromycin; Stationary phases; Macrolide; Antibiotics

1. Introduction

Dirithromycin (DIR) is a semi-synthetic oxazine derivative of erythromycin A that exhibits antibacterial activity against a variety of gram-positive and gram-negative bacteria [1–12]. The structures of DIR and related substances are shown in Fig. 1. Dirithromycin B (DIR B) and dirithromycin C (DIR C), respectively described by the European Pharmacopoeia (Ph. Eur.) as impurity C and impurity D, are produced from the corresponding erythromycin B and erythromycin C present in the starting material. Erythromycin A hydrazone (EAH) and erythromycylamine A (ERYL), described as impurity A by the Ph. Eur., are precursors in the synthesis of DIR and may be present at low levels in samples. ERYL may also arise from hydrolysis of DIR in neutral or slightly acidic solution. DIR forms an equilibrium amount of epi-dirithromycin (EDIR) in solution with

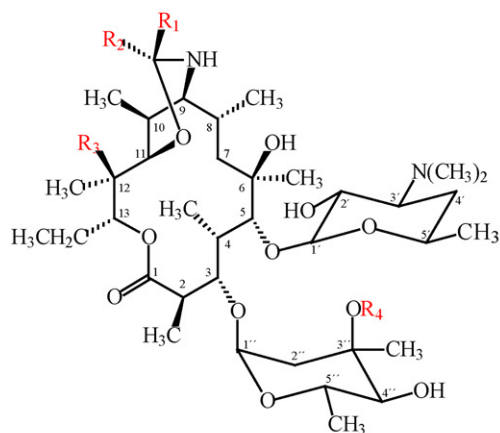
epimerization accelerated under acidic conditions [13–15]. Erythromycin B hydrazone (EBH), erythromycylamine B (ERYL B), erythromycylamine C (ERYL C) and erythromycylamine A acetone adduct (EAA) are other potential impurities. It is therefore important to have good analytical methods for the determination of DIR and its related substances.

Liquid chromatographic (LC) methods have been used for the analysis of DIR because of its ability to separate closely related compounds arising from biosynthetic, synthetic or metabolic processes [16–18]. Most of these methods were used to monitor the concentration of DIR in blood. The method described by Olsen et al. [16] allows the purity control of DIR. This method was the basis of the current official method in the Ph. Eur. [19] and in the United States Pharmacopoeia (USP) [20]. With this method, the separation of EDIR from the main component DIR is not complete. Moreover, this method suffers sometimes from drift of the baseline and from subsequent quantitation problems.

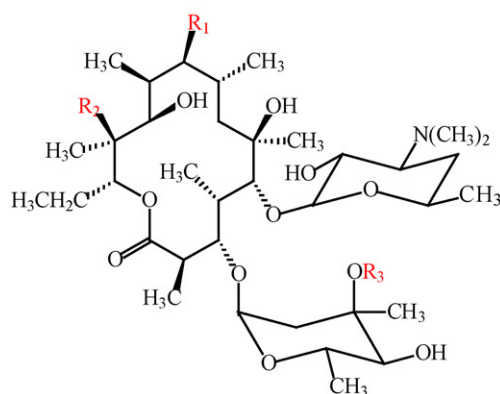
In this work, a more performant LC method suitable for the determination of DIR and related substances was developed. The method allows a good separation of dirithromycin components, which is much better than that obtained with existing methods.

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Compound	R ₁	R ₂	R ₃	R ₄
Dirithromycin (DIR)	H	CH ₃ OCH ₂ CH ₂ OCH ₂	OH	CH ₃
Epi-dirithromycin (EDIR)	CH ₃ OCH ₂ CH ₂ OCH ₂	H	OH	CH ₃
Dirithromycin B (DIR B)	H	CH ₃ OCH ₂ CH ₂ OCH ₂	H	CH ₃
Dirithromycin C (DIR C)	H	CH ₃ OCH ₂ CH ₂ OCH ₂	OH	H



Compound	R ₁	R ₂	R ₃
Erythromycylamine A (ERYL)	NH ₂	OH	CH ₃
Erythromycin A hydrazone (EAH)	NNH ₂	OH	CH ₃
Erythromycylamine B (ERYL B)	NH ₂	H	CH ₃
Erythromycin B hydrazone (EBH)	NNH ₂	H	CH ₃
Erythromycylamine C (ERYL C)	NH ₂	OH	H
Erythromycylamine acetone adduct (EAA)	NC(CH ₃) ₂	OH	CH ₃

Fig. 1. Chemical structures of dirithromycin and related substances.

The selectivity of a set of more than 40 reversed-phase columns towards DIR components was also investigated.

2. Experimental

2.1. Reagents and samples

Acetonitrile HPLC grade was purchased from Biosolve LTD (Valkenswaard, The Netherlands). 2-Propanol was obtained from Sigma–Aldrich (Steinheim, Germany). Methanol HPLC grade was purchased from VWR (Leuven, Belgium). 2-

Methyl-2-propanol (Merck Eurolab, Leuven, Belgium) was distilled before use. Dipotassium hydrogen phosphate was from Applichem (Darmstadt, Germany) and potassium dihydrogen phosphate was from BDH (Poole, England). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to purify glass-distilled water. Reference substances of DIR, EDIR, DIR B, DIR C, ERYL, ERYL B, ERYL C, EAA, EAH and EBH, as well as a DIR bulk sample were obtained from Eli Lilly and Company (Lafayette, IN, USA). Sample concentration and injection volume were chosen so as to provide adequate response without overloading the column and degrading the resolution.

A concentration of 10 mg/ml and an injection volume of 10 μ l were used. For identification purpose, solutions of reference substances were prepared at a concentration of about 0.5 mg/ml. The samples and the reference substances were dissolved in a mixture of methanol–acetonitrile (30:70). Fresh solutions of DIR sample were prepared just before injection as the amount of EDIR in the sample solutions increased gradually during storage. A refrigerated autosampler set at 4 °C was used to preserve the stability of these solutions during analysis. Under these conditions, sample solutions were stable over 6 h.

2.2. LC instrumentation and chromatographic conditions

The LC apparatus consisted of a L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany), a TSP Spectra Series

AS 100 autosampler (San Jose, CA, USA) equipped with a 10 μ l loop, a variable wavelength Merck Hitachi L-4200 UV–vis detector set at 205 nm and Chromeleon 6.50 software (Dionex Corporation, Sunnyvale, CA, USA) for data acquisition. Specifications about the different columns examined are given in Table 1. In the final method, a Zorbax Extend C18 column (250 mm $\times$ 4.6 mm i.d.), 5 μ m (Agilent Technologies), maintained at 30 °C in a water bath heated by means of a Julabo EC thermostat (Julabo, Seelbach, Germany), was used. A mobile phase containing acetonitrile–methanol–2-propanol–0.2 M potassium phosphate buffer pH 7.5–water (425:135:45:100:295, v/v/v/v) was used at a flow rate of 1.0 ml/min. The mobile phase was degassed by sparging helium. The 0.2 M potassium phosphate buffer was prepared by mixing 0.2 M potassium dihydrogen phosphate solution with 0.2 M

Table 1
Specifications for the examined columns

Column number	Name of the column	Length (mm)	Particle size (μ m)	Manufacturer/supplier
c1	Ace	250	5	Advanced Chrom. Tech./Achrom
c2	Alltima	250	5	Alltech
c3	Apex Basic	250	5	Jones Chromatography/Sopachem
c4	Apex ODS II	250	5	Jones Chromatography/Sopachem
c5	Astec	250	5	Advanced Separations Technologies
c6	Bondapak	250	10	Jones Chromatography/Sopachem
c7	Brava BDS	250	5	Altech
c8	Discovery	250	5	Supelco
c9	Hypersil BDS	250	5	ThermoQuest
c10	Hypersil BDS	250	5	ThermoQuest
c11	Hypersil ODS (New)	250	5	ThermoQuest
c12	Kromasil (EKA)	250	5	Akzo Nobel/SerCoLab
c13	Kromasil (MN)	250	5	Macherey-Nagel/Filter service
c14	LiChrospher	250	5	Merck
c15	Luna	150	5	Phenomenex/Bester
c16	Nucleosil	250	5	Macherey-Nagel/Filter service
c17	Nucleosil HD	250	5	Macherey-Nagel/Filter service
c18	Nucleosil Nautilus	250	5	Macherey-Nagel/Filter service
c19	OmniSpher	250	5	Varian
c20	Platinum	250	5	Alltech
c21	Platinum EPS	250	5	Alltech
c22	Prevail	250	5	Alltech
c23	Purospher	250	5	Merck
c24	Purospher endcapped	250	5	Merck
c25	Purospher Star	250	5	Merck
c26	Spheri	250	5	Perkin-Elmer
c27	Spherisorb ODS2	250	5	Waters
c28	Supelcosil LC	250	5	Supelco
c29	Supelcosil LC-DB	250	5	Supelco
c30	Supelcosil LC-ABZ	250	5	Supelco
c31	Superspher	250	5	Merck
c32	Symmetry	250	5	Waters
c33	Synergi	150	4	Phenomenex/Bester
c34	TracerExcel ODS	250	5	TeknoKroma/SerCoLab
c35	Uptisphere HDO	250	5	Interchrom/Achrom
c36	Uptisphere ODB	250	5	Perkin-Elmer
c37	Validated	250	5	Perkin-Elmer
c38	Validated (New)	250	5	Perkin-Elmer
c39	Wakosil HG	250	5	SGE/Achrom
c40	XTerra	250	5	Waters
c41	YMC-Pack Pro	150	5	YMS Sep. Techn./ThermoQuest
c42	Zorbax Eclipse XDB	250	5	Agilent Technologies
c43	Zorbax Extend	250	5	Agilent Technologies
c44	Zorbax SB	250	5	Agilent Technologies

dipotassium hydrogen phosphate solution until the required pH was reached.

2.3. Experimental design

A robustness study was performed by means of an experimental design and multivariate analysis using Modde 5.0 software (Umetrics, Umea, Sweden). A central composite design was applied. The central composite design is composed of a full or fractional factorial design, star points and replicated centre point. The star points enable the model to estimate the curvature response. These star points are located at the centre and both extreme levels of the experimental domain. For a complete central composite design, which includes points of a two level full factorial design, the number of runs is equal to $2^k + 2k + n$, where k is the number of parameters and n is the number of centre points. In this study, five parameters (percentage of acetonitrile, percentage of 2-propanol, percentage of methanol, column temperature and mobile phase pH) were investigated. With this number of parameters and three centre points, a complete central composite design would result in a number of runs equal to 45. In order to reduce the number of runs, a central composite design which includes points of a two level half fractional factorial design was chosen, with a number of runs equal to $2^{k-1} + 2k + n = 29$. The statistical relationship between a response Y and the experimental variables $X_i, X_j, \dots$ is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \dots + E \quad (1)$$

where the β 's are the regression coefficients and E is the overall experimental error.

The linear coefficients β_i and β_j describe the quantitative effect of the respective variables. The cross-coefficient β_{ij} measures the interaction effect between the variables and the square terms $\beta_{ii} X_i^2$ and $\beta_{jj} X_j^2$ describe the nonlinear effects on the response.

3. Results and discussion

3.1. Preliminary investigation

At the first stage of the method development, DIR samples were analysed using the official method currently prescribed in the Ph. Eur. [19] and in the USP [20]. This method uses a C18 (250 mm $\times$ 4.6 mm i.d.), 5 μ m, stationary phase. The column temperature is set at 40 °C and the UV detection is performed at 205 nm. The mobile phase consists of 9 volumes of water; 19 volumes of methanol; 28 volumes of a solution containing 1.9 g/l of potassium dihydrogen phosphate and 9.1 g/l of dipotassium hydrogen phosphate adjusted to pH 7.5 using potassium hydroxide solution (100 g/l); 44 volumes of acetonitrile. The flow rate is 2.0 ml/min.

A typical chromatogram obtained with a DIR sample under these conditions, using a Hypersil BDS C18 column, is shown in Fig. 2. As can be seen, EDIR is not completely separated

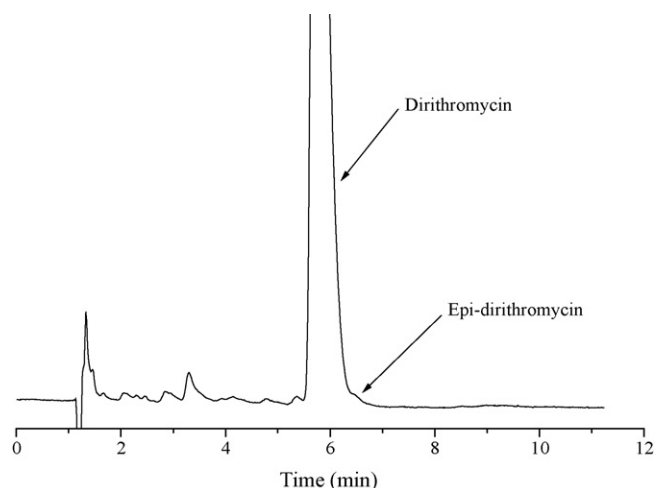


Fig. 2. Typical chromatogram obtained by analysing a DIR bulk sample according to the current Ph. Eur. or USP method. Stationary phase: Hypersil BDS C18 (ThermoQuest, Cheshire, UK). Column temperature: 40 °C; mobile phase: potassium phosphate buffer pH 7.5–acetonitrile–methanol–water (28:44:19:9, v/v/v/v), at a flow rate of 2 ml/min; UV detection: 205 nm; 10 μ l of 10 mg/ml solution were injected.

from the main compound DIR. Analysing the system suitability solution (2 mg/ml solution of DIR in the mobile phase, allowed to stand at room temperature for 24 h), the prescribed resolution between DIR and EDIR (resolution of at least 2) was not reached, even after adjustment of the organic modifiers in the mobile phase. This poor resolution was observed with other batches of the Hypersil BDS column and on other C18 reversed-phase columns as well.

Several changes were introduced in the chromatographic parameters to improve the separation of the critical pair of peaks DIR–EDIR. Varying the pH in the range from 6.0 to 8.0 and the temperature in the range from 25 to 60 °C did not improve the separation. Three other reversed-phase columns (XTerra, Prevail and Astec) were also investigated. The Xterra column was chosen since this stationary phase previously allowed good separation of erythromycin and its related substances [21]. The Prevail column, which is reported to have similar selectivity as the Xterra column was also included. Conditions of high pH and elevated column temperature have been reported to improve the separation and the peak shape of macrolide antibiotics [16]. Therefore, the use of stationary phases with high stability, such as polymer columns, was considered. However, poor resolution and peak shape were observed by Kibwage et al. [22], using conventional polymer columns. Since Astec, a C18 derivatized polyvinyl alcohol column gave good separation for erythromycin [23], this stationary phase was also investigated for its suitability in the analysis of DIR components. However, none of the different columns investigated resulted in an improved separation.

3.2. Selectivity of reversed-phase columns towards DIR components

As none of the conditions described above allowed good separation of DIR and related substances, it was decided to check

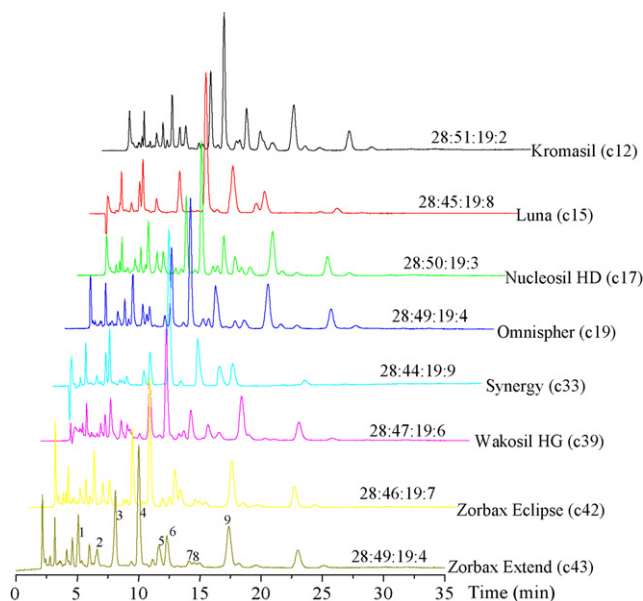


Fig. 3. Separation of DIR and related substances using the selected columns. Mobile phase: potassium phosphate buffer pH 7.5–acetonitrile–methanol–water in the ratios indicated, at a flow rate of 1 ml/min. A mixture containing ERYL C (1), DIR C (2), ERYL (3), EAH (4), ERYL B (5), DIR (6), EDIR (7), EBH (8) and EAA (9) was analysed.

a wider number of C18 columns (44 columns; see Table 1) for their selectivity towards DIR components, using an LC method derived from the one prescribed by the Ph. Eur. and the USP. The content of acetonitrile in the mobile phase was adjusted for each column to have similar retention times of DIR for the different columns. A mixture containing DIR, EDIR, ERYL, ERYL B, ERYL C, EAA, EAH and EBH was analysed. The selectivity between closely eluting peaks was monitored to select the best columns.

Among the 44 columns examined, 17 columns (c1, c2, c8, c10, c12, c15, c17, c19, c24, c25, c32, c33, c38, c39, c41, c42, and c43) gave clearly better separation of DIR components compared to the others. None of these 17 columns showed superior selectivity over the others. Decreasing the flow rate down to 1 ml/min and adjusting the acetonitrile content improved the separation. Under these conditions, the separation obtained with the columns c12, c15, c17, c19, c33, c39, c42 and c43 was superior to the others and these columns were selected for further investigations. Typical chromatograms are shown in Fig. 3. The order of elution for the different compounds was the same, except for ERYL B, which was eluted just before the main peak DIR using a Zorbax Extend column and just after using the other columns. The selection of the most suitable column was achieved by the chromatographic response function (CRF) approach [24–27].

The CRF is calculated as follows:

$$\text{CRF} = \prod_{i=1}^{n-1} \frac{f_i}{g_i}$$

where n is the number of compounds taken into consideration, g the interpolated peak height, i.e. the distance from the baseline to the line connecting two peak tops at the location of the valley, and f is the depth of the valley, measured from the line connecting

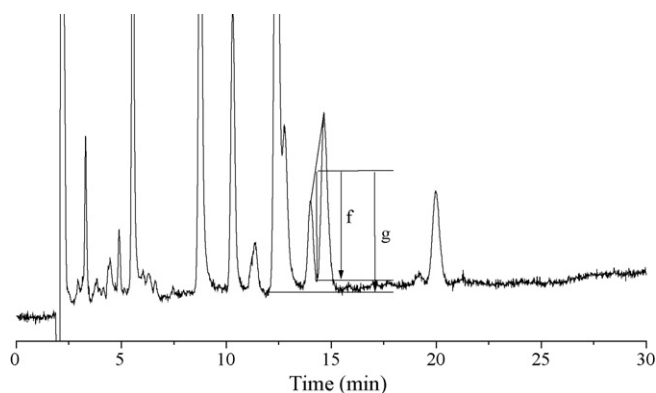


Fig. 4. A typical chromatogram showing the measurement of f and g for the calculation of the chromatographic response function (CRF) for one pair of peaks. Column: Omnispher (250 mm $\times$ 4.6 mm i.d.), 5 μ m, maintained at 40 °C. Mobile phase and sample are as indicated for Omnispher in Fig. 3.

the two peak tops (see Fig. 4). CRF values always lie between 0 (two or more peaks not separated from each other) and 1 (all peaks completely separated from each other). The use of these values is described for thin layer-chromatographic methods [25], but they can be used in LC as well [26,27].

For the above selected columns, the CRF values for dirithromycin and related substances, present in the mixture shown in Fig. 3, were calculated (Table 2). It is observed that all CRF values are well below 1. This is due to the poor separation of the peak pairs ERYL B–DIR and EDIR–EBH. Moreover, CRF values of 0 were found for Luna, Omnispher, Synergi and Wakosil columns, as no separation was obtained for the pairs ERYL B–DIR and/or EDIR–EBH. The highest CRF value was obtained with Zorbax Extend column. Moreover, this column is reported to be stable in the pH range from 2 to 11.5. It was therefore selected for further investigations.

3.3. Improvement of the liquid chromatographic method

A good overall separation of DIR components was obtained, using a Zorbax Extend column maintained at 40 °C and a mobile phase consisting of acetonitrile–methanol–phosphate buffer pH 7.5–water (49:19:28:4, v/v/v/v). However, the pairs ERYL B–DIR and EDIR–EBH were insufficiently separated. On the other hand, under these conditions, a drift of the baseline was observed.

Table 2
Chromatographic response function (CRF) for dirithromycin and related substances obtained with different columns

Column	CRF
Kromasil (c12)	0.316
Luna (c15)	0.000
Nucleosil HD (c17)	0.237
Omnispher (c19)	0.000
Synergi (c33)	0.000
Wakosil (c39)	0.000
Zorbax Eclipse (c42)	0.230
Zorbax Extend (c43)	0.321

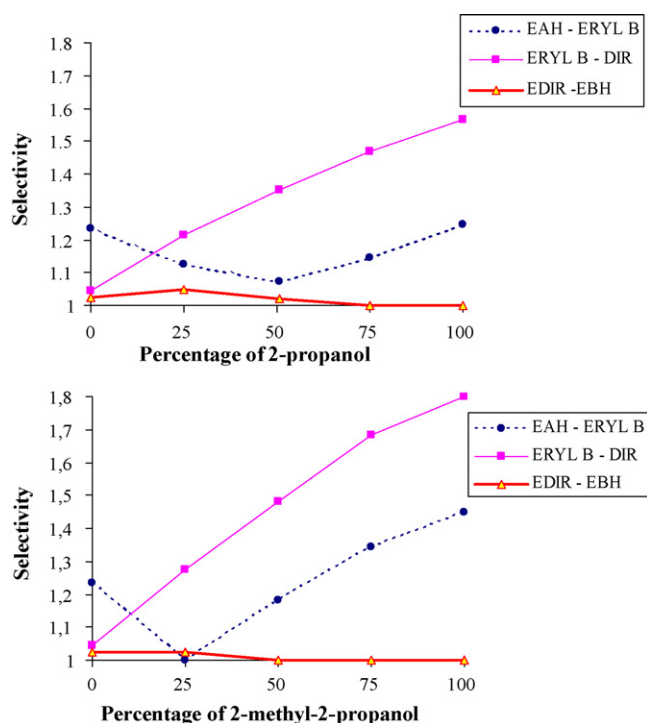


Fig. 5. Effect of increasing amounts of 2-propanol or 2-methyl-2-propanol in the “alcohol” part of the mobile phase on the selectivity for the pairs EAH–ERYL B, ERYL B–DIR and EDIR–EBH. The selectivity was calculated as the relative retention. The percentages indicate the amount of methanol in the mobile phase which was replaced by 2-propanol or 2-methyl-2-propanol.

The use of comodifiers such as 2-propanol or 2-methyl-2-propanol has been shown to improve the selectivity of erythromycin components [28]. The effect of these alcohols on the separation of DIR components was therefore investigated by gradually replacing methanol in the mobile phase. The ratio acetonitrile–“alcohol” was kept constant (49:19) for all these experiments, but the mobile phase strength was adapted to have similar retention times for the main component DIR. The column temperature was lowered down to 30 °C; resulting in a more stable baseline. Most of the conditions investigated allowed good separation of DIR components, except for the pairs EAH–ERYL B, ERYL B–DIR and EDIR–EBH. Fig. 5 shows the effect of increasing amounts of 2-propanol or 2-methyl-2-propanol in the mobile phase on the separation of these pairs of peaks. It is observed that EDIR and EBH were coeluted with most of the conditions investigated. The best selectivity (expressed as the relative retention) [19] for these 2 peaks was obtained when 25% of methanol in the mobile phase were replaced with 2-propanol. Although little improvement of the selectivity of these peaks was achieved under these conditions, the separation of all the components, including the pair ERYL B–DIR, was much better, thanks to a better peak shape. These conditions were therefore selected. Typical chromatograms obtained by analysing a dirithromycin bulk sample and a mixture of dirithromycin and related substances are shown in Fig. 6. The method allows a good separation of dirithromycin components, which is much better than that obtained with existing methods. Several impurities of unknown identity are also separated. This method should

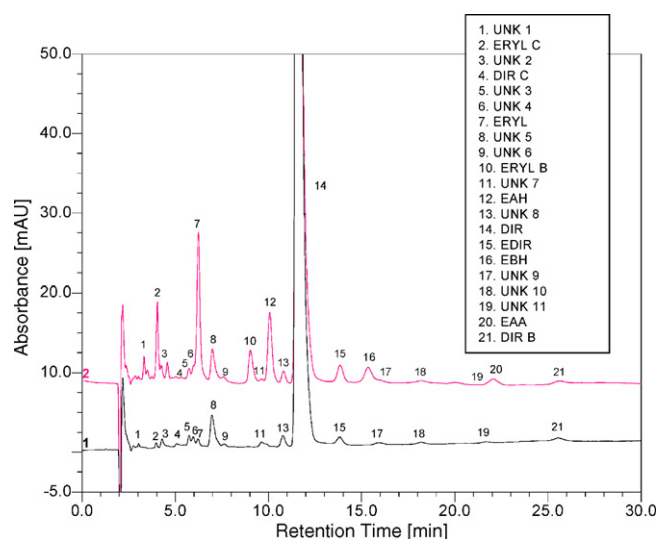


Fig. 6. Typical chromatograms of a DIR bulk sample (1) and the same sample spiked with ERYL C, ERYL, ERYL B, EAH, EBH and EAA (2). Column: Zorbax Extend. Mobile phase: acetonitrile–methanol–2-propanol–0.2 M potassium phosphate buffer pH 7.5–water (425:135:45:100:295, v/v/v/v/v). UV detection: 205 nm. Flow rate: 1 ml/min. Injection volume: 10 µl. Column temperature: 30 °C. UNK (unknown): unidentified peak.

allow a more correct quantitation of DIR components, as they are now better separated. In further investigations of the intermediate precision of the method, it was observed by analysing a bulk sample that the use of different batches of the Zorbax Extend column (brand-new column and old columns) gave very similar results. The old column gave somewhat better separation. This phenomenon was already observed with erythromycin analyses [29,30]. It was believed that a certain level of silanol activity might be beneficial to the chromatography or that removal of residual metals through aging might also play a role since this can influence the acidity of silanols. The use of a different LC equipment and the use of different brands of columns (namely those described in Fig. 3 and Table 2) also gave good results.

3.4. Robustness study

The effect of small changes of the chromatographic parameters on the separation was investigated by means of an experimental design using Modde 5.0 software. The different chromatographic parameter settings in the design are given in Table 3. The individual and interaction effects on the resolution for the most important separations, namely ERYL B–EAH (Rs_1), EAH–DIR (Rs_2), DIR–EDIR (Rs_3) and EDIR–EBH (Rs_4), are

Table 3

Chromatographic parameter settings applied in the robustness investigation, corresponding to low (–), central (0) and high (+) levels

Parameter	Low value (–)	Central value (0)	High value (+)
pH	7.3	7.5	7.7
Temperature (°C)	27	30	33
Acetonitrile (ml)	415	425	435
Methanol (ml)	130	135	140
2-Propanol (ml)	42	45	48

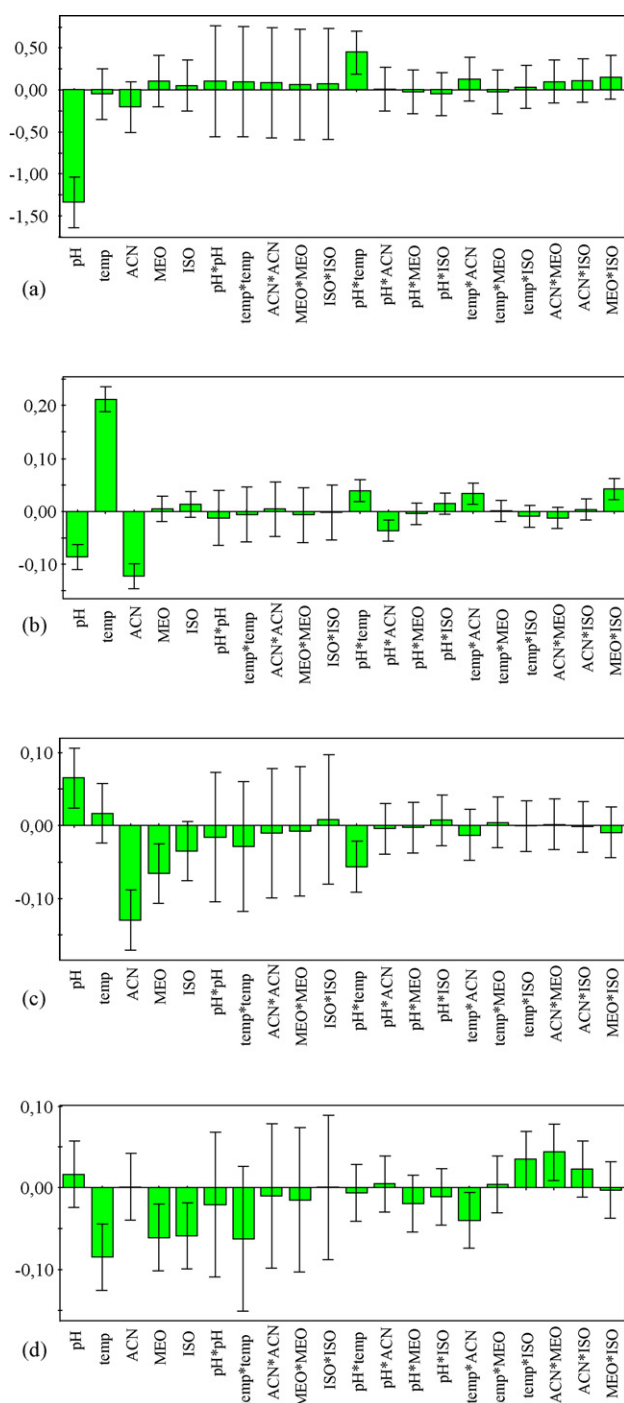


Fig. 7. Regression coefficient plots for the separation of the pairs (a) ERYL-B-EAH (Rs_1), (b) EAH-DIR (Rs_2), (c) DIR-EDIR (Rs_3) and (d) EDIR-EBH (Rs_4): temp, temperature; CAN, acetonitrile; MEO, methanol; ISO, 2-propanol.

summarised in Fig. 7. The plot (Fig. 7) consists of bars, which correspond to the regression coefficients. The bars denoted by variable * variable reflect the regression coefficient for the non-linear effect of that particular variable, whereas the bars denoted by variable 1 * variable 2 reflect the interaction between the two variables concerned. The 95% confidence interval is expressed in terms of an error line over the coefficient. When the coefficient is smaller than the interval, the variation of the response

caused by changing the variable is smaller than the experimental error and the effect is considered not to be significant. The magnitude of the effect is proportional to the regression coefficient (see Eq. (1)). A positive regression coefficient stands for a positive effect on the separation, while a negative regression coefficient indicates a negative effect. It is observed that the pH has the most important effect on Rs_1 . This effect is negative. The effects of the other parameters are not significant. A positive interaction is observed between pH and temperature. All the other interaction effects and all the quadratic effects are found to be not significant. The prediction plot of Rs_1 as a function of pH (data not shown) indicated that an increase of pH might decrease Rs_1 down to 1.5. The temperature has the most important effect on Rs_2 , followed by acetonitrile and pH. This effect is positive for the temperature, while it is negative for pH and acetonitrile. A positive interaction between methanol and 2-propanol is also observed. Acetonitrile has the most important effect on Rs_3 , followed by pH and methanol. This effect is negative for acetonitrile and methanol, while it is positive for pH. A negative interaction is observed between pH and temperature. All the other interaction effects and all the quadratic effects are found to be not significant. For Rs_4 , the temperature has the most important effect, followed by methanol and 2-propanol. No distinctly important interaction or quadratic effects are observed. In order to estimate better the influence of the parameters with the most important effects on Rs_2 , Rs_3 and Rs_4 , response surface plots were constructed. Fig. 8 shows, in the top row, the variation of Rs_2 as a function of temperature, pH and acetonitrile, while the other parameters are kept constant at their central values. The middle row shows the variation of Rs_3 as a function of acetonitrile, methanol and pH, while the lower row shows the variation of Rs_4 as a function of temperature, methanol and 2-propanol; the other parameters being kept constant at their central values. It is observed that in the ranges examined, Rs_2 , Rs_3 and Rs_4 are always well above 2. This means that small variations in the chosen chromatographic conditions will not have a harmful effect on the resolution for the different pairs investigated. It can therefore be concluded that the developed method is robust.

3.5. Quantitative aspects

The precision, the sensitivity and the linearity of the method were evaluated using DIR reference substance and a DIR bulk sample. To evaluate the precision of the method, intra-day and

Table 4
Repeatability data for DIR and its impurities

	ERYL	DIR	EDIR	DIR B	Number of experiments
Component level (%)	0.4	91.1	0.9	0.4	
Intra-day R.S.D. (%)					
Day 1	2.6	0.7	1.4	2.1	6
Day 2	2.6	0.6	1.4	3.6	6
Day 3	4.3	0.7	1.5	1.2	6
Inter-day R.S.D. (%)	3.2	0.8	2.4	3.1	18

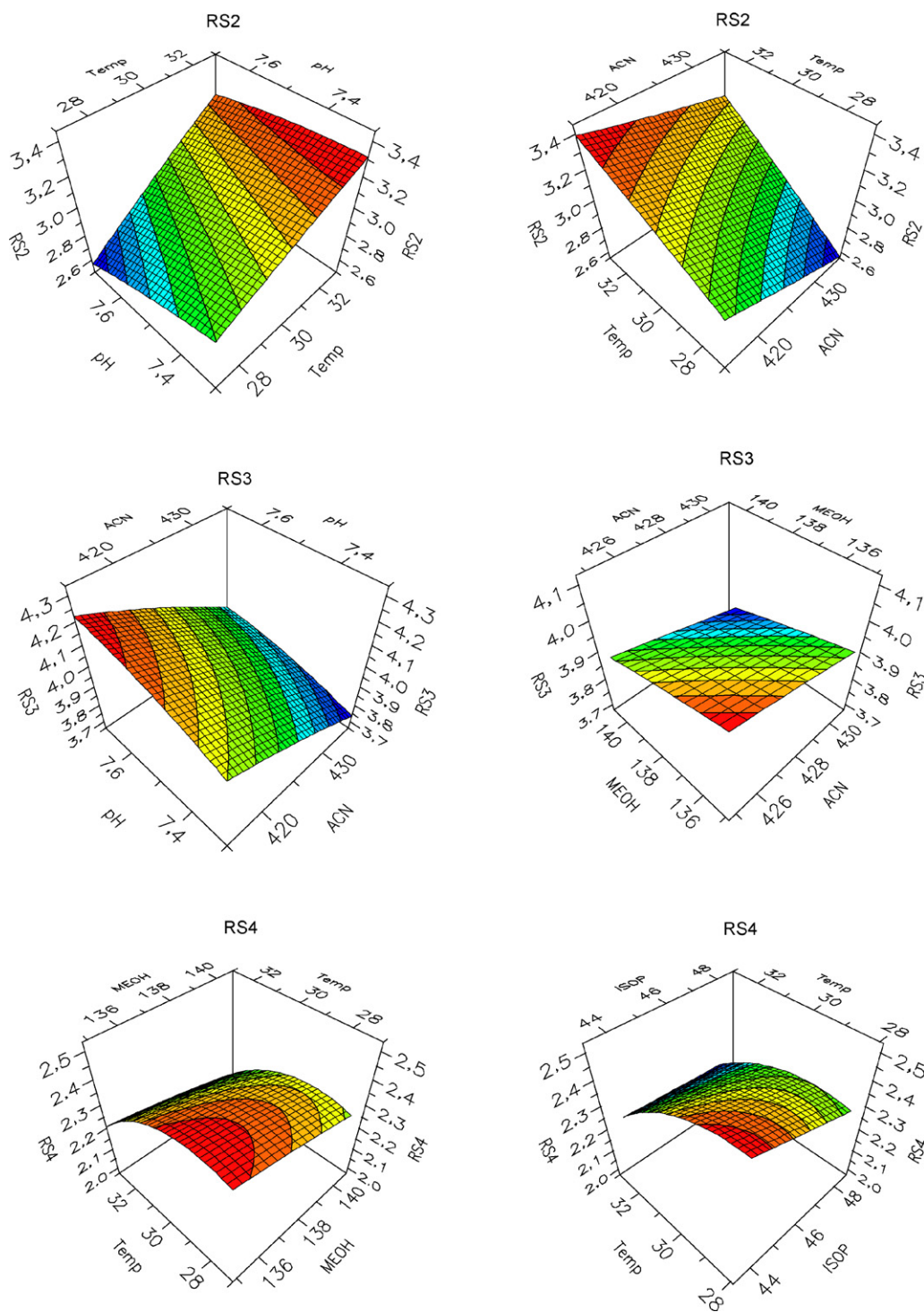


Fig. 8. Response surface plots for the separation of the pairs EAH–DIR (RS₂), DIR–EDIR (RS₃) and EDIR–EBH (RS₄): temp, temperature; CAN, acetonitrile; MEOH, methanol; ISOP, 2-propanol.

inter-day assays were run. The DIR bulk sample was assayed in replicate ($n=6$) in a single day and on each of three successive days. The relative standard deviation (R.S.D.) values for both intra-day and inter-day repeatability are summarized in Table 4. The results obtained demonstrate the good precision of the method. The limits of quantitation (LOQ) (corresponding to a signal-to-noise ratio of 10) were assessed for the main

compound DIR. An LOQ value of 0.05% (R.S.D. = 4.6%, $n=6$), calculated versus the amount of substance brought onto the column ($100\text{ }\mu\text{g} = 100\%$), was found. Comparing these data to those reported by Olsen et al. [16], the LC method developed here shows better sensitivity. For the main compound, the linearity of the method was checked over a concentration range of 25–125% (25–125 μg injected). For the related substances, dilute stan-

Table 5
Linearity data for DIR and related substances

	Concentration range	Regression equation	$S_{y,x}$	R^2	n_c	n_i
DIR	25–125%	$y = 1.3247x + 1.4863$	1.0167	0.9998	5	3
Related substances	LOQ–5%	$y = 1.3357x - 0.0615$	0.1036	0.9991	6	3

R^2 : coefficient of determination; $S_{y,x}$: standard error of estimate; n_c : number of experimental concentrations studied; n_i : number of injections for each concentration; y : peak area; x : concentration (%).

dards of DIR were used in the range from LOQ to 5%. Data shown in Table 5 demonstrate the good linearity of the method.

3.6. System suitability

A system suitability test that is based on the same approach employed in the current method [16,19,20] is proposed. It is developed so that no sample of related substances is needed. The system suitability solution is prepared by dissolving 20 mg of DIR sample in 10 ml of mobile phase and allowing to stand at room temperature for 24 h. Under these conditions, DIR is partially converted into EDIR (relative retention time = 1.2) and ERYL (relative retention time = 0.4). The symmetry factor for the main peak DIR and the resolution between ERYL and DIR as well as the resolution between DIR and EDIR were established as system suitability parameters. The proposed limits are 1.3 for the symmetry factor, 10 for the resolution between ERYL and DIR and 2.5 for the resolution between DIR and EDIR. When these criteria are not met, the organic modifier content in the mobile phase should be adjusted slightly.

4. Conclusion

An improved LC method was developed for the determination of DIR and related substances. The method allows good separation of DIR components, which is much better than that obtained with existing methods. This method shows better selectivity and higher efficiency than the Ph. Eur. and USP method, especially towards EDIR and the main component DIR that are now well separated. The method is also linear, repeatable and sensitive. In addition, this method proved to be robust and it is a suitable alternative to official methods for the analysis of DIR.

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Ion-selective electrodes for determination of organic ammonium ions: Ways for selectivity control

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Abstract

The influence of the ISE membrane composition on the selectivity for primary, secondary, tertiary, and quaternary alkylammonium cations, as well as for cations of physiologically active amines, has been investigated. Factors studied include the effect of plasticizer (2-nitrophenyl octyl ether, *o*-NPOE; dibutyl phthalate, DBP; dinonyl adipate, DNA; tris(2-ethylhexyl) phosphate, TEHP) and ion exchanger (potassium tetrakis(4-chlorophenyl)borate, K(TpClPB); potassium tris(nonyloxy)benzenesulfonate, K(TNOBS)), as well as that of the lipophilic cationic additive (tetradecylammonium nitrate, (TDA)NO₃) and neutral carrier (dibenzo-18-crown-6) presence in membrane. It has been established that plasticizer nature affects $K_{i,j}^{\text{pot}}$ values both when the target and/or foreign ions have non-ionic polar groups capable of specific interaction with plasticizer, and when the only difference consists in the substitution degree of their ionic groups. $K_{i,j}^{\text{pot}}$ values for quaternary alkylammonium cations over primary–tertiary ones change in the following order: TEHP > DBP $\approx$ DNA > *o*-NPOE. The highest $K_{i,j}^{\text{pot}}$ value change is achieved for the primary–quaternary alkylammonium cation pair, amounting to 3 and 4.7 orders for membranes containing K(TNOBS) and K(TpClPB) as ion exchangers, respectively. In its turn, the ion exchanger influence on the selectivity depends on plasticizer nature, it being maximal for *o*-NPOE (about 2 orders) and practically non-existent for TEHP. On the whole, as compared to K(TpClPB)-based ISEs, those based on K(TNOBS) show higher selectivity for primary–tertiary alkylammonium cations over quaternary ones. Incorporation of (TDA)NO₃ into membrane causes further improvement of selectivity for primary–tertiary alkylammonium cations in the case of K(TNOBS) only. The maximal total effect of the ion exchanger and lipophilic ionic additive is observed for ISEs with DNA-plasticized membranes and is over 3 orders. The influence of crown ether on the selectivity also depends significantly upon ion exchanger and plasticizer nature. For ISEs with *o*-NPOE-plasticized membranes the $K_{i,j}^{\text{pot}}$ value changes can be as great as 3 (ion exchanger K(TNOBS)) and even 4.5 (ion exchanger K(TpClPB)) orders. On the contrary, for ISEs with TEHP-plasticized membranes the crown ether effect on the selectivity is unessential. The results obtained are explained by peculiarities of organic ammonium cations solvating by plasticizer and association of cations with ion exchangers.

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Keywords: Cation-selective electrodes; Physiologically active amines; Potentiometric selectivity; Ion exchanger influence; Ionic additive

1. Introduction

Development of ion-selective electrodes (ISEs) responsive to amine-type substances continues to be of interest, on the one hand, due to obvious advantages of a potentiometric analysis technique (simplicity, speed, sensitivity, selectivity, applicability to turbid and colored solutions, etc.) and, on the other hand,

for the fact that many drugs used widely in modern pharmacy, are, from the chemical viewpoint, primary–tertiary amines or quaternary ammonium salts (QASes).

By now, many ISEs with liquid and film polymeric membranes responsive to organic ammonium cations have been described [1–8]. The key factor determining the possibility of using such ISEs is their sufficient selectivity to a principal ion over other sample solution components. However, despite the crucial importance of this characteristic, ways for controlling selectivity of such ISEs (in contrast to ISEs sensitive to inorganic ions) have not been studied enough and are not utilized fully.

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Varying plasticizers is quite an effective way of controlling ISE selectivity for amine cations containing non-ionic polar groups capable of being solvated [9]. For instance, plasticizers, which are strong Lewis bases, improve selectivity to amine cations with hydroxy groups whereas those containing “acid” hydrogen atoms increase selectivity to ions with basic polar groups. Besides, highly basic plasticizers can improve selectivity for primary amine cations over quaternary ones. It can sometimes cause inversion of selectivity series [9–11].

Besides, various ionophores (crown ethers, substituted calixarenes, cyclodextrins and their derivatives, and other macrocyclic or open-chained polydental ligands) were reported to be used in ISEs responsive to several QAS cations and some primary–tertiary amine cations [12]. The effect of selectivity increase caused by macrocyclic ionophores is best known for primary ammonium cations and is explained by neutral carrier complexing a protonated primary amine group (by analogy with ammonium and alkali metal cations) [13–16]. Nevertheless, no systematic investigation of the plasticizer and ion exchanger nature influence on this effect has been carried out yet.

Noticeably less attention is paid to the choice of liquid ion exchanger, although in some papers [17,18], it is noted that ion exchanger nature somewhat affects the selectivity of those ISEs. At the same time, it is well known that the selectivity of interaction between ion exchanger and analyte does not transform fully into potentiometric selectivity [19]. In ref. [20], it has been shown, using ISEs responsive to aliphatic amine cations as an example, that if an ion exchanger forms more stable ion associates with primary ions against foreign ones, significant selectivity increase can be achieved by incorporating lipophilic cationic sites into membrane. However, unlike anion-selective electrodes [21–24], in the case of such ISEs this approach has not received proper attention.

In the current paper, results of comprehensive systematic investigation of ISEs selectivity for simple alkylammonium cations with varied substitution degree, as well as for cations of physiologically active amines (PhAAs), are presented. Both “purely ion exchange” electrodes and ISEs containing a neutral carrier (dibenzo-18-crown-6) have been studied. Factors investigated include effects of plasticizer and ion exchanger nature, as well as (for ISEs without a neutral carrier) that of lipophilic cationic additive, on the selectivity.

2. Experimental

2.1. Reagents and solutions

For making ISE membrane the following reagents were used: 2-nitrophenyl octyl ether (*o*-NPOE), dibutyl phthalate (DBP), dinonyl adipate (DNA) and tris(2-ethylhexyl) phosphate (TEHP) as plasticizers; potassium tris(nonyloxy)benzenesulfonate (K(TNOBS)), potassium tetrakis(4-chlorophenyl)borate (K(TpClPB)) as ion exchangers; dibenzo-18-crown-6 as a neutral cation carrier; tetradecylammonium nitrate (TDA)NO₃ as a cationic site additive; poly(vinyl chloride) (PVC) as polymeric matrix; tetrahydrofuran (THF) as a solvent. All the chemicals were purchased from Fluka AG (Selectophore®), with the excep-

tion of K(TNOBS) synthesized at the Department of Analytical Chemistry of the Belarussian State University according to ref. [25]. Doubly distilled water was used throughout.

Stock aqueous solutions of di- and triethylammonium chlorides, and tetraethyl- and tetrabutylammonium bromides (all of analytical grade) were prepared by dissolving appropriate salt amounts in water. Solutions of other alkylammonium chlorides were prepared by the addition of a slightly excessive amine amount to an aliquot of HCl aqueous solution, followed by titrating the emulsion thus obtained with the same acid solution up to pH 6. In the case of benzyl-, methylbenzyl-, *iso*-butyl-, and butylamine 0.1 M HCl solution was used whereas for diamyl- and tributylamine the concentration of hydrochloric acid was 1×10^{-3} M. Dilute solutions of necessary concentrations were freshly prepared by diluting stock ones.

PhAA salts used, namely gangleronum, spasmolytinum, papaverinum, dimedrolum, remantadinum, chininum, trimecainum, novocainum (all hydrochlorides), and pachycarpinum hydroiodide, were of pharmaceutical grade (at least 99% purity). They were obtained from local manufacturers. Their solutions were freshly prepared by dissolving the appropriate salt amount either in water or in corresponding HCl aqueous solution, depending upon working pH range (see below).

2.2. Membranes and ISEs

Film polymeric membranes contained plasticizer and PVC in 2:1 ratio and ion exchanger (1×10^{-2} mol/kg). When used, TDA and crown ether were of 5×10^{-3} and 5×10^{-2} mol/kg concentrations, respectively. The components of each membrane (totaling ~0.6 g) were dissolved in THF (6 ml) and poured into a glass ring (i.d., 35 mm) fixed on a glass plate. Overnight evaporation yielded membranes of ~0.6 mm thickness. A 10 mm diameter disc was cut out from the parent membrane and glued to the end of the electrode PVC body with THF/PVC adhesive. 0.1 M KCl was used as an internal filling solution, except for the membranes containing crown ether. In the latter case ISE tubes were filled with 0.01 M NaCl. An Ag/AgCl internal reference wire electrode was immersed in the internal solution.

2.3. Procedures and apparatus

Prior to selectivity coefficient measurements the selectivity series were determined for each electrode investigated. To do this, using membranes of one composition, emf values were measured for each ion of interest. Based on the values obtained, the ions were ordered according to their lipophilicity. Then, using newly cut membranes, potentiometric selectivity coefficients were determined by the modified separate solution method [26], in the order of the ion lipophilicity increase. All measurements were performed at room temperature (20–21 °C) with cells of the following type:

Ag, AgCl|KCl (satd.); sample solution|

ISE membrane|0.1 M KCl|Ag, AgCl

and, in the case of ISEs with membranes containing crown ether:

Ag, AgCl|KCl (satd.):1 M LiOAc:samplesolution|

ISE membrane|0.01 M NaCl|Ag, AgCl

A conventional silver chloride electrode was used as an external reference one. To ensure full amine conversion into the cation form pH of all alkylammonium cation solutions were adjusted to 5–6. For each PhAA cation, the pH range was determined where the potential is not pH-dependent, and thereafter pH was kept within this range. Potential measurements and pH control of the solutions were performed using I-130 ion meter with input resistance $>10^{12} \Omega$.

Activity coefficients were calculated by the Debye–Hückel approximation. The potential readings were recorded after stabilization to ± 0.2 mV within 1 min, and near-Nernstian slopes were obtained for all the cations investigated. To calculate selectivity coefficient values potential readings at the concentration of either 1×10^{-2} M (for alkylammonium cations) or 1×10^{-3} M (for PhAA cations) were used, except for tributylammonium (1×10^{-3} M) and diamylammonium (1×10^{-4} M). The $\log K_{i,j}^{\text{pot}}$ values given in tables and figures were calculated using the potentials of not less than three electrodes made with the same parent membrane. The greatest difference of $\log K_{i,j}^{\text{pot}}$ values determined as a result of parallel measurements, did not exceed 0.15.

3. Results and discussion

3.1. Influence of plasticizer and ion exchanger nature on the selectivity of ISEs responsive to organic ammonium cations

3.1.1. ISEs responsive to alkylammonium cations

Influence of plasticizer: For Bu_4N^+ -selective electrodes (Bu_4N^+ -SEs) based on K(TpCIPB), the selectivity to alkylammonium cations with varied substitution degree has been investigated; logarithmic values of potentiometric selectivity coefficients ($\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$) are given in Table 1. It is evident that the plasticizer nature effect on the selectivity of the ISEs stud-

ied depends primarily upon the nitrogen substitution degree in amine molecules. For instance, the Bu_4N^+ -SE selectivity coefficients for Et_4N^+ (i.e., quaternary ammonium cation, just like the principal ion) are almost the same for all the ISEs investigated. Gradual plasticizer change in the order *o*-NPOE-DBP-DNA-TEHP causes virtually the same selectivity changes towards tertiary amine cations: Et_3NH^+ , Bu_3NH^+ , and dimethylbenzylammonium. The similar situation is observed for secondary alkylammonium cations (Am_2NH_2^+ , Et_2NH_2^+ , and methylbenzylammonium) as well as for the primary ones (BuNH_3^+ , *i*- BuNH_3^+ , and benzylammonium). There are certain regularities in the plasticizer nature effect upon the Bu_4N^+ -SE selectivity. In particular, $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values for mono-, di-, and trisubstituted alkylammonium cations increase orderly from *o*-NPOE-plasticized membranes to either DBP- or DNA-plasticized ones, and then, to TEHP-plasticized ones; the lesser the amine group substitution, the stronger $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values change. Evidently, the facts noted are mainly caused by peculiarities of amine cations solvating by plasticizer.

The efficiency of the alkylammonium cation solvation depends upon that of hydrogen bonding between the Lewis base plasticizer and “acidic” hydrogen atoms of amine groups. *o*-NPOE ability to form hydrogen bonds is strongly weakened by the NO_2 -group, therefore, this plasticizer cannot interact efficiently with primary–tertiary amine cations. The selectivity of ISEs with *o*-NPOE-plasticized membranes is mainly determined by relative lipophilicity of alkylammonium cations and, hence, is worse to amine cations than to QAS cations. Being stronger Lewis base than *o*-NPOE, DBP interacts more efficiently with alkylammonium cations. It is known that the amine cation ability to be solvated by basic solvents improves regularly with the increase of the hydrogen atom number in the amine group [27,28]. For this reason, if *o*-NPOE is replaced by DBP, it is for primary alkylammonium cations that $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ value changes are most significant as compared to secondary and tertiary ones. Since basicities of DNA and DBP are pretty close, then there is little difference in $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values to the same cations between electrodes with these two plasticizers. The solvation effect upon the selectivity is most pronounced for the membranes plasticized with TEHP, the strongest Lewis base among the plasticizers involved. As would be expected, the greatest rise in $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values (up to 4.7 orders) is observed for primary amine cations.

Ion exchanger nature influence: The influence of ion exchanger on potentiometric selectivity is caused by its specific interaction with target and foreign ions and, as a rule, is exhibited when corresponding ionic groups are of different nature.

In Fig. 1, the cation exchanger nature effect on $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values is shown for the membranes with varied plasticizers. It can be seen that the selectivity of K(TNOBS)-based ISEs for primary–tertiary amine cations is higher than that of K(TpCIPB)-based ones. The only exceptions are ISEs with TEHP-plasticized membranes for which $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values do not depend on the ion exchanger choice. Interestingly, $\log K_{\text{Bu}_4\text{N}^+,\text{Et}_4\text{N}^+}^{\text{pot}}$ value is virtually constant for all

Table 1

The plasticizer influence on the selectivity ($\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$) of the tetrabutylammonium-selective electrode for alkylammonium cations of varied substitution degree (ion exchanger K(TpCIPB))

Cation	<i>o</i> -NPOE	DBP	DNA	TEHP
Tetrabutylammonium (Bu_4N^+)	0.00	0.00	0.00	0.00
Tributylammonium (Bu_3NH^+)	−2.28	−1.57	−1.45	−0.14
Diamylammonium (Am_2NH_2^+)	−3.54	−2.07	−1.84	0.38
Tetraethylammonium (Et_4N^+)	−3.79	−3.80	−3.86	−4.01
Methylbenzylammonium	−4.91	−3.81	−3.47	−1.77
Triethylammonium (Et_3NH^+)	−5.07	−4.51	−4.34	−3.31
Benzylammonium	−6.26	−4.36	−3.85	−1.56
butylammonium (BuNH_3^+)	−6.72	−5.14	−4.45	−2.22
<i>iso</i> -Butylammonium (<i>i</i> - BuNH_3^+)	−6.80	−5.05	−4.39	−2.07
Diethylammonium (Et_2NH_2^+)	−6.38	−5.21	−4.92	−3.01
K^+	−9.05	−6.54	−5.73	−5.07

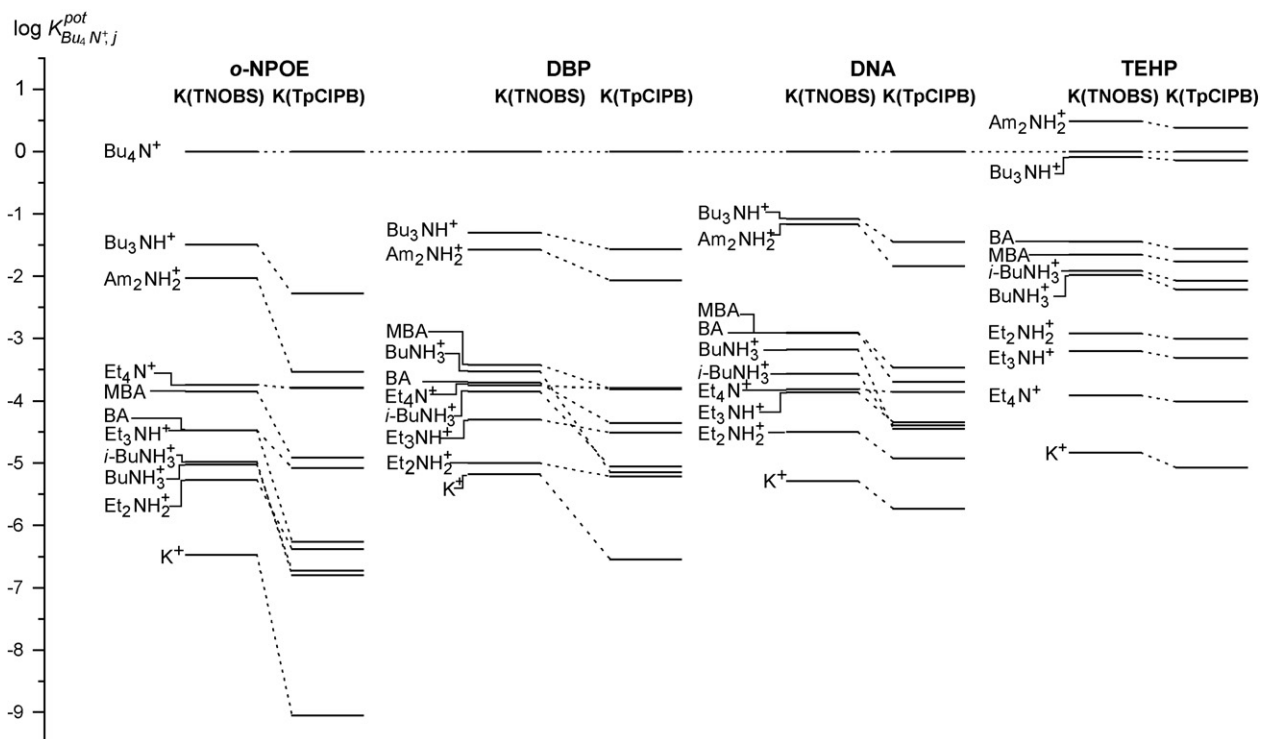


Fig. 1. The ion exchanger influence on the selectivity ($\log K_{Bu_4N^+,j}^{pot}$) of the tetrabutylammonium-selective electrode for alkylammonium cations of different substitution degree.

the ISEs used. The selectivity coefficient differences between K(TNOBS)- and K(TpCIPB)-based ISEs tend to diminish with the increase of the amine group substitution degree. The same trend is observed in the following plasticizer series: *o*-NPOE > DBP $\approx$ DNA > TEHP. The greatest $\log K_{Bu_4N^+,j}^{pot}$ value change (about 2 orders) induced by the ion exchanger replacement is observed towards primary alkylammonium cations for ISEs with *o*-NPOE-plasticized membranes. The regularities noted can be explained by peculiarities of the principal (Bu_4N^+) and interfering (primary–tertiary ammonium) cation association with $TpCIPB^-$ and $TNOBS^-$ anions.

According to ref. [21], the selectivity coefficients determined by the separate solution method are described by the equation:

$$K_{ij}^{pot} = \frac{k_j^{z_i/z_j}}{k_i} \cdot \frac{\bar{C}_i}{\bar{C}_j^{z_i/z_j}} \quad (1)$$

where k_i, k_j are the individual distribution coefficients of primary (i) and foreign (j) ions and $\bar{C}_i, \bar{C}_j$ are the concentrations of “free” (not bound with the ion exchanger) i and j ions in membrane, provided that all cationic sites are occupied with ions of only one kind— i or j , respectively.

In the case of ISEs based on liquid ion exchangers for single-charged i and j ions Eq. (1) takes the form:

$$K_{ij}^{pot} = \frac{k_j}{k_i} \cdot \left(\frac{(k_{ass})_{jR}}{(k_{ass})_{iR}} \right)^{1/2} \quad (2)$$

where $(k_{ass})_{jR}, (k_{ass})_{iR}$ are the ion-pair formation constants with an ion exchanger (R). In Eq. (2), the first term implies the contribution of hydration and solvation processes to potentiometric

selectivity, and the second one—that of the ion association factor.

It is known that constants of primary–tertiary amine ion association with the same anion are 3–6 orders (depending on solvent nature) higher than those of QAS cations [29,30], both the steric factors governing the closest approach parameter and hydrogen bond (H-bond) formation abilities being essential. According to Fuoss theory [31,32], the role of the steric factor is most significant in media with low dielectric constants while in the case of moderate and high dielectric constants ($\epsilon > 10$) this role is relatively negligible. Hence, in *o*-NPOE-plasticized membranes ($\epsilon \approx 14$ [33]) the steric factor will not influence the selectivity substantially whereas hydrogen bonding will. As for $TNOBS^-$ anion, its negative charge is located predominantly on oxygen atoms of the sulfonic group thus making it most possible to form quite stable H-bonds with alkylammonium cations. In contrast, in $TpCIPB^-$ anion the negative charge is delocalized, and therefore, H-bonds do not form. Consequently, K(TNOBS)-based ISEs are expected to show better selectivity for primary–tertiary amine cations (especially for primary) than K(TpCIPB)-based ones, and they do.

As it follows from Fuoss theory, in membranes plasticized with DNA and DBP (both possess low dielectric constants) the steric factor has to be manifested more strongly than in *o*-NPOE-plasticized ones. This was supposed to have led to an increase in ion exchanger affecting k_{ass} values and potentiometric selectivity thereby. In fact, the opposite effect is observed: for ISEs with DNA- and DBP-plasticized membranes, $\log K_{Bu_4N^+,j}^{pot}$ value variances caused by ion exchanger nature are somewhat

Table 2

The plasticizer and ion exchanger influence on the selectivity ($\log K_{i,j}^{\text{pot}}$) of the gangleronum-selective electrode for PhAA cations of different structure

PhAA cation	<i>o</i> -NPOE		DBP		DNA		TEHP	
	K(TNOBS)	K(TpClPB)	K(TNOBS)	K(TpClPB)	K(TNOBS)	K(TpClPB)	K(TNOBS)	K(TpClPB)
Gangleronum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spasmolytinum	−1.44	−1.56	−1.32	−1.36	−1.47	−1.48	−1.41	−1.42
Pachycarpinum	−1.07	−0.56	−1.46	−1.29	−1.99	−1.65	−2.61	−2.54
Papaverinum	−1.15	−1.29	−1.09	−1.27	−1.41	−1.53	−1.63	−1.63
Dimedrolum	−1.72	−1.63	−1.43	−1.50	−1.53	−1.54	−1.74	−1.75
Remantadinum	−2.98	−3.87	−2.10	−2.81	−1.83	−2.38	−1.16	−1.17
Chininum	−2.43	−3.08	−1.92	−2.40	−2.05	−2.39	−1.37	−1.43
Trimecainum	−3.40	−3.23	−3.09	−2.96	−2.87	−2.89	−2.24	−2.31
Novocainum	−3.86	−3.74	−3.43	−3.35	−3.36	−3.40	−2.90	−2.97
K ⁺	−6.55	−8.64	−6.64	−6.61	−5.92	−6.08	−6.31	−6.49

lower than for ISEs with *o*-NPOE-plasticized ones. In this case, better solvation (compared to *o*-NPOE) of primary–tertiary amine cations by DNA and DBP may be the key factor weakening the efficiency of hydrogen bonding between TNOBS[−]

and those cations. TEHP possessing high solvation ability, the ion exchanger influence upon selectivities of ISEs with membranes plasticized with this compound is almost utterly leveled.

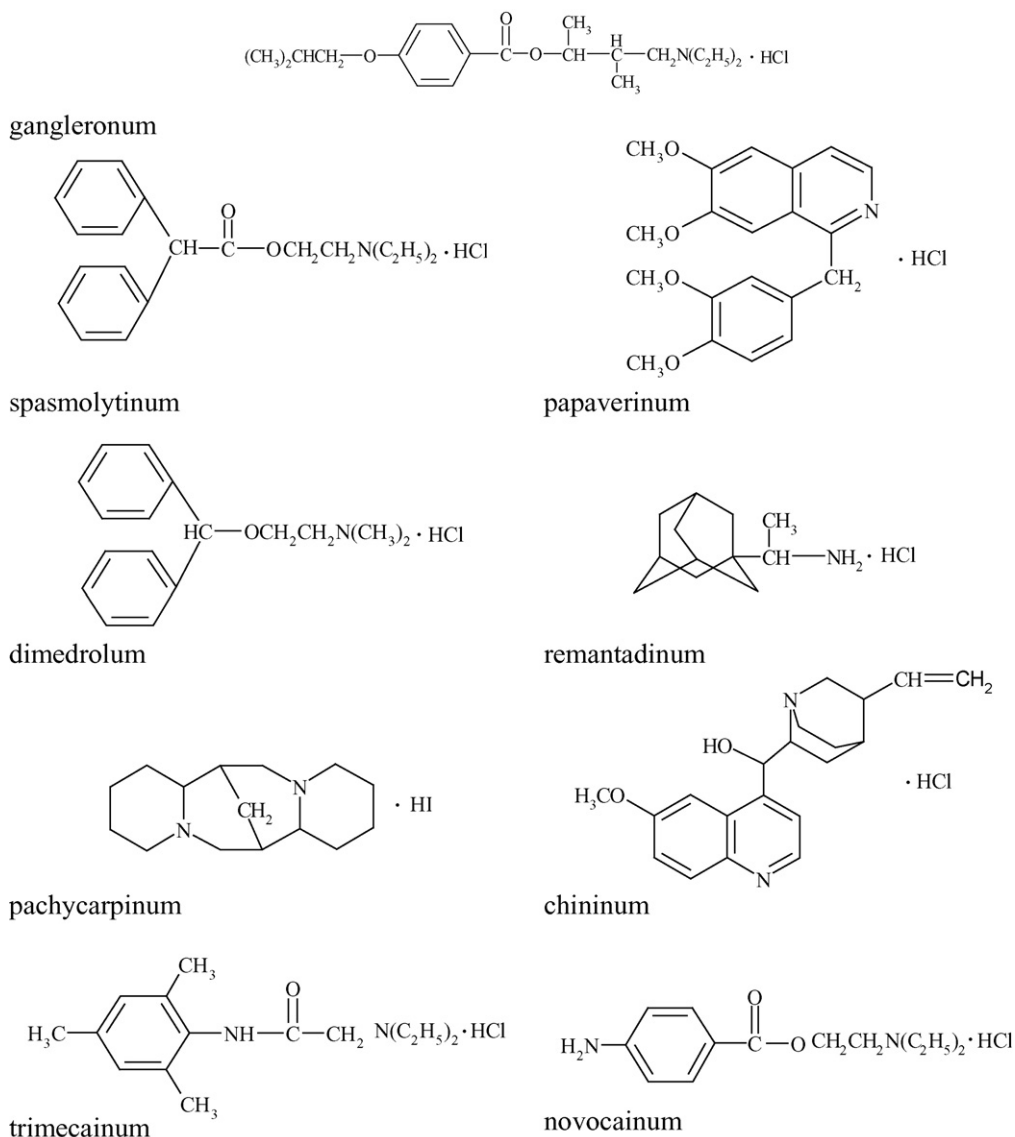


Fig. 2. Physiologically active amine formulas.

Table 3

The influence of the cationic lipophilic additive ((TDA)NO₃) on log $K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values

Cation	<i>o</i> -NPOE				DNA			
	K(TNOBS)	K(TNOBS) with TDA	K(TpCIPB)	K(TpCIPB) with TDA	K(TNOBS)	K(TNOBS)with TDA	K(TpCIPB)	K(TpCIPB) with TDA
Bu ₄ N ⁺	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bu ₃ NH ⁺	−1.49	−0.36	−2.28	−2.30	−1.08	0.74	−1.45	−1.50
Am ₂ NH ₂ ⁺	−2.03	−0.80	−3.54	−3.46	−1.17	0.95	−1.84	−1.84
Et ₄ N ⁺	−3.75	−4.07	−3.79	−3.85	−3.81	−3.13	−3.86	−3.89
MBA ^a	−3.84	−2.75	−4.91	−4.95	−2.90	−0.72	−3.47	−3.55
Et ₃ NH ⁺	−4.47	−3.99	−5.07	−5.16	−3.86	−2.04	−4.34	−4.48
BA ^b	−4.47	−3.26	−6.26	−6.05	−2.92	−0.97	−3.69	−3.78
BuNH ₃ ⁺	−5.02	−3.69	−6.72	−6.42	−3.18	−1.04	−4.45	−4.59
<i>i</i> -BuNH ₃ ⁺	−4.98	−3.74	−6.80	−6.67	−3.57	−1.23	−4.39	−4.49
Et ₂ NH ₂ ⁺	−5.27	−4.40	−6.38	−6.57	−4.50	−2.60	−4.92	−5.03
K ⁺	−6.47	−4.98	−9.05	−9.24	−5.28	−3.32	−5.73	−5.87

^a Methylbenzylammonium cation.^b Benzylammonium cation.

3.1.2. ISEs responsive to PhAA cations

Plasticizer nature influence: As data given in Table 2 show, gangleronum-SE selectivity for spasmolytinum, papaverinum, and dimedrolum ions exhibits little dependence upon plasticizer nature. In contrast, log $K_{i,j}^{\text{pot}}$ values for chininum, remantadinum, novocainum, and trimecainum ions increase regularly in the following plasticizer series: *o*-NPOE < DBP ≈ DNA < TEHP. At the same time, log $K_{i,j}^{\text{pot}}$ value for pachycarpinum is maximal for ISEs with *o*-NPOE-plasticized membranes and minimal for ISEs with TEHP-plasticized ones. The facts noted can be explained as follows.

In contrast to alkylammonium cations, plasticizer nature can affect the selectivity of ISEs responsive to PhAA cations not only when target and foreign ions have different substitution degrees of the cationic nitrogen, but also when they bear different non-ionic polar groups capable of acid–base interaction with plasticizer. The principal ion, the gangleronum cation, has no such polar groups capable of being solvated efficiently by Lewis bases (see Fig. 2); neither do spasmolytinum, papaverinum and dimedrolum ions. Consequently, gangleronum-SE selectivity for these ions is virtually independent of plasticizer nature. At the same time, chininum, trimecainum, and novocainum ions have hydrogen atoms with positive charge. Therefore, the use of plasticizers that are strong Lewis bases improves the corresponding ISE selectivity for the three ions, for ISEs with TEHP-plasticized membranes the effect being most visible. Interestingly, although there are no non-ionic polar groups in the remantadinum molecule, the log $K_{i,j}^{\text{pot}}$ value increase towards the cation is the greatest one (more than 2.5 orders from *o*-NPOE- to TEHP-plasticized membranes). This is due to remantadinum being a primary amine, unlike all the other PhAAs containing a tertiary nitrogen atom. Hence (by analogy with alkylammonium cations), much more significant selectivity change occurs. As for pachycarpinum, though it is a tertiary amine, its protonated amino group is much less solvated due to steric hindrance. Thereby, the best selectivity for pachycarpinum cations occurs when the opportunity for competing ions to be solvated is mini-

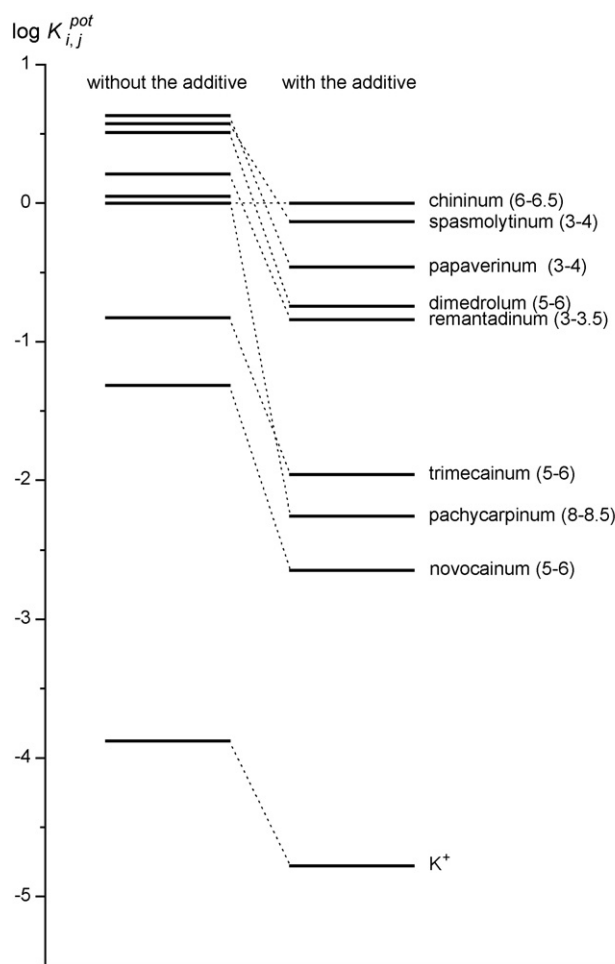


Fig. 3. The influence of the cationic lipophilic additive ((TDA)NO₃) on chininum-selective electrode selectivities for PhAA cations (membranes are plasticized with DNA and contain K(TNOBS)). pH of solutions are given in parentheses.

mal, that is, in the case of ISEs with *o*-NPOE-plasticized membranes.

Ion exchanger nature influence: Almost all the PhAAs, namely spasmolytinum, papaverinum, dimedrolum, novocainum, trimecainum contain tertiary cationic nitrogen just like gangleronum. Therefore, the selectivity coefficients for them are virtually independent of ion exchanger nature. When K(TNOBS) is used, selectivity for the remantadinum (a primary amine) cation increases as expected. At first glance, it seems surprising that using K(TNOBS) causes the similar selectivity improvement for chininum cation as well, whereas $\log K_{i,j}^{\text{pot}}$ values for pachycarpinum are somewhat increased for K(TpCIPB)-based ISEs, though both PhAA cations are tertiary amines. Nonetheless, such results are quite reasonable if one takes into account that chininum forms abnormally durable ion associates with anions [34] (additional H-bond may form between a chininum hydroxy group and an anion) and that pachycarpinum associates are weaker than those of other tertiary amines [34] because of steric hindrance.

3.2. Lipophilic cationic additive influence on ISEs selectivity to organic ammonium cations

3.2.1. ISEs responsive to alkylammonium cations

The incorporation of lipophilic ionic sites of the charge sign opposite to that of the ion exchanger is known to be rather an effective way to improve the selectivity for ions forming more stable ion associates with ion exchanger [21,22]. In such a case, Eq. (1) for single-charged *i* and *j* ions takes the form:

$$K_{i,j}^{\text{pot}} = \frac{k_j}{k_i} \cdot \left(\frac{(k_{\text{ass}})_{jR}}{(k_{\text{ass}})_{iR}} \right) \quad (3)$$

that is, the selectivity coefficient change is proportional to the ratio of ion associate constants. As a result, the contribution of the ion association factor to potentiometric selectivity rises, as compared to membranes without additive (see Eq. (2)).

As seen from Table 3, when incorporated into the K(TpCIPB)-containing membrane, (TDA)NO₃ induces no visible selectivity change for any of the ions studied. This provides the evidence that TpCIPB[−] itself cannot discriminate between them. This situation seems to be quite understandable, taking into account, first, TpCIPB[−] being incapable of hydrogen bonding with amine cations; second, the fact that the effect of cation size on association constant values is highly leveled due to TpCIPB[−] considerable effective radius (e.g., this value for tetraphenylborate is 4.21 Å [35]). On the contrary, upon incorporating (TDA)NO₃ into membranes containing K(TNOBS), evident increase in $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values can be seen for primary–tertiary amine cations.

An interesting fact is observed for ISEs with *o*-NPOE-plasticized membranes. For K(TNOBS)-based electrodes the changes in $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values caused by (TDA)NO₃ are fairly close to $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ value differences between K(TpCIPB)- and K(TNOBS)-based ISEs without the additive. This follows directly from the comparison between Eqs. (2) and (3), provided there is no selectivity in TpCIPB[−] interacting with cations under question.

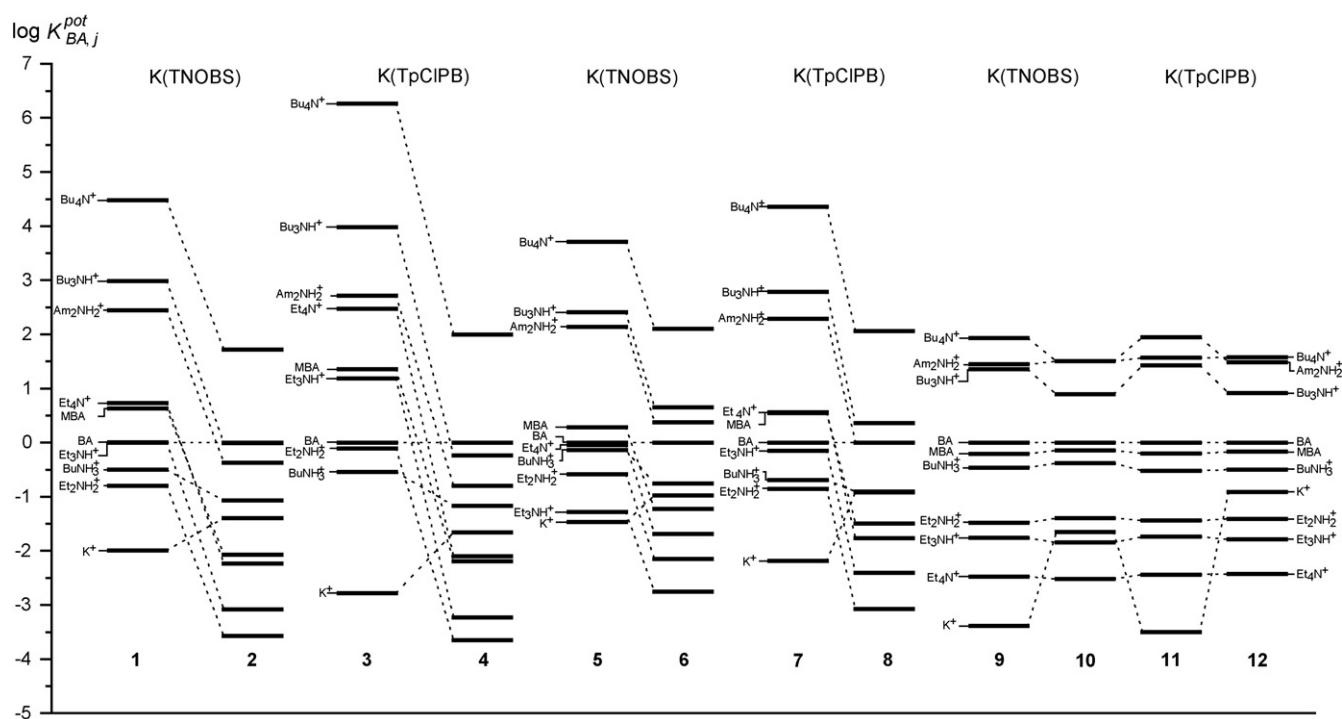


Fig. 4. The crown ether influence on the selectivity ($\log K_{\text{BA},j}^{\text{pot}}$) of the benzylammonium-selective electrode for alkylammonium cations. Membranes are plasticized with *o*-NPOE (1–4), DBP (5–8), and TEHP (9–12). The membranes with even numbers contain crown ether, others do not.

However, in the case of ISEs with DNA-plasticized membranes the situation is quite different. (TDA)NO₃ affects $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values much stronger than it would be expected on comparing $\log K_{\text{Bu}_4\text{N}^+,j}^{\text{pot}}$ values for K(TpCIPB)- and K(TNOBS)-containing membranes without the additive. The effect found cannot be adequately explained in the framework of the simple model of potentiometric selectivity [21–24]. However, according to ref. [36] the ionic site influence can vary strongly, depending upon the stoichiometry of complexes formed. So, the observed effect is likely to be governed by significant deviation of DNA-plasticized membrane properties from those of an ideally associated solution, in particular, by the formation of ion associates more complicated than ion pairs. Nevertheless, this effect is of good reproducibility and thus might be of practical importance, for example, in developing ISEs with improved selectivities for secondary and tertiary ammonium cations over QASes.

3.2.2. ISEs responsive to PhAA cations

The influence of cationic additive on chininum-SE selectivity for PhAA cations is shown in Fig. 3. (TDA)NO₃ incorporation induces decrease in $\log K_{i,j}^{\text{pot}}$ values by 1 order on average for all the PhAA cations but pachycarpinum (for which it is over 2 orders).

The effects can be explained by abovementioned peculiarities of the chininum and pachycarpinum ion association (see Section 3.1.2). Since chininum k_{ass} values are abnormally high, then (TDA)NO₃ leads to relative decrease in selectivities for the rest of PhAA cations, especially for pachycarpinum forming the weakest ion associates.

3.3. Dibenzo-18-crown-6 influence on ISE selectivity: relation to plasticizer and ion exchanger nature

3.3.1. ISEs responsive to alkylammonium cations

The neutral carrier influence on the selectivity depends strongly upon plasticizer and ion exchanger used. Data given in Fig. 4 illustrate the ambiguity of such an effect on the benzylammonium(BA)-SE selectivity; both K(TpCIPB)- and K(TNOBS)-based ISEs with *o*-NPOE-, DBP-, and TEHP-plasticized membranes have been studied.

The greatest selectivity change caused by crown ether introduction can be seen when using *o*-NPOE, the weakest Lewis base among the plasticizers involved (Fig. 4, membranes 1–4). For K(TNOBS)-based ISEs $\log K_{\text{BA},j}^{\text{pot}}$ values for all the ammonium cations but primary ones are diminished by almost 3 orders. Crown ether more strongly affects selectivities of K(TpCIPB)-based ISEs, for this ion exchanger, unlike K(TNOBS), is not capable of specific interactions with cations: $\log K_{\text{BA},j}^{\text{pot}}$ value drop amounts to 3.5 orders for secondary and almost 4.5 orders for tertiary amine and QAS cations. Seemingly, poor *o*-NPOE solvation of BA cations (and, in the membranes containing K(TpCIPB), absence of hydrogen bonding with ion exchanger as well) is beneficial for BA forming a complex with crown ether. Consequently, this decreases the selectivity for amine cations of higher substitution degree. It should be pointed out, however,

that it is difference in selectivities of initial (in the absence of crown ether) membranes that cause different crown ether effects, depending on the ion exchanger choice. As for the resulting selectivity achieved by crown ether incorporation, it depends slightly upon the ion exchanger nature.

In contrast to ISEs with *o*-NPOE-plasticized membranes, for those with TEHP-plasticized ones (based both on K(TNOBS) and K(TpCIPB)) the selectivity is virtually independent of the crown ether introduction (Fig. 4, membranes 9–12). Obviously, the P=O group efficient solvation of primary amine cations prevents BA from interacting with crown ether, leading to $\log K_{\text{BA},j}^{\text{pot}}$ constancy.

DBP, according to its solvation ability, is positioned between *o*-NPOE and TEHP. For this reason, crown ether does affect the selectivity of corresponding ISEs (Fig. 4, membranes 5–8), as compared to ISEs with TEHP-plasticized membranes, but the effect is less than that for ISEs with *o*-NPOE-plasticized membranes. Just as in the latter case, $\log K_{\text{BA},j}^{\text{pot}}$ to secondary and tertiary amine and QAS cations decrease more strongly for K(TpCIPB)-based ISEs (over 2 orders) than for K(TNOBS)-based ones (1.5 orders on average).

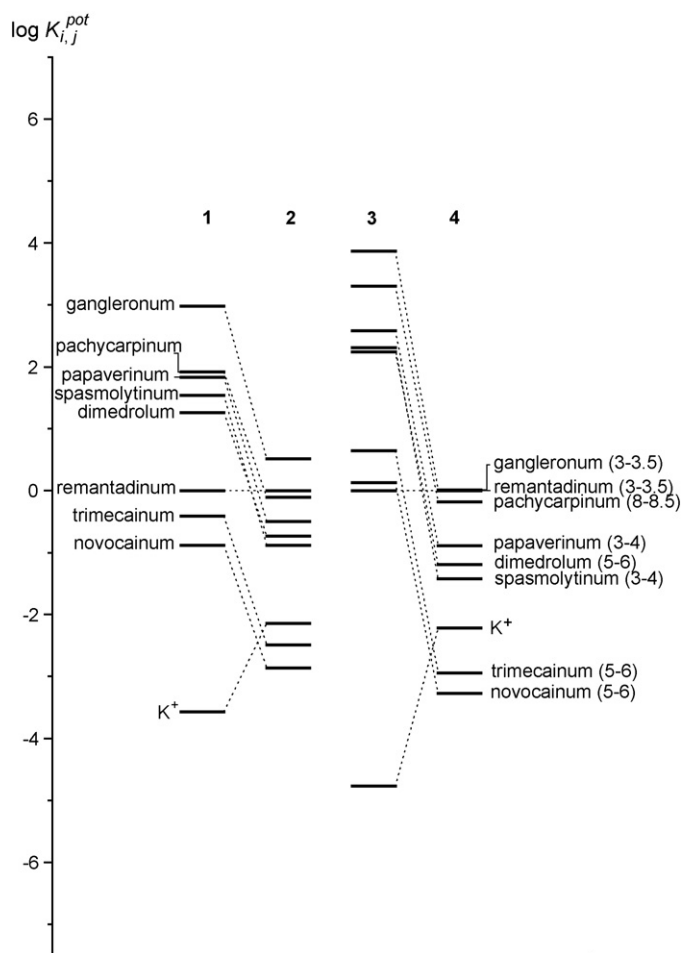


Fig. 5. The crown ether influence on the selectivity ($\log K_{i,j}^{\text{pot}}$) of the remantadinum-selective electrode with *o*-NPOE-plasticized membranes for PhAA cations. 1, K(TNOBS); 2, K(TNOBS) with crown; 3, K(TpCIPB); 4, K(TpCIPB) with crown. pH of solutions are given in parentheses.

It is interesting that for all four ISEs (based on both K(TpCIPB) and K(TNOBS) with *o*-NPOE- and DBP-plasticized membranes) crown ether causes a little (up to 0.5 orders) decrease of $\log K_{\text{BA},j}^{\text{pot}}$ values towards BuNH_3^+ and *i*- BuNH_3^+ (i.e., primary amine cations, just like BA). In this case, some kind of specific interaction may occur, caused by structural compatibility between the BA benzene ring and that of the crown ether; it somewhat improves the selectivity for BA against BuNH_3^+ and *i*- BuNH_3^+ .

3.3.2. ISEs responsive to PhAA cations

The above-described rules of the crown ether effect on selectivity hold true for ISEs responsive to PhAA cations as well. In Fig. 5, the crown ether influence on the remantadinum-SE selectivity is shown (membranes are plasticized with *o*-NPOE and contain either K(TpCIPB) or K(TNOBS)). Being a primary amine, only remantadinum can form quite stable bonds with crown ether. Just like in the alkylammonium-ISE case, the effect depends on ion exchanger nature: $\log K_{i,j}^{\text{pot}}$ values for all PhAA cations decrease by 2–2.5 and by more than 3.5 orders for the ISEs based on K(TNOBS) and K(TpCIPB), respectively. Note that, although ion exchanger influence on selectivity is leveled in presence of crown ether, the K(TNOBS)-based ISE possesses somewhat worse selectivity (by 0.3–0.5 orders) for remantadinum. It can be explained by hydrogen bonding between TNOBS[−] and interfering ammonium cations not complexing with the crown ether.

4. Conclusions

The data obtained provide the evidence that effective ways for controlling the selectivity of ISEs responsive to organic ammonium cations can include, along with those widely used (either plasticizer varying or neutral carrier introduction into membranes), careful selection of ion exchanger and incorporation of lipophilic cationic sites as well. It has been shown that plasticizer nature can strongly affect the ISE selectivity both when the target ion contains non-ionic polar groups capable of being solvated by plasticizer, and when target and foreign ions have different substitution degree of a salt-forming nitrogen atom. The selectivity for amine cations of low substitution degree improves with plasticizer basicity increase. For the membranes containing “non-complexing” ion exchanger (K(TpCIPB)), for the primary–quaternary alkylammonium cation pair, selectivity coefficient change, caused by plasticizer variation, may be up to 4.7 orders. It has been shown that ion exchanger effect upon selectivity of such ISEs may occur when target and foreign ions differ in nitrogen substitution degree, or when one of the ions is capable of additional (non-Coulomb) interaction with ion exchanger. For chininum, for example, hydrogen bond formation between the hydroxy group adjacent to cationic nitrogen and TNOBS[−] anion leads to strengthening of ion associate formed. Selectivity coefficient change, caused by ion exchanger nature, depends significantly upon plasticizer used, increasing with the decrease of its basicity, and for the primary–quaternary alkylammonium cation pair, this effect can be as great as 2 orders.

Incorporation of a lipophilic cationic additive into membrane containing a “selective” ion exchanger causes further improvement of selectivity for the ammonium cation forming more stable ion associates with the ion exchanger. The total effect of selectivity change for the primary–quaternary alkylammonium cation pair exceeds 3 orders.

It has been shown that the effect caused by crown ether introduction into membrane, depends upon both plasticizer and ion exchanger nature. The greatest effect (about 4.5 orders) of selectivity change for the primary–quaternary alkylammonium cation pair is attained when membranes plasticized with *o*-NPOE (weak Lewis base) and containing non-complexing ion exchanger (K(TpCIPB)) are used. On the contrary, for the membranes plasticized with TEHP (strong Lewis base), neither crown ether incorporation nor variation of ion exchangers virtually affects ISE selectivity. At the same time, if crown ether is used, plasticizer and ion exchanger influences are negligible.

The data obtained make it possible to outline ways of optimizing the membrane composition for ISEs responsive to amine cations, tailored to the particular analytical problem being solved. For instance, to determine quaternary ammonium cations over primary–tertiary ones K(TpCIPB)-based ISEs with *o*-NPOE-plasticized membranes seem to be most preferable. For determining primary amine cations over those with higher substituted cationic nitrogen, neutral carrier (e.g., dibenzo-18-crown-6) should be introduced into membrane plasticized with *o*-NPOE and containing K(TpCIPB)-type ion exchanger. To determine secondary or tertiary amine cations over quaternary ones membranes containing plasticizer with low dielectric constant (e.g., DNA), complexing ion exchanger (of K(TNOBS) type) and lipophilic cationic sites (e.g., TDA) could be recommended.

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Hg(II)-imprinted thiol-functionalized mesoporous sorbent micro-column preconcentration of trace mercury and determination by inductively coupled plasma optical emission spectrometry

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Abstract

Hg(II)-imprinting thiol-functionalized mesoporous sorbent was prepared by a sol–gel method and characterized by X-ray diffraction (XRD), FT-IR spectroscopy and nitrogen gas adsorption–desorption. The static adsorption capacity of the Hg(II)-imprinted and non-imprinted sorbent was 78.5 and 26.6 mg g^{−1}, respectively. The breakthrough capacity was 4.46 mg g^{−1}, and the relative selectivity coefficient for Hg(II) in the presence of Cd and Pb was 3.3 and 3.9, respectively. A new method using a micro-column packed with Hg(II)-imprinting thiol-functionalized mesoporous sorbent has been developed for preconcentration of trace mercury prior to its determination by inductively coupled plasma optical emission spectrometry (ICP-OES). The effects of pH, sample flow rate and volume, elution solution and interfering ions on the recovery of the analyte have been investigated. The limit of detection was 0.39 ng ml^{−1} with a concentration factor of 150 times. The developed method has been applied to the determination of trace mercury in some biological and environmental samples with satisfactory results. The accuracy was assessed through recovery experiments and analysis of certified reference material.

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Keywords: Ionic imprinting; Thiol-functionalized mesoporous sorbent; Mercury; ICP-OES

1. Introduction

Mercury is an environmentally and toxicologically important element and is one of the most widely studied heavy metals because of the toxicological and biogeochemical behavior [1,2]. Therefore, the determination of trace amounts of mercury is of particular significance in environmental and toxicological studies. One of the routes of incorporation of mercury into the human body is water and food. Hence, its determination in this type of sample becomes very important.

Since concentration of mercury in natural waters is very low, of the order of a few μg l^{−1}, hence, powerful techniques are required and only a few of them show sufficient sensitivity. In order to achieve accurate, reliable and sensitive results, separation and preconcentrations step is often necessary prior to analysis.

Nowadays, many separation and preconcentration techniques for metal ions have been developed, and these include liquid–liquid extraction [3], coprecipitation [4] and solid phase extraction (SPE) [5–8]. Of all these methods, SPE was used widely in separation and preconcentration systems. Compared with traditional extraction technologies, it is simple, fast, inexpensive and less polluting to the environment. Numerous substances have been proposed and applied as SPE sorbents, such as organic chelate resin [9,10], modified silica gel [11,12], carbon nanotubes [13], polytetrafluorethylen (PTFE) beads and turnings [14,15] and polyurethane foam [16], etc.

Ion imprinting polymers (IIP) is an attractive method for the preparation of selective sorbents [17–19]. The selectivity of IIP arises because the polymer synthesized in the presence of a template ion and a functional monomer. Then, template ions are removed from the polymer matrix. As a consequence, the IIP is able to selectively recognize the template ion or molecule from other components in a sample. Ersöz et al. [20] have developed a polymer obtained by imprinting with Ni(II) ion as an ion-imprinted sorbent. The K_d (distribution coefficient) values

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for the Ni(II)-imprinted polymer show increase in K_d for Ni(II) with respect to both K_d values of Zn(II), Cu(II) and Co(II) ions and non-imprinted polymer. Bae et al. [21] have also described the synthesis of ion imprinted polymer and have been successfully used for enrichment of uranyl ion from aqueous and sea water solutions. Liu et al. [22] have reported the synthesis of Hg(II)-imprinted copolymers by copolymerizing mercury chloride, diazoaminobenzene and vinylpyridine and its analytical applications for column preconcentrative separation of Hg(II) from natural water. Rao et al. [23] have reviewed the preparation of tailored materials for preconcentration of metal ions by IIP. However, the main problems of IIPs are: (i) the slow rate of mass transfer in binding and release of template from the sites; (ii) the bleeding of unleached template.

The sol–gel route provides a useful method to prepare glass-like or ceramic materials through the hydrolysis and condensation of suitable metal alkoxides [24]. There are several advantages associated with using sol–gel materials for ion-imprinted sorbents over polymers. For example, large surface areas, excellent metal ion transfer kinetics, high metal adsorption capacity and high selectivity. Dai et al. [25] showed that sol–gel glasses prepared with the template $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ exhibit enhanced affinity and selectivity for the uranyl ion relative to that observed for a similarly prepared control blank gel. Recently, Dai et al. [26] proposed a novel method for template-selective recognition sites on mesoporous sorbents that is referred to as “surface imprinting” in ordered mesoporous materials. Yan and co-workers [27] have also described the synthesis ion-imprinted functionalized silica gel sorbent by a surface imprinting technique and have been successfully used for selective solid phase extraction of Cd. The essence of this approach is to coat the mesopore surface with complexes of the ligands and target metal ions instead of the free ligands. This technique has been shown to be successful in creating selective recognition sites on mesoporous sorbents and precise control of stereochemical arrangement of ligands on the adsorption sites.

3-Mercaptopropyltrimethoxysilane (MPTMS) is a kind of coupling agent, which has often been used to modify the surface of inorganic materials. In this work, we have prepared an Hg(II)-imprinted thiol-functionalized mesoporous sorbent by a sol–gel process and developed a new method involving a microcolumn packed with Hg(II)-imprinted thiol-functionalized mesoporous sorbent combined with ICP-OES for the determination of trace Hg in biological and environmental samples.

2. Experimental

2.1. Apparatus

Atom Scan 25 inductively coupled plasma optical emission spectrometry (TJA, USA) was used for the determination of metal ion concentrations. The operating conditions were as follows: wavelength 194.2 nm, incident power of 1.2 kW, observation height of 13 mm, integration time of 3 s, plasma gas flow rate of 15 l min^{-1} , auxiliary gas flow rate of 0.5 l min^{-1} and nebulizer gas flow rate of 0.8 l min^{-1} . Continuous nebulization was realized by means of a concentric glass nebulizer with

cyclonic chamber. An X-ray diffractometer (Bruker, Germany) was used to measure the crystallinity of the prepared sorbents. Fourier transform infrared spectroscopy (FT-IR) (Nicolet, USA) was used to identify the functional groups in the sorbents. N_2 adsorption–desorption analysis was performed on each sorbent by ASAP2010 system (Micromeritics, USA). Surface areas were obtained using the Brunauer–Emmett–Teller (BET) method [28]. The pore size calculated using the Barrett–Joyner–Halenda (BJH) formula [29]. All pH measurements were carried out using a pHs-3TC digital pH meter equipped with a combined glass-calomel electrode (Shanghai Tianda, Shanghai, China). A HL-2 peristaltic pump (Shanghai Qingpu Huxi Instrument Factory, Shanghai, China) was used in separation and preconcentration process. A self-made PTFE micro-column ($20 \text{ mm} \times 3.0 \text{ mm i.d.}$) was used for separation and preconcentration. PTFE tubing with 0.5 mm i.d. was used for all connections.

2.2. Reagent

Tetraethylorthosilicate (TEOS), 3-mercaptopropyltrimethoxysilane (MPTMS) (Wuhan University Chemical Factory, Wuhan, China) and Cetyltrimethylammonium bromide (CTAB), $\text{Hg}(\text{NO}_3)_2 \cdot 0.5\text{H}_2\text{O}$ (Beijing Chemical Co., Beijing, China) were used in this work. All metal stock solutions (1000 mg l^{-1}) were purchased from the National Research Center for standard materials (NRCSM, Beijing, China). Doubly deionized water (DDW) was used throughout this work. All reagents used were of at least analytical grade. The certified reference materials used in this work are human hair GBW 09101 and soil GBW 08303 (both obtained from National Institute of Standards and Technology, Beijing, China).

2.3. Preparation of the Hg(II)-imprinted mesoporous sorbent

2.3.1. Mesoporous sorbent

The mesoporous material was prepared according to the literature [30]. The typical procedure involves mixing CTAB, water and $\text{NH}_3\text{H}_2\text{O}$. TEOS was added to this solution at room temperature and the mixture was then refluxed for 10 h. The molar ratio of TEOS:CTAB: H_2O : $\text{NH}_3\text{H}_2\text{O}$ was 1.0:0.12:150:1.8. The solid product was recovered via vacuum filtration. This was then refluxed in ethanol to extract the surfactant, filtered, washed with deionized water.

2.3.2. Hg(II)-imprinted mesoporous sorbent

0.5890 g of $\text{Hg}(\text{NO}_3)_2 \cdot 0.5\text{H}_2\text{O}$ was added in 100 ml of methanol under stirring. After the $\text{Hg}(\text{NO}_3)_2 \cdot 0.5\text{H}_2\text{O}$ had completely dissolved, MPTMS was added (5.0 ml). The solution was stirred and refluxed for 2 h, mesoporous sorbent (4.0 g) was added. After 10 h of stirring, the product was evaporated to dryness. The residue was washed with copious amounts of 0.6 mol l^{-1} HNO_3 to remove all Hg(II) and any excess ligands. The final product was recovered by filtration, washed with deionized water up to the eluent pH 5–6 and dried under vacuum at 60°C for 24 h. The control blank samples were pre-

pared using an identical procedure, but without the addition of $\text{Hg}(\text{NO}_3)_2 \cdot 0.5\text{H}_2\text{O}$.

2.4. Micro-column preparation

A total of 50 mg of $\text{Hg}(\text{II})$ -imprinted thiol-functionalized mesoporous sorbent was filled into a PTFE micro-column ($20\text{ mm} \times 3.0\text{ mm}$ i.d.) plugged with a small portion of glass wool at both ends. Before use, 5.0 ml of methanol and 10 ml of DDW were passed through the column in sequence in order to clean it. Then, the column was conditioned to the desired pH with buffer solution.

2.5. Analytical procedure

2.5.1. Batch operation mode

Aliquots of 50 ml of sample solutions containing $100\text{ }\mu\text{g ml}^{-1}$ of the analytes of interest were prepared and the pH value was adjusted to ~ 6 with ammonium hydroxide or acetic acid solution. Then, 50 mg of thiol-functionalized mesoporous sorbent was added into the solution, shaken for 10 min and centrifuged. The supernatant liquid was detected with ICP-OES. The adsorption rate of the elements was calculated from the subtraction of the concentration between initial sample and the supernatant liquid. The batch operation mode was used only in the study of the static adsorption capacity and selectivity of thiol-functionalized mesoporous sorbent.

2.5.2. Column operation mode

Aliquots of sample solutions containing the interesting analytes were prepared and the pH value was adjusted to the desired value with ammonium hydroxide or acetic acid solution. The solution was passed through the column by using a peristaltic pump at the desired flow rate. After the sample loading, air was passed through the column for 2 min. Then, the retained metal ion was eluted with 1.0 ml of 0.6 mol l^{-1} HNO_3 solution. The analytes in the effluents were determined by ICP-OES.

2.6. Sample preparation

Water samples (river water and seawater) were collected in Nalgene bottles and filtered through $0.45\text{ }\mu\text{m}$ pore size membrane filter to remove the suspended particles. The filtrates acidified to $\text{pH} < 2$ with 0.1 mol l^{-1} HNO_3 and kept in a refrigerator.

Portions (0.2500 g) of GBW009101 human hair and GBW08303 soil were transferred into PTFE beaker, 8 ml of concentrated HNO_3 or in combination with 2 ml HF (for soil) was added and the solution was kept standing overnight. Then, 2 ml of H_2O_2 was added to the sample and the solution was heated almost to dryness. The residue was dissolved with 0.2 mol l^{-1} HNO_3 , and then diluted to 50 ml. Before use, the pH value was adjusted to 6.0 with ammonium hydroxide or acetic acid solution.

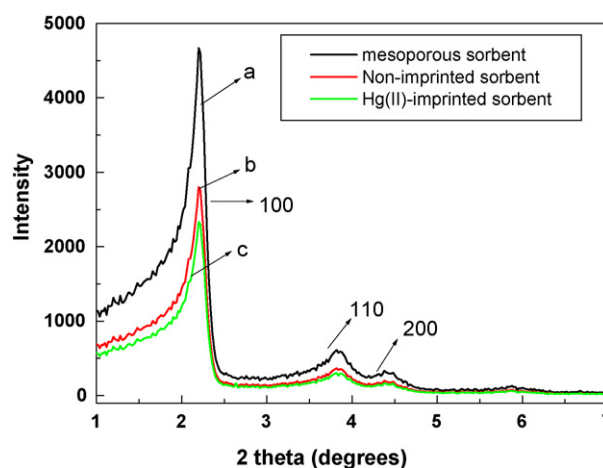


Fig. 1. SAXS patterns of (a) mesoporous sorbent; (b) non-imprinted mesoporous sorbent; (c) $\text{Hg}(\text{II})$ -imprinted mesoporous sorbent.

3. Results and discussion

3.1. Characterization studies

3.1.1. X-ray diffraction analysis

The X-ray diffraction patterns of mesoporous sorbent, non-imprinted and $\text{Hg}(\text{II})$ -imprinted mesoporous sorbents were showed in Fig. 1. As can be seen, a very intense peak (1 0 0) and two additional high-order peaks (1 1 0) and (2 0 0) with lower intensities, indicating 2θ values of 2.2, 3.88 and 4.4, the ratio of which was 1:1.73:2. This result was characteristic of a hexagonal pore structure. It should be noted that the decrease in the (1 0 0) intensity for non-imprinted and $\text{Hg}(\text{II})$ -imprinted mesoporous sorbents provides further evidence that grafting occurred inside the mesopore channels because attachment of the organic functional groups onto the surface of the mesopore channels tends to reduce the scattering power. These results indicate the maintenance of the structural orderness of the mesoporous sorbent after functionalization.

3.1.2. IR spectra

In order to ascertain the presence of $-\text{SH}$ in the matrix, FT-IR spectra were obtained from mesoporous sorbent, non-imprinted and $\text{Hg}(\text{II})$ -imprinted mesoporous sorbents (Fig. 2a–c). The features around 798 and 1090 cm^{-1} indicate $\text{Si}-\text{O}$ and $\text{Si}-\text{O}-\text{Si}$ stretching vibrations, respectively. The $-\text{OH}$ bond stretching band of the mesoporous sorbent were observed at $3200\text{--}3600\text{ cm}^{-1}$. After grafting of the thiol functionality, a very weak $-\text{SH}$ stretching peak can be seen at $2560\text{--}2580\text{ cm}^{-1}$ for non-imprinted and $\text{Hg}(\text{II})$ -imprinted mesoporous sorbent.

3.1.3. Surface area, pore size and adsorption capacity

The physical properties of these sorbent, such as the surface areas, pore diameter and adsorption capacity of $\text{Hg}(\text{II})$, were summarized in Table 1. The surface areas and pore diameter of $\text{Hg}(\text{II})$ -imprinted and non-imprinted mesoporous sorbent were less than the mesoporous sorbent as a result of the grafting of the organic functional group. Both $\text{Hg}(\text{II})$ -imprinted and non-

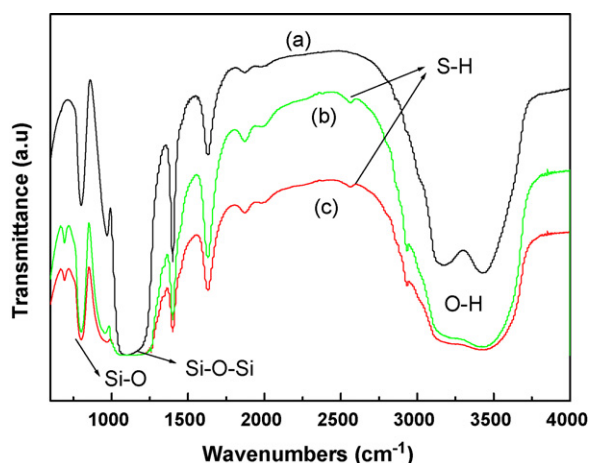


Fig. 2. FT-IR spectra (a) mesoporous sorbent, (b) non-imprinted mesoporous sorbent, and (c) Hg(II)-imprinted mesoporous sorbent.

imprinted mesoporous silica sorbents have very similar surface areas and pore diameter.

The static adsorption capacity of the Hg(II)-imprinted and non-imprinted mesoporous sorbent for Hg(II) was 78.5 and 26.6 mg g⁻¹, respectively, where the mesoporous sorbent no adsorption for Hg(II). The maximum Hg(II) adsorption capacity of the Hg(II)-imprinted mesoporous sorbent was about three time than that of non-imprinted mesoporous sorbent.

The breakthrough curve was obtained by pumping solution containing 5.0 µg ml⁻¹ Hg(II) at pH 6.0 through the column at flow rate 5.0 ml min⁻¹, continuously collecting each 5 ml of the column effluent to determine of Hg(II) by ICP-OES, then plotting the extraction ratio against the volume of effluent. The extraction ratio was equal to (C/C_0), where C was the concentration of the column effluent and C_0 was that of the column influent [20]. The breakthrough capacity curve for mercury ion was given in Fig. 3. As can be seen, the mercury breakthrough column started after 45 ml. The breakthrough capacity was found 4.46 mg g⁻¹ (the breakthrough capacity was calculated with the assumption that breakthrough occurred at $C/C_0 = 0.01$).

3.2. Analytical studies

3.2.1. Effect of pH on adsorption

Sample acidity plays an important role in complex adsorption extraction because it affects the complexing reaction between metal ions and ligands. In order to evaluate the effect of pH, the pH values of sample solutions was adjusted to a range of 1–9. The results of the effect of pH on the recoveries of studied ions were shown in Fig. 4. As can be seen, the Hg(II)-imprinted mesoporous sorbent exhibited a low affinity in acidic conditions,

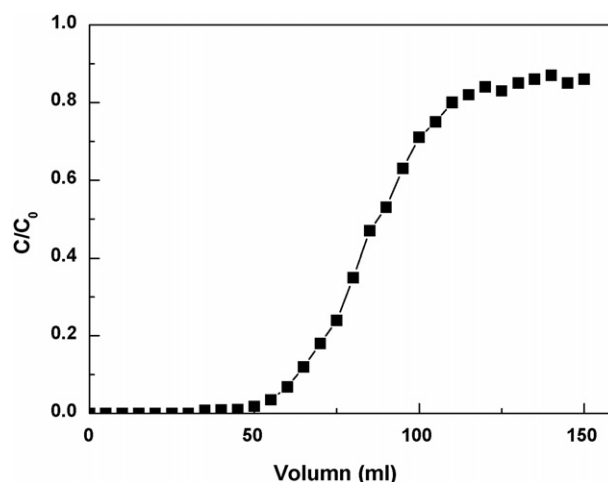


Fig. 3. The breakthrough curve for mercury ion. Hg(II): 5.0 µg ml⁻¹; pH 6.0; flow rate: 5.0 ml min⁻¹.

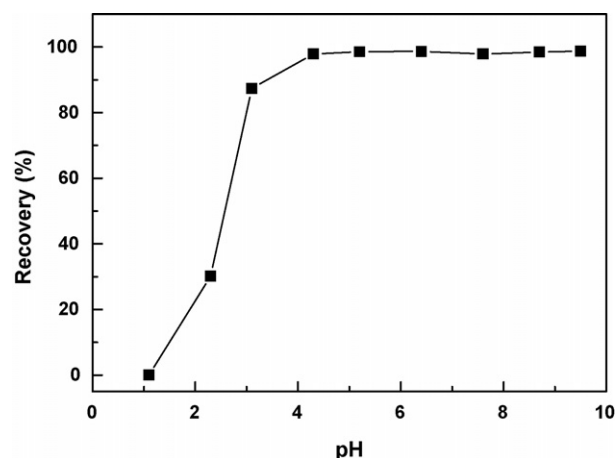


Fig. 4. Effect of pH on sorption of mercury on Hg(II)-imprinted mesoporous sorbent. Hg(II): 0.5 µg ml⁻¹; sample volume: 50 ml.

below pH 2.0, no Hg(II) was adsorbed onto the imprinted sorbent. A quantitative recovery (>90%) was found for Hg(II) at the pH range of 4–8.

3.2.2. Effect of flow rate of sample

The sample flow rate through the micro-column was very important parameter, since this is one of the steps that control the time of analysis. Therefore, the effect of the flow rate of sample solution was examined under the optimum conditions (pH, the sample volume and eluent, etc.). The flow rate was adjusted in a range of 1–8 ml min⁻¹. It was found that the analyte retention was not changed up to 6.0 ml min⁻¹ flow rate. The recovery of the analyte decreases slowly when the flow rate was over

Table 1
Physicochemical characteristics of Hg(II)-imprinted mesoporous sorbent

Material	Surface area (m ² g ⁻¹)	Pore diameter (nm)	Hg(II) adsorption capacity (mg g ⁻¹)
Mesoporous sorbent	983	2.7	0
Non-imprinted sorbent	638	1.7	26.6
Hg(II)-imprinted sorbent	626	1.6	78.5

6.0 ml min⁻¹. Thus, a flow rate of 5.0 ml min⁻¹ was employed in this work.

3.2.3. Elution

In order to recover the analytes retained in the column quantitatively, diluted HNO₃ was used for elution of the retained analyte from the micro-column. Effects of HNO₃ concentration and volume on the recovery of the Hg(II) were examined at a flow rate of 2.0 ml min⁻¹. The results indicate that 1.0 ml of 0.5–1.0 mol l⁻¹ HNO₃ was sufficient for quantitative elution of the adsorbed Hg(II) from the micro-column packed with the imprinted mesoporous sorbent. Therefore, the 1.0 ml of elution solution containing 0.6 mol l⁻¹ HNO₃ was used in the following experiments.

3.2.4. Effect of the sample volume

The effect of the sample volume on the retention of Hg(II) was also investigated. For this purpose, 25, 50, 100, 150, 200 and 250 ml of sample solutions containing 10 µg of Hg(II) were passed through the micro-column at a flow rate of 5.0 ml min⁻¹. It was found that the quantitative recovery (>90%) of Hg(II) were obtained for sample volumes up to 150 ml, so the enrichment factor achieved by this method was 150. In this work, 50 ml of sample solution was adopted for the preconcentration of analytes from water sample.

3.2.5. Selectivity

Competitive adsorption of Hg(II), Cd(II) and Pb(II) from their mixture was also investigated in a batch system. A 10 ml of aqueous solution containing 100 µg ml⁻¹ from each metal ion was treated with the Hg(II)-imprinted and non-imprinted mesoporous sorbent at a pH of 6.0 at room temperature, in the flasks stirred magnetically at 500 rpm. After adsorption equilibrium 10 min, the concentration of the metal ions in the remaining solution was measured by ICP-OES.

The effect of imprinting on selectivity was defined as $K_d = \{(C_0 - C_f)/C_f\} \{(\text{solution volume (ml)}/\text{mass sorbent (g)})\}$, where K_d is distribution coefficient, C_0 and C_f are the initial and final concentrations of the given ion in solution, respectively [25]. The selectivity coefficient k for the binding of a specific metal ion in the presence of competitor species can be obtained from equilibrium binding data according to $k = K_d(M_1)/K_d(M_2)$. The relative selectivity coefficient ($k' = k_{\text{imprinted}}/k_{\text{non-imprinted}}$) results from the ratio of the k values of the Hg(II)-imprinted mesoporous sorbent with non-imprinted mesoporous sorbent. The results of competitive ion binding bath tests are summarized in Table 2. A comparison of the selec-

Table 2
The effect of imprinting on selectivity

Metal type	Initial concentration (mg l ⁻¹)	Hg(II)-imprinted		Non-imprinted		k'
		K_d	k	K_d	k	
Hg(II)	100	13500	–	2731	–	–
Pb(II)	100	66	205	52	52.5	3.9
Cd(II)	100	98	138	65	42.0	3.3

tivity coefficient of Hg(II)-imprinted mesoporous sorbent with the selectivity coefficient of non-imprinted mesoporous sorbent showed that the imprinted matrix for Hg(II)/Pb(II), Hg(II)/Cd(II) and was 3.9 and 3.3 times greater than non-imprinted matrix, respectively.

3.2.6. Interference studies

For the study of interference by other coexisting ions, such as K(I), Na(I), Ca(II), Mg(II), Fe(III) and Al(III), a 50 ml of sample solutions containing 0.5 µg of Hg(II), and vary amounts of other cations, was taken and operated according to the proposed procedure. The tolerance limits of the coexisting ions, defined as the largest amount making the recovery of the studied element less than 90%, were found to be 5000 mg l⁻¹ for Na(I) and K(I), 2500 mg l⁻¹ for Ca(II) and Mg(II), 1000 mg l⁻¹ for Al(III), 200 mg l⁻¹ for Fe(III).

3.3. Analytical performance

The analytical figures of merit of the present micro-column separation and preconcentration of trace amounts of Hg using Hg(II)-imprinted mesoporous sorbent and its determination by ICP-OES were evaluated under optimal experimental conditions. According to the IUPAC definition, the detection limit (3σ) was 0.059 µg ml⁻¹. Given the 150-fold concentration factor for Hg, the detection limit of the method was 0.39 ng ml⁻¹. The relative standard deviation (R.S.D.) was 4.8% ($c = 0.01$ µg ml⁻¹, $n = 7$, sample volume = 50 ml).

3.4. Sample analysis

The proposed method was applied to the determination of Hg in the seawater sample (Tianjin, China) and natural river water sample (Fen River, Linfen, China). The results given in Table 3 show that the recovery of spiked sample was in a range of 93.3–104%.

In order to establish the validity of the proposed procedure, the method has been applied to the determination of the content of Hg in certified reference materials (GBW09101 human hair, GBW08303 soil). The results were listed in Table 4. As can be seen, a good agreement between determined values and the certified values could be obtained.

Table 3
Determination of Hg(II) in river water and seawater ($n = 3$)^a

Sample	Added (µg l ⁻¹)	Found (µg l ⁻¹) ^b	Recovery (%)
River water	0.00	0.49 ± 0.03	–
	0.25	0.73 ± 0.08	96.0
	0.50	1.02 ± 0.10	104
Seawater	0.00	0.33 ± 0.01	–
	0.30	0.61 ± 0.04	93.3
	0.60	0.90 ± 0.05	95.0

^a Sample volume: 50 ml.

^b Average of three runs ± S.D.

Table 4

Analytical results of Hg(II) in certified materials of GBW009101 human hair, and GBW08303 soil samples ($n=3$)

Sample	Certified ($\mu\text{g g}^{-1}$)	Determined ($\mu\text{g g}^{-1}$) ^a
GBW09101 human hair	2.16 ± 0.21	2.08 ± 0.19
GBW08303 soil	2.15 ± 0.06	2.12 ± 0.22

^a Average of three runs $\pm$ S.D.

4. Conclusions

A the Hg(II)-imprinting thiol-functionalized mesoporous sorbent was prepared by a surface imprinting techniques in combination with a sol–gel process and used in a micro-column for trace Hg enrichment. The adsorption capacity of Hg(II)-imprinting for Hg(II) ion was three time than that of non-imprinted thiol-functionalized mesoporous sorbent. In addition, Hg(II)-imprinting thiol-functionalized mesoporous sorbent exhibited good selectivity for Hg(II) even in the presence of complicated matrix like seawater.

Acknowledgment

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Evaluation of two commercial capillary columns for the enantioselective gas chromatographic separation of organophosphorus pesticides

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Abstract

The separation of the enantiomers of 13 organophosphorus pesticides (OPPs) has been investigated by gas chromatography (GC) with flame ionisation detection (FID) using two different commercially available chiral columns, Chirasil-Val (L-valine-*tert*-butylamide) and CP-Chirasil-Dex CB (heptakis (2,3,6-tri-*O*-metil)- β -cyclodextrin). Using the Chirasil-Val column no chiral resolution was obtained for the OPPs investigated under any tested experimental condition. The use of the CP-Chirasil-Dex CB stationary phase enabled good individual enantiomeric separation of two OPPs, ruelene and trichlorfon and partial separation of naled, chloretoxypfos, isophenphos and metamidophos. Also, the obtained chromatographic results showed that Chirasil-Dex could resolve enantiomers through the combination of different mechanism (e.g. formation of inclusion complexes and/or interactions outside the cyclodextrin cavity).

Under optimised conditions, precision, linearity range and detection limits were evaluated for the enantiomers of ruelene and trichlorfon using CP-Chirasil-Dex CB column and electron capture detection (ECD). By using the GC-ECD method the enantiomers of these OPPs could be satisfactorily detected at very low concentration levels. The detection limits observed were 1.5 ng mL^{-1} and 11.5 ng mL^{-1} for the enantiomers of trichlorfon and ruelene, respectively.

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1. Introduction

The development of analytical methods for the efficient separation of enantiomers is one of the most important tasks in several research fields, particularly in pharmaceutical and agrochemical research. This is mainly due to the different biological activity that can be exhibited by a pair of enantiomers. Most published papers have focused on the separation of chiral pharmaceutical products as a consequence of the more severe guidelines for marketing new chiral drugs. However, it should be recognized that the same chiral principles apply to pesticides containing stereogenic centers, e.g. in many organophosphorus compounds.

Organophosphorus pesticides (OPPs) are among the most widely employed world-wide. Their large use, especially for crop protection, may result in the presence of residues of these substances in a wide range of surface and ground waters, drink-

ing waters, fruits, vegetables and foodstuff in general [1–4]. The OPPs act as pesticides because of their property of inhibiting acetylcholinesterase (AChE) in insects. Although these compounds show preferential toxicity to insects, they are also toxic to mammals by the same mode of action [5]. Regulatory limits for human exposure are based on inhibition of AChE either in experimental animals or in humans [6,7]. Moreover, some OPPs have been shown to produce long term neurological damage known as organophosphate-induced delayed polyneuropathy (OPIDP) [5].

Numerous OPPs are enantiomeric compounds with phosphorus or carbon atoms as chiral centers. They are introduced into the environment as racemic mixtures despite that the pesticidal activity is usually due to the preferential reactivity of one enantiomer, while the other may have toxic effects on nontarget organisms [8,9]. Additionally, the microbial degradation and the metabolic pathways of chiral pesticides can be stereoselective [10]. Thus, it would be desirable to use single enantiomers as pesticides.

In order to evaluate the real environmental toxicity of chiral pesticides, to understand their chiral discrimination in biolog-

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ical processes and to assess the enantiopurity of commercial pesticide formulations, analytical separation methods of high stereoselectivity are required. Such enantioselective methodologies will be also useful to control the industrial production of enantiomerically pure agrochemicals.

In this regard, the enantiomeric separation of a limited number of OPPs has been performed mainly by high-performance liquid chromatography (HPLC) [8,10–14] and capillary electrophoresis (CE) [9,15–17]. There are very few publications concerning the use of gas chromatography (GC) in the enantiomeric separation of OPPs [18,19], in spite of the high efficiency and sensitivity advantages of enantioselective GC [20]. Many existing chiral OPPs have not been enantiomerically separated yet by GC, specially using commercial chiral stationary phases (CSPs).

Enantiomeric separation by GC is still based on an empirical selection of the stationary phase. In fact, at present it is not possible to predict the exact “enantiomer-selective” behaviour of a particular stationary phase. Thus, most practical chiral analysis depends on the experience obtained in a case by case basis.

In this line, the aim of this work was to carry out a systematic analytical study of the enantioselective gas chromatographic separation of several chiral OPPs, of present importance and with different structures, via the use of two commercially available chiral stationary phases. The two CSPs selected have different separation mechanisms based on hydrogen-bonding (Chirasil-Val column) [20] and (inter alia) inclusion (CP-Chirasil-Dex CB column) interactions [20]. The results obtained could be of interest to select and adequate CSP for eventual application to the determination of this particular racemic OPPs in real samples. Moreover, the GC determination of those two chiral OPPs separating completely on the CP-Chirasil-Dex CB column has been also investigated here.

2. Experimental

2.1. Chemicals

The chiral organophosphorus pesticide standards selected in this work: naled, metamidophos, trichlorfon, malaoxon, malathion, isofenphos, phentoate, phenamiphos, leptophos and dialiphos were purchased from Riedel de Haën (Seelze, Germany) while chloretoxypfos, ruelene and pyraclophos were obtained from Chem Service (New Haven, CT, USA). All pesticides were only available as racemates. The pesticides can be divided in two groups, one group containing a carbon atom as chiral center (Fig. 1) and the other group with a stereogenic phosphorus atom (Fig. 2). Stock standard solutions of each OPP at a concentration of 5000 $\mu\text{g mL}^{-1}$ were prepared in *n*-hexane or acetone and stored at -20°C . These solutions were used for the preparation of different working standard racemic mixtures of the OPPs in *n*-hexane. Acetone and *n*-hexane for pesticides residue analysis were supplied by Riedel de Haën and methanol for ultratrace analysis was obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Gas chromatography was carried out with a HP-5890 Series II gas chromatograph equipped with an electron capture detector (GC-ECD) and a flame ionisation detector (GC-FID) (Hewlett-Packard, Avondale, PA, USA). Helium was used as carrier gas at a pressure of 10 psi. Split/Splitless injections (splitless time 2 min) of 1 μL were carried out. An injector temperature of 260°C (200°C in the case of the trichlorfon separation) and a detector temperature (ECD and FID) of 300°C were used.

Enantiomeric separations were investigated using two chiral columns. The first one, a Chirasil-Val column (25 m $\times$ 0.25 mm I.D., Macherey-Nagel, Düren, Germany) with a stationary phase consisted in *L*-valine-*tert*-butylamide directly bonded to dimethylpolysiloxane; this column has a maximum temperature of 190 – 200°C . The second chiral column used was a CP-Chirasil-Dex CB, (25 m $\times$ 0.25 mm I.D. $\times$ 0.25 μm film thickness, Varian, Walnut Creek, CA) which has a maximum temperature of 200 – 225°C . The stationary phase of this column consisted in a heptakis (2,3,6-tri-*O*-metil)- β -cyclodextrin molecule directly bonded to dimethylpolysiloxane.

3. Results and discussion

As it was mentioned before, the selection of a suitable GC stationary phase which can undergo enantioselective interactions with the desired enantiomers is still highly empirical. Thus, most practical chiral analysis depends on the experience obtained in a case by case basis. In this work, two commercially available chiral columns were selected to investigate the separation of a wide range of chiral OPPs containing carbon (Fig. 1) or phosphorus (Fig. 2) as the sole asymmetric atom.

3.1. Enantiomeric separation of OPPs on a Chirasil-Val column

The Chirasil-Val column was first selected because this stationary phase has excellent properties with respect to thermal stability and separating power for enantiomers able to form hydrogen-bonding interactions. In fact, the enantiomers of some organophosphoroamidothioates have been separated in a column with a similar separation mechanism [21]. Therefore, the GC resolution of only the chiral OPPs containing phosphoroamidate (ruelene, phenamiphos and metamidophos), phosphonate (trichlorfon) or phosphorothioate (malathion and malaoxon) groups in their structures (see Figs. 1 and 2) were attempted with the Chirasil-Val column. Detection was carried out by flame ionisation detector.

Enantioseparations of chiral compounds in gas chromatography are usually governed by enthalpy control and so, the best chiral resolution can be obtained with isothermal elution at low temperatures or slow GC heating rate [20]. Thus, the separation was carried out under isothermal elution at temperatures of 190°C , 160°C and 140°C . The following temperature programs: 50°C for 1 min, then raised to 190°C at a rate of 1 – 4°C min^{-1} were also assayed. Unfortunately, using the above

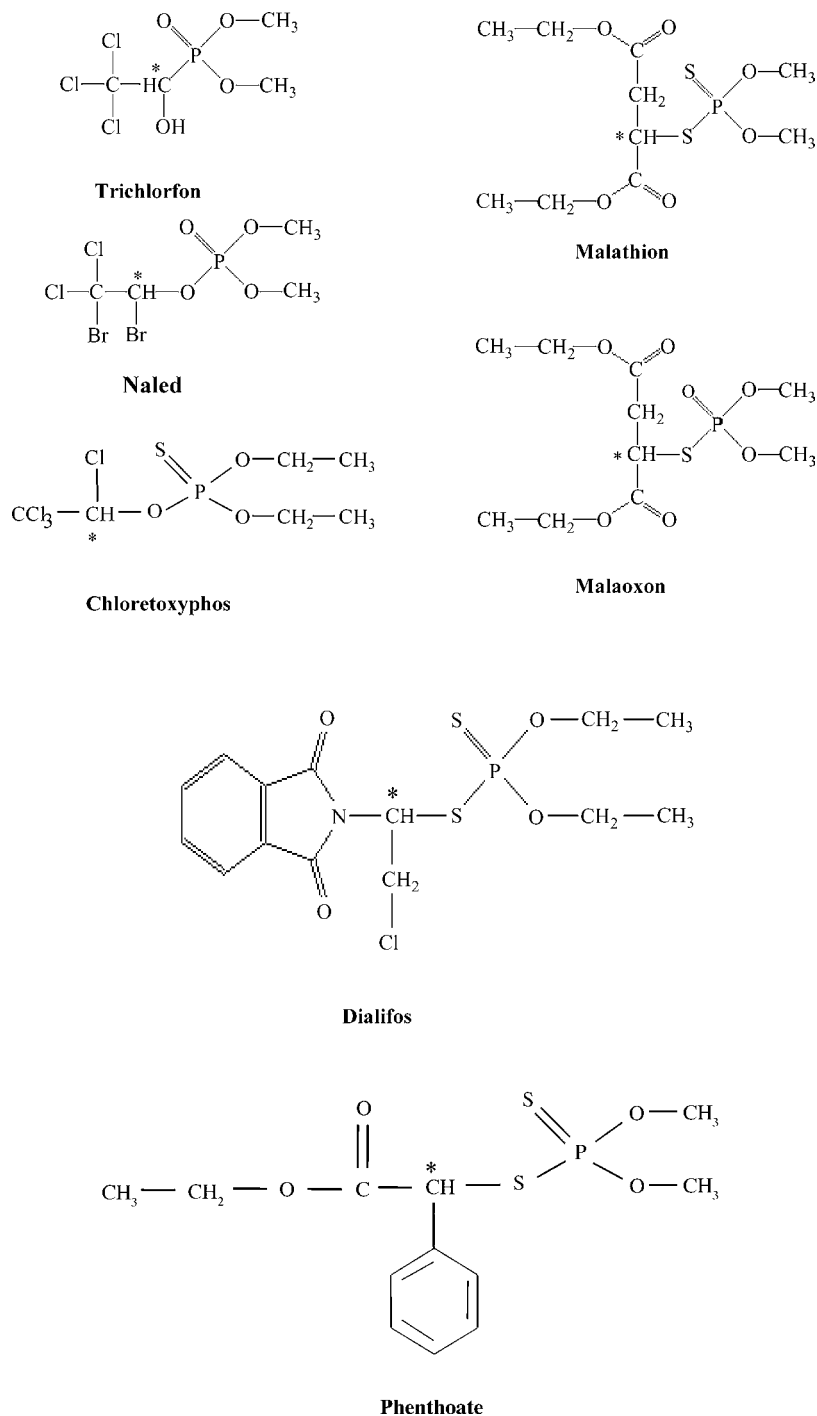


Fig. 1. Chemical structures of the OPPs with an asymmetric carbon atom.

mentioned conditions and after many attempts we were not able to resolve any enantiomers of the OPPs investigated.

3.2. Enantiomeric separation of OPPs on a CP-Chirasil-Dex CB column

It has been demonstrated that polysiloxane-anchored heptakis (2,3,6-tri-*O*-metil)- β -cyclodextrin (Chirasil-Dex) represent useful stationary phases for the GC enantiomer separation of many chiral compounds with different geometries and

functionalities [20,22]. The mechanism of chiral recognition includes a reversible diastereomeric association between the enantiomer and the chiral selector. For modified cyclodextrins (CDs), host–guest interaction via inclusion complex formation is usually accepted as an explanation for the chiral resolution. However, systematic studies of the enantioselectivity of modified CDs showed that most racemic mixtures can be resolved through external or multiple association mechanisms than through pure inclusion processes [23–25]. Therefore, multimodal recognition processes, which may involve inter alia

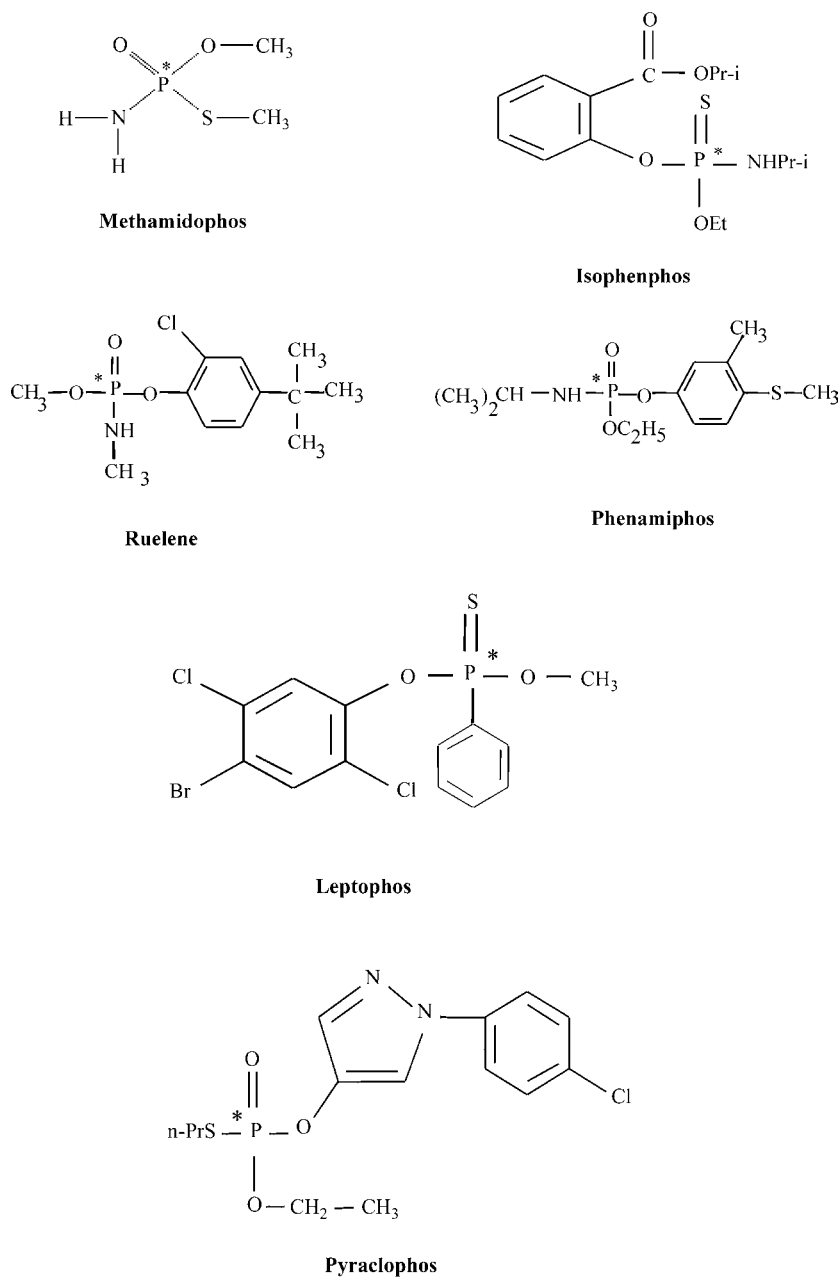


Fig. 2. Chemical structures of the OPPs with an asymmetric phosphorus atom.

inclusion, hydrogen-bonding, dispersion forces, dipole–dipole interactions, electrostatic interactions and hydrophobic interactions, are important [20].

Taking into account the versatility of the CP-Chirasil-Dex CB column, all the OPPs of Figs. 1 and 2 were submitted to GC-FID on this column under isothermal elution at temperatures ranging from 120 °C to 190 °C. The chiral separation was also investigated under different temperature programs, such as 50 °C for 1 min, then raised to 190 °C at different rate from 0.2 °C min⁻¹ to 40 °C min⁻¹.

Table 1 summarises the chromatographic parameters obtained such as retention time, enantioselectivity (α) and resolution factor (R_S) for the 13 OPPs under study. It has to be emphasized that the chromatographic conditions had been opti-

mised for the individual OPPs. As shown in Table 1 only ruelene and trichlorfon are completely separated into enantiomers. While, metamidophos, isophenphos, naled and chloretoxypfos are slightly separated and the rest of the studied OPPs are not separated under any of the chromatographic conditions assayed.

Fig. 3a shows a representative chromatogram of the separation obtained for the enantiomers of trichlorfon. As it can be seen, the enantiomers are baseline separated ($R_S = 2.20$) in 30 min under isothermal conditions at 160 °C. Fig. 3b and c shows the partial separation obtained at very long retention times for the enantiomers of naled ($R_S = 0.89$) and chloretoxypfos ($R_S = 0.69$), respectively, under optimised experimental conditions: 50 °C for 1 min, raised to 110 °C at 10 °C min⁻¹ and then raised at 0.2 °C min⁻¹ to 190 °C for 10 min.

Table 1

Retention time, resolution factor (R_S) and enantioselectivity (α) of OPPs studied under the chromatographic conditions providing best resolution

	t_1	t_2	R_S^a	α^b
Compound asymmetric carbon				
Trichlorfon	28.81	29.90	2.20	1.04
Naled	135.16	135.83	0.89	1.00
Chloretoxyphos	92.10	92.62	0.69	1.00
Malathion	49.05	—	—	—
Maloxon	44.04	—	—	—
Dialiphos	62.50	—	—	—
Phentoate	59.57	—	—	—
Compound asymmetric phosphorus				
Metamidophos	109.84	111.76	0.67	1.02
Ruelene	46.56	47.51	2.01	1.02
Isofenphos	202.87	203.77	1.03	1.00
Phenamiphos	50.76	—	—	—
Leptophos	86.4	—	—	—
Pyraclophos	139.05	—	—	—

t_2 : retention time of the second eluted enantiomer. t_1 : retention time of the first eluted enantiomer.

^a $R_S = (t_2 - t_1)/(w_{b1} + w_{b2}) \times 0.5$; w_{b1} : peak width at the base of the first eluted enantiomer; w_{b2} : peak width at the base of the second eluted enantiomer.

^b $\alpha = k_2/k_1$; t_0 : retention time of solvent; k : capacity factor; $k_2 = (t_2 - t_0)/t_0$, $k_1 = (t_1 - t_0)/t_0$.

A careful look to the structures of trichlorfon, naled and chloretoxyphos (see Fig. 1) reveals that these OPPs, which are completely or partially separated on the CP-Chirasil-Dex CB column, have not functional groups able to form inclusion complexes with β -cyclodextrins. However, dialiphos and phentoate could not be resolved (see Table 1) in spite of the fact that they have a functional group bound to the chiral carbon atom able to form an inclusion complex (see Fig. 1). Thus, the presence, in the structures of trichlorfon, naled and chloretoxyphos, of chlorine or bromine substituents at the carbon atom bound to the asymmetric carbon seems to have some effect on the subtle stereoselective interactions between the racemic compounds to be separated on the β -cyclodextrin column. This fact can contribute to achieve a successful enantiomeric separation of such pesticides.

Compounds such as malathion and malaoxon with no halogenated substituents and no functional groups able to undergo inclusion interactions with β -cyclodextrins (see Fig. 1) cannot be separated into the corresponding enantiomers on the Chirasil-Dex column (see Table 1).

Other OPPs investigated such as ruelene, isophenphos, fenamiphos, leptophos and pyraclophos have similar structures (see Fig. 2), with a phosphorus atom as chiral center and a phenyl ring which might be able to form an inclusion complex with β -cyclodextrin. However, the conducted experiments showed that only the enantiomers of ruelene and isophenphos, respectively, could be completely or partially separated.

A typical chromatogram obtained under optimal conditions (50 °C for 1 min, raised at 20 °C min⁻¹ to 190 °C for 50 min) for the chiral separation of ruelene is shown in Fig. 4a. As it can be seen, very good resolution ($R_S = 2.01$) was obtained in about 46 min. The enantiomers of isophenphos could be just partially separated ($R_S = 1.03$) by using long chromatographic pro-

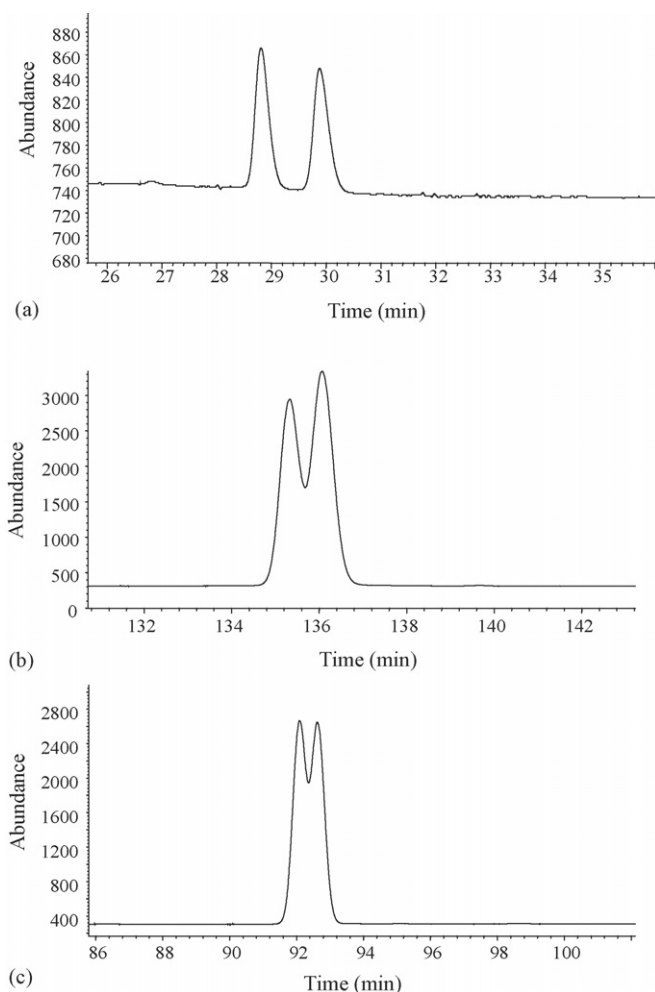


Fig. 3. Enantiomeric separation obtained by GC-FID on a CP-Chirasil-Dex CB column of standard racemic solutions of: (a) Trichlorfon (200 µg mL⁻¹); (b) Naled (200 µg mL⁻¹); (c) Chloretoxyphos (200 µg mL⁻¹). Experimental conditions in the text.

grams such as: 50 °C for 1 min, raised to 125 °C at 10 °C min⁻¹ and then raised to 190 °C at 0.2 °C min⁻¹. The obtained chromatogram is shown in Fig. 4b.

From the results described above, for the OPPs with a stereogenic phosphorus atom, it is clear that the formation of an inclusion complex with CDs does not seem sufficient to achieve acceptable enantiomeric resolution of most pesticides. The presence of a chlorine atom in the phenyl ring, together with an amide group attached to the phosphorus chiral center, may be additional factors to explain the excellent resolution obtained for ruelene (see Fig. 4a). In fact, this CSP has proved excellent enantiomer separations ability for compounds with halogen atoms in their phenyl ring [26,27].

Finally, the resolution obtained for the enantiomers of metamidophos under isothermal elution (120 °C) is very poor ($R_S = 0.67$) as can be seen in Fig. 4c. This compound cannot form an inclusion complex with CDs but it has a phosphoroamidate group (see Fig. 2) with a phosphorus chiral center which seems to be able to suffer stereoselective interactions with the CDs.

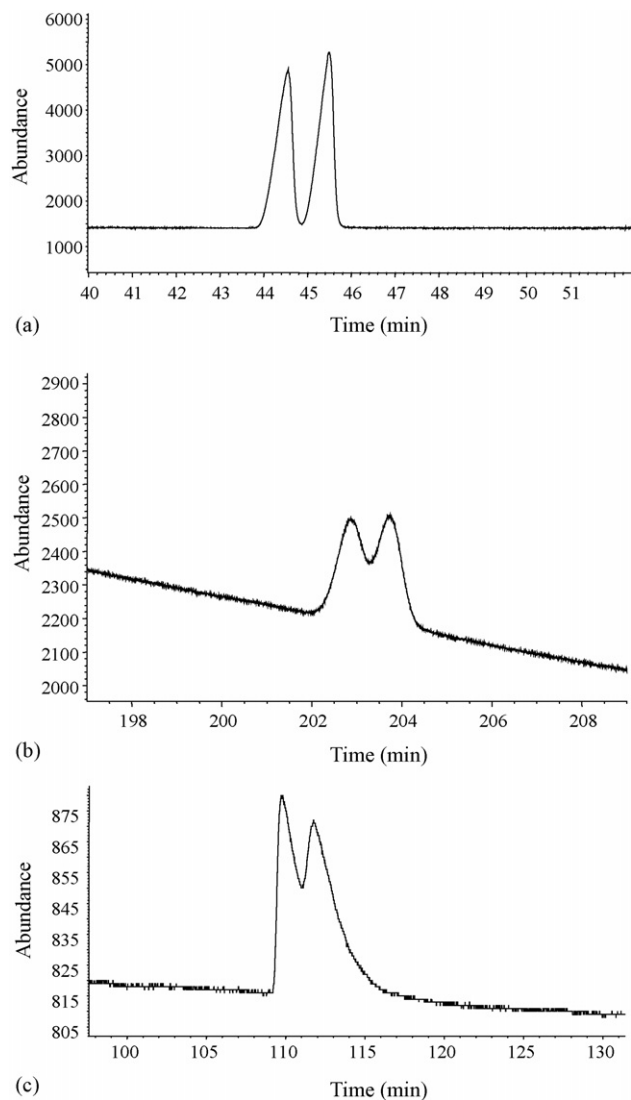


Fig. 4. Enantiomeric separation obtained by GC-FID on a CP-Chirasil-Dex CB column of standard racemic solutions of: (a) Ruelene ($200 \mu\text{g mL}^{-1}$); (b) Isophenphos ($20 \mu\text{g mL}^{-1}$); (c) Metamidophos ($200 \mu\text{g mL}^{-1}$). Experimental conditions in the text.

It should be pointed out that the retention order of the individual separated enantiomers of OPPs could not be determined because such individual enantiomers were not available.

3.3. Analytical characteristics

The analytical performance characteristics of the GC-ECD system using the Chirasil-Dex column for the separation and detection of the enantiomers of ruelene and trichlorfon were also investigated (linearity range, precision and detection limits). Since these two compounds possess chlorine atoms in their chemical structure (see Figs. 1 and 2) the final analytical characteristics of the GC method were evaluated using the electron capture detector (ECD) (more sensitive and selective detector than the FID previously used).

The linearity of the proposed GC-ECD method was tested using standard solutions of racemic mixtures of ruelene and

trichlorfon with increasing concentrations up to $10 \mu\text{g mL}^{-1}$ (maximum concentration assayed). The calibration curves (peak area versus concentration) for the two OPPs studied showed a good linear behaviour in that tested range, with correlation coefficients (R^2) ranging from 0.994 to 0.999. The repeatability, in terms of relative standard deviation (R.S.D.) of peak area, calculated by three individual injections of the standard solutions at a concentration level of 5 ng mL^{-1} , was 2% for the enantiomers of ruelene and 6% for the enantiomers of trichlorfon.

The detection limits, calculated as the lowest concentration of an analyte giving a signal of three-times the base line noise of the chromatogram, were 1.5 ng mL^{-1} and 11.5 ng mL^{-1} for the enantiomers of trichlorfon and ruelene, respectively.

4. Conclusions

A systematic study of the separation of several chiral OPPs on Chirasil-Val and CP-Chirasil-Dex CB columns has shown that only the β -cyclodextrin column enable the enantiomeric separation of ruelene and trichlorfon, and the partial enantiomeric separation of naled, chloretoxyphos, metamidophos and isophenphos. The results obtained seem to confirm that at least two different enantioselective retention mechanisms exist for the cyclodextrin GC stationary phases. Ruelene containing a phenyl group in their structure may be resolved through a classic inclusion complex formation, while the enantiomeric separation of trichlorfon seems to involve interactions outside the cyclodextrin cavity. However, other OPPs studied with structures similar to that of ruelene and trichlorfon were not separated on the Chirasil-Dex column. Thus, it appears that the mechanism of the enantiomeric resolution of the Chirasil-Dex column is rather complex and may involve a combination of multiple associations between the analyte and the chiral selector. Therefore, as expected, to predict or even extrapolate the results obtained for a particular chiral compound on a CSP to other analytes of similar structures is not straight forward.

On the other hand, application of the commercially available β -cyclodextrin column in combination with a sensitive and selective detector such as ECD has allowed the development of a method with adequate analytical performance in terms of linearity range, precision and detection limits for the chiral discrimination and detection of ruelene and trichlorfon enantiomers. The GC-ECD method could be now applied to the analysis of environmental samples as both enantiomers of ruelene are insecticidal and the (+)enantiomer is four times more toxic than the (–)enantiomer.

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Determination of epichlorohydrin in water and sewage samples

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Abstract

The simple, quick and effective methods for the analysis of epichlorohydrin (ECH) in water and sewage samples with the use of gas chromatography have been presented. From among all the methods developed, the procedures for monitoring drinking-water quality and the methods which allow the determination of epichlorohydrin in sewage samples have been selected.

The limits of ECH detection have been determined by direct aqueous injection (DAI) into the chromatographic column and an analysis with the application of a flame ionization detector (FID), a mass spectrometry detector (MS), an electron capture detector (ECD) and atomic emission detection (AED) detectors. The method allows the determination of ECH in water samples at the concentration level of 0.1 mg l^{-1} . Moreover, the developed methods of water samples preparation for chromatographic analysis using the following extraction methods: headspace (HS), stripping with adsorption on solid phase, liquid–liquid extraction (LLE), solid phase extraction (SPE) and solid phase microextraction (SPME) have been evaluated. The limits of ECH detection for each procedure with the application of gas chromatography (GC) combined with various detectors have been determined and their statistical evaluation has been presented. The SPME method allowed us to determine ECH in water samples at the concentration levels of 1.0 ng l^{-1} .

The results of studies on the choice of the selective methods allowing ECH analysis in sewage samples have been demonstrated. The applied SPME method was found to be a quick and effective technique to determine micro trace amounts of ECH in samples containing high amounts of various organic compounds.

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1. Introduction

Epichlorohydrin (ECH), 1-chloro-2,3-epoxypropane, is a raw material used in the production of epoxy resins, synthetic glycerol, elastomers; and it is also applied in paper and pharmaceutical industries [1–3]. In addition, it is widely employed in the production of drinking-water pipes as well as in the synthesis of cationic polyelectrolytes, which are used in surface water and wastewater clarification [4,5]. It can be released from food packaging, e.g. cardboard boxes for drinks, and then it can migrate into juices, milk and other foodstuffs [6]. The migration of ECH, used as a cross-linking agent in packaging materials, into food, drinking water and epoxy resins is possible but it is expected to be low [5]. Recently, ECH has been listed among relevant to

human health compounds that have to be monitored. According to European Council Directive 98/83/EC on the quality of waters intended for human consumption, its acceptable limit in drinking water is $0.1 \text{ } \mu\text{g l}^{-1}$ [4,7,8].

ECH is released into the environment during its manufacturing process together with industrial sewage and wastes as well as during distribution, storage and handling. In consequence of its widespread uses, low concentrations of ECH in wastewater, ground and surface water have been observed [4]. ECH can be lost primarily by evaporation (half-life 29 h in a typical river) and hydrolysis (half-life 8.2 days) [9].

ECH has been shown to be a carcinogen in experimental animals. It is classified in group 2A, which contains chemicals probably carcinogenic to humans, by the International Agency for Research on Cancer (IARC) [1,2,10–12].

Due to its toxicity, ECH has been listed among compounds dangerous to the water environment. No acceptable means of detecting epichlorohydrin are currently available and the Envi-

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ronmental Protection Agency (EPA) requires water suppliers to use special treatment techniques to control its release into the environment [1].

At present no practical routine analytical technique is available to determine epichlorohydrin in water samples at such low concentrations. The problem with the analysis of ECH in water samples arises from its high solubility in water (66 g l^{-1}), volatility and polar character [8].

The determination of ECH and other volatile organic compounds (VOCs) is carried out by gas chromatography which requires multi-stage and time-consuming procedures of sample preparation for the analysis.

ECH can be determined by various analytical techniques (spectrophotometry, spectroscopy); however, the most applicable method is gas chromatography coupled with specific detectors [6,13,14].

The methods of ECH determination in water samples described in literature are most often based on the following methods of isolation and/or enrichment: liquid–liquid extraction (LLE) [15], solid phase extraction (SPE) [8,15], gas extraction – static headspace (HS) [4,15], dynamic headspace – purge and trap (P&T) [4,16].

Michael et al. developed a method based on GC–MS preceded by purge and trap (P&T), enrichment for the analysis of ECH in 200-ml aqueous samples. Nevertheless, the LOD ($0.1 \mu\text{g l}^{-1}$) was inadequate for regulatory purpose [16].

Lucentini et al. [4] developed some methods based on GC-ECD for ECH analysis in drinking-water samples: static headspace (LOD = $40 \mu\text{g l}^{-1}$) and purge and trap (LOD = $0.01 \mu\text{g l}^{-1}$). P&T is a sensitive, simple and rapid method; however, it requires some automatic equipment, which renders it expensive.

Recently, a new method for ECH determination by suppressed ion chromatography has been described in literature. The technique is based on the reaction between an analyte and sulfur(IV) to form a product with a terminal sulfonate group that can be analyzed by anion-exchange chromatography [1,5]. The method (LOD = $0.07 \mu\text{g l}^{-1}$) is, however, cumbersome and time-consuming and also needs a derivatization step.

For several years organic compounds in water matrices have often been determined by solid phase microextraction (SPME) allowing a precise determination of VOCs in water samples at low concentration levels [17–19]. SPME is a fast, solventless alternative to conventional sample extraction techniques. To our knowledge, no SPME method is available for the determination of epichlorohydrin.

The aim of this study was to develop a cheap, simple and precise method allowing quick determination of ECH in drinking-water samples (LOD < $0.1 \mu\text{g l}^{-1}$).

For that purpose, the limits of ECH detection in water solutions determined by a direct aqueous injection (DAI) into the chromatographic column were compared. The possibilities to apply various extraction methods (LLE, SPE, HS, stripping, SPME) and feasible limits of detection with the application of various detectors (FID, MS, ECD, AED) were investigated.

The most applicable technique (SPME) for the determination of trace amounts of ECH in sewage samples was selected from

among all the developed methods. The sewage was sampled from a sewage-treatment plant supplied with a mixture of municipal and chemical plant sewage. ECH was determined in a matrix composed of organic and inorganic compounds characterized by various physicochemical properties, various volatility and polarity as well as different levels of concentrations.

2. Experimental

2.1. Chemicals

Epichlorohydrin (>99%) was purchased from Sigma–Aldrich (Steinheim, Germany). Methanol, tetrachloromethane, dichloromethane, chloroform, water (analytical-reagent grade) were used as solvents (Merck Darmstadt, Germany). Sodium chloride that was used was purchased from POCH Gliwice (Poland).

2.2. GC analysis

In our studies, the following equipment was applied: gas chromatograph HP 5890 (Hewlett Packard, CA, USA) fitted with detectors: FID (flame ionization detector), ECD (electron capture detector), MS (mass spectrometry detector) (HP 5972) and split–splitless injector and gas chromatograph 6890 (Hewlett Packard, CA, USA) equipped with detectors: FID and AED (atomic emission detection) and on-column injector. The chromatographs applied were joined with a connector in order to enable the operation of various injectors with the detectors.

The temperature of the connector (precolumn, $2 \text{ m} \times 0.25 \text{ mm}$) was 200°C .

HP-1 columns (Crosslinked Methyl Siloxane) $30 \text{ m} \times 0.53 \text{ mm} \times 2.65 \mu\text{m}$ and $25 \text{ m} \times 0.32 \text{ mm} \times 0.17 \mu\text{m}$ (for MS detector) were used.

The volumes of the injected solution were: $2 \mu\text{l}$ for on-column and $5 \mu\text{l}$ for split/splitless injector.

Oven temperature program for solvent solutions (split/splitless injector): 50°C (4 min), $10^\circ\text{C min}^{-1}$ to 150°C (0 min).

Oven temperature program for water solutions (on-column injector): 100°C (2 min), $10^\circ\text{C min}^{-1}$ to 200°C (0 min).

Oven temperature program for SPME method (sewage samples): 50°C (4 min), $10^\circ\text{C min}^{-1}$ to 250°C (8 min).

The carrier gas was helium at a constant flow rate of 1.3 ml min^{-1} .

The temperatures of FID and ECD detectors were 220 and 320°C , respectively.

The temperature of the on-column injector was 103°C and of the split/splitless injector was 230°C and the latter one was operated in split mode with a split ratio of 5:1.

MS detector: time-lag in data collection, 3 min; auxiliary temperature, 280°C ; multiplier voltage, 2094 V. In the SCAN operating mode of the MS detector, the ions characteristic for ECH were selected and they were applied in SIM mode: 57, 62 m/z .

Operating parameters of AED detector (Hewlett Packard, CA, USA) (model G2350A): auxiliary temperature, 280°C ;

time-lag in data collection, 3 min. Spectral lines for the individual elements: carbon 496 nm, hydrogen 486 nm, oxygen 171 nm, chlorine 479 nm. Auxiliary gases supplied to plasma burner: oxygen 37.48 psi, hydrogen 6.30 psi, nitrogen 22.40 psi (1 psi = 6894.4 Pa).

2.3. Methods of analyte isolation and concentration

2.3.1. Static headspace (HS)

A thermostat, a gas-tight syringe of 1 ml (Hamilton) and some vials of 4 ml fitted with silicone seals were applied in our tests. The syringe was thermostated at the temperature of the extraction in order to avoid the condensation of the analyte on the walls. The optimum parameters of HS procedure applied in our studies were as follows: the time of sample thermostating, 35 min; the temperature of thermostating, 70 °C; the volume of the sample, 2 ml. A 1 ml volume of gas phase after the extraction was injected into the injector port of the gas chromatograph.

2.3.2. Stripping with adsorption on solid phase

An absorption bulb of 150 ml and an extraction column (J.T. Baker SDB, styrene and divinylbenzene copolymer) of 3 ml with packing mass of 200 mg were used for the extraction. The tested sample of 130 ml was introduced into the absorption bulb and then nitrogen was passed through the inlet at the rate of 50 ml min⁻¹; whereas the outlet was connected to an earlier conditioned extraction column (3 ml methanol). The prepared bulb was placed in a thermostat at the temperature of 60 °C and subjected to stripping for 40 min. After the extraction, the analytes were washed out from the column with methanol (2 ml × 1 ml). Two fractions of the collected extract were subjected to chromatographic analysis.

2.3.3. Liquid–liquid extraction (LLE)

Liquid–liquid extraction (LLE) was performed in separatory funnels at the ratio of the water phase to the solvent of 100:1. The degree of the extraction was determined by using the following solvents: tetrachloromethane, dichloromethane, chloroform. The effect of salting out on the effectiveness of the extraction was determined by introducing sodium chloride into the solution in the following amounts: 150, 200, 250 and 300 g l⁻¹, respectively.

2.3.4. Solid phase extraction (SPE)

Solid phase extraction (SPE) of ECH from water solution was performed by means of J.T. Baker assembly and SDB extraction columns of 3 ml and packing mass of 200 mg. Two columns connected in a series (analytical and control) were applied and they were conditioned with methanol in the amounts of 3 ml × 1 ml. About 1000 ml of solution was passed through the system at the rate of 5 ml min⁻¹. Methanol was applied for elution and it was passed through the columns in the volumes of 1 ml and then two fractions of eluate were collected. The obtained eluates were separately subjected to chromatographic analysis.

2.3.5. Solid phase microextraction (SPME)

Solid phase microextraction was performed by means of SPME assembly (Supelco, Bellefonte, PA, USA) composed of a syringe and a magnetic stirrer with a heated plate.

The prepared water solutions of ECH were introduced into some glass vessels (nut with septum) of 4 ml. Then, the vessels were filled with the tested solution. In the case of SPME the vessels were filled to the top, while in the case of HS-SPME 2 ml of solution was introduced.

Optimization studies allowed us to select the following parameters of the procedure: sorption fiber: 75 µm CAR/PDMS (carboxen-polydimethylsiloxane); sorption time, 15 min; sorption temperature, 50 °C; stirrer revolutions, 1000 rpm.

Parameters of desorption: temperature of split/splitless injector, 240 °C; time, 60 s.

2.3.6. Sampling and storage of sewage samples

Sewages were sampled from the municipal treatment plant which was also supplied with the pre-treated sewage from a chemical plant. The samples were drawn into dark glass bottles of 2 l with PTFE-faced silica septum. The bottles were washed with soap and water, rinsed with tap water, ultra pure water and acetone and placed in an oven at 150 °C for 2 h. The bottles were filled to the top and tightly closed. The samples were stored at the temperature of 4 °C and were subjected to analytical procedures on the day of sampling.

3. Results and discussion

3.1. Direct aqueous injection (DAI)

The limits of epichlorohydrin detection (LOD) were determined by applying FID, ECD, MS and AED detectors, and water solution was directly injected into the chromatographic column.

The obtained limits of ECH detection in water solution with the application of DAI technique and precision for the individual detectors are summarized in Table 1. The limits of detection were estimated by accepting 3:1 signal-to-noise ratio (S/N).

The lowest limits of ECH detection in a water sample on the concentration level of 0.10 mg l⁻¹ were obtained when applying ECD and AED detectors for chlorine analysis. MS detector operating in SIM mode demonstrated quantitation on the similar concentration level. DAI technique is characterized by a suitable precision and accuracy (R.S.D. 2.1–5.3%). The described analytical technique can be applied to determine ECH in water solutions on the concentration levels above 0.1 mg l⁻¹.

The range of DAI technique applicability is limited to the analysis of the so called “pure samples”. The method is applicable in the determination of analytes in samples of drinking water. Its main advantage is the elimination of the stage of sample preparation for an analysis.

Therefore it is considered little time-consuming and low labour-intensive method and the results are burdened with a minor measuring error. However, it is not advisable to apply it for the analysis of environmental samples. Suspensions, inorganic substances and non-volatile organic compounds present in environmental samples can have unfavourable effect on the

Table 1

Limits of ECH detection in water with application of DAI technique and various methods of detection

FID		ECD		AED			MS-SCAN		MS-SIM	
LOD (mg l ⁻¹)	R.S.D. (%) (n = 7)	LOD (mg l ⁻¹)	R.S.D. (%) (n = 7)		LOD (mg l ⁻¹)	R.S.D. (%) (n = 7)	LOD (mg l ⁻¹)	R.S.D. (%) (n = 7)	LOD (mg l ⁻¹)	R.S.D. (%) (n = 7)
3.5	2.9	0.1	3.3	C	14.1	4.6	17.2	2.1	0.2	3.1
				H	1.1	3.5				
				O	3.5	5.3				
				Cl	0.1	2.1				

final result of the analysis or they can cause clogging of the chromatographic column, which results in cutting down its service life.

3.2. Static headspace (HS)

Due to ECH properties (b.p. = 115 °C), attempts to apply the headspace method to ECH determination in water solutions were undertaken. Studies on the procedure of optimization were carried out. The temperature (70 °C) and time of sample thermostating required to attain the state of phase equilibrium (35 min) were determined. The developed method was subjected to statistical evaluation. The precision of the method was determined by means of FID detector. The relative standard deviation (R.S.D.) of the technique was 7.48% (n = 7) and the plotted calibration curve was characterized by good linearity ($R^2 = 0.9902$) within the range of concentrations from 100 to 1000 mg l⁻¹.

The limits of ECH detection in water solutions were determined by means of developed HS technique with the application of various methods of detection. The lowest limit of ECH detection in water (LOD = 0.20 mg l⁻¹) were obtained by the headspace method with MS detector operating in SIM mode. The application of this technique allows the detection of ECH in water solutions in trace amounts; however, its unquestionable advantage is simplicity of the procedure, high sensitivity, good precision and the fact that it is a solventless method. The procedure can be successfully applied to analyze higher contents of ECH in water and environmental samples.

3.3. Stripping with adsorption on solid phase

In order to quantitatively determine ECH in water solutions by stripping with adsorption on the solid phase, the equipment consisting of a glass absorption bulb enabling barbotage and an extraction column connected to it was assembled (Section 2.3.2).

Optimization studies were carried out, i.e. the time and temperature of the process which allow obtaining the lowest limits of detection were determined.

Statistical evaluation of the method was also conducted. The prepared solution (2.79 mg l⁻¹) was subjected to the procedure (n = 7) and was analyzed by means of GC-FID method. The relative standard deviation (R.S.D.) for the results obtained was calculated (6.78%). Moreover, the recovery of the procedure

was determined by calculating from the mean values (n = 7) (104.2%).

The developed method enables quantitative determination of ECH in water solutions on the concentration level of 0.8 µg l⁻¹ by means of DAI-GC and ECD or AED(Cl) detectors.

3.4. Liquid–liquid extraction (LLE)

Studies on the possibilities of applying liquid–liquid extraction method for the isolation and concentration of ECH from water samples were carried out. The preliminary studies comprised the selection of extractant which would enable ECH extraction from water solution with high yield. From all the extractants investigated, chloroform was chosen due to suitable resolution of chromatographic peaks and relatively high recoveries in comparison to other solvents. Moreover, the influence of salting out of solution on ECH concentration was determined in order to obtain better effects of ECH concentration.

ECH can be extracted from water with a very low recovery and in the case of low (12 mg l⁻¹) concentration it is equal to 31.1%. These results are insufficient to achieve satisfactory limits of detection. Due to the volatility of ECH, the application of higher amounts of a solvent or several extractions and evaporation of the solvent excess are not advisable.

The application of salting the sample out caused a significant increase in the recovery (up to about 60%). The addition of NaCl in the amounts of 150 and 300 g l⁻¹ increased the recovery up to 50.7% and 60%, respectively. The progressive increase in the amount of salt caused an increase in the extraction efficiency, which enables to achieve the limit of ECH detection in water of 1.4 µg l⁻¹ (DAI-GC-AED(Cl)).

3.5. Solid phase extraction (SPE)

The determination of ECH in water by applying solid phase extraction (SPE) was carried out according to the procedure described in Section 2.3.4. Statistical evaluation of the method was carried out and R.S.D. was calculated to be equal to 8.6% (n = 7). The calibration curve for SPE-GC/MS method was determined from the solutions of the concentrations from 3.5 to 110 µg l⁻¹. The results obtained ($R^2 = 0.9959$) showed a good linear relationship. Moreover, the recovery of ECH from water for the concentrations from 2.5 to 110 µg l⁻¹ was determined from the mean values obtained for seven measurements at 110%.

Table 2

Limits of detection for ECH in water determined by SPME-GC method

FID		ECD		MS-SCAN		MS-SIM	
LOD ($\mu\text{g l}^{-1}$)	R.S.D. (%) ($n=7$)	LOD ($\mu\text{g l}^{-1}$)	R.S.D. (%) ($n=7$)	LOD ($\mu\text{g l}^{-1}$)	R.S.D. (%) ($n=7$)	LOD ($\mu\text{g l}^{-1}$)	R.S.D. (%) ($n=7$)
0.3	3.9	0.001	4.4	0.2	5.1	0.003	4.9

The developed method enables quantitative determination of ECH in water solutions at concentration levels above $0.1 \mu\text{g l}^{-1}$ by applying DAI-GC and ECD or AED(Cl) detectors.

3.6. Solid phase microextraction (SPME)

Investigations on the determination of limits of detection in water solution were carried out prior to the optimization studies. The optimization of the procedure comprised the selection of GC operating parameters, such as: the kind of extraction fiber, the time of fiber exposure in the sample, the manner of exposure (SPME or HS-SPME), the time of analyte desorption from the fiber, the method of stirring and the effect of salting the sample out. The optimum parameters of the applied SPME procedure are presented in Section 2.3.5.

The determination of the limits of detection and the quantitation was conducted by the analysis of the successive dilutions of solutions. Simultaneously, the calibration curve was plotted at the range of concentrations from 4.8 to $400 \mu\text{g l}^{-1}$ and linear correlation coefficient was determined ($R^2 = 0.9978$).

On the basis of the results obtained, it could be asserted that the method was found to be reliable and characterized by a good linearity.

The limits of the detection and precision of ECH determinations in water samples by SPME technique with the application of various detectors are summarized in Table 2.

The results presented in Table 2 show that the developed SPME technique is applicable for the determination of micro trace amounts of ECH in water solutions (min. LOD = 1.0 ng l^{-1}). The obtained low R.S.D. values and perfect linearity of the method within a wide range of concentrations prove the technique to be precise and the quantitative results to be reliable. The applied method allows the tens increase in sensitivity with respect to analytical methods described earlier. Its advantages over the other methods of analysis include: the limitation of the analytical operations i.e. shorter time of the analytical procedure and the minimization of possible analytical errors which can occur during the successive stages of the procedure.

3.7. Determination of epichlorohydrin in sewage samples

From all the methods studied, the developed procedure for the determination of ECH by SPME technique was selected for the determination of ECH in sewage samples. Waste waters were sampled from the municipal sewage-treatment plant supplied with sewage from a chemical plant producing epichlorohydrin, nitro compounds, dyes and other organic compounds. In order

to assess the usefulness of the developed procedure for the determination of low contents of ECH, the purified waste waters were sampled on the outflow from the sewage-treatment plant. The preliminary studies comprised a selection of the most favourable extraction parameters for ECH on the sorption fiber (Section 2.3.5).

The analyses carried out by means of FID detector showed no differences between the quantities and the qualities of the analytes determined by SPME and HS-SPME techniques. However, taking into account the fact that various organic substances which are present in real water (humic and fulvic acids) are also present in sewage samples, we decided to apply HS-SPME technique in further studies in order to avoid a premature destruction of the fiber. The studies were repeated by making preliminary qualitative analyses using MS detector. The chromatogram obtained as a result of the analysis was presented in Fig. 1.

As a result of GC–MS analysis, the following compounds were identified: (1) phenol, (2) 1,2,3-trichloropropane, (3) 1,4-dinitrobenzene, (4) 1-methyl-4-nitrobenzene, (5) 2-methyl-1,3-dinitrobenzene, (6) 1,3,5-trinitro-2-methylbenzene.

Due to low ECH concentrations in the sewage sample, the quantitative determination of the analyte was impossible by means of FID and MS detectors.

The only possibility to analyze ECH quantitatively at such low concentration levels was to apply more sensitive detector i.e. ECD. The chromatogram obtained is illustrated in Fig. 2.

The application of HS-SPME-GC/ECD technique produced the expected results. The identified peak at the retention time of 8.11 was derived from ECH. The developed method was the basis for quantitative determination of ECH in the tested sewage samples.

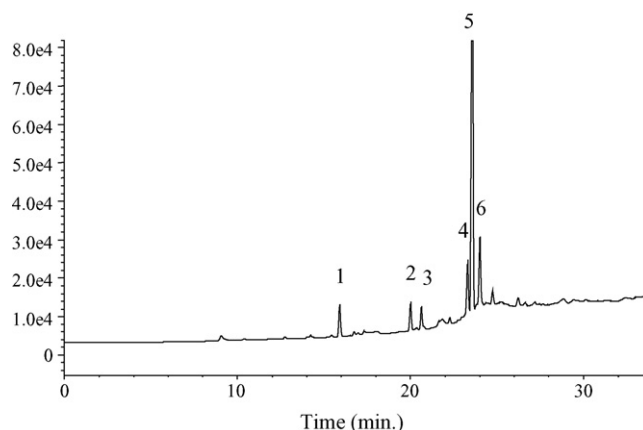


Fig. 1. Chromatogram of sewage sample obtained by SPME-GC/MS technique.

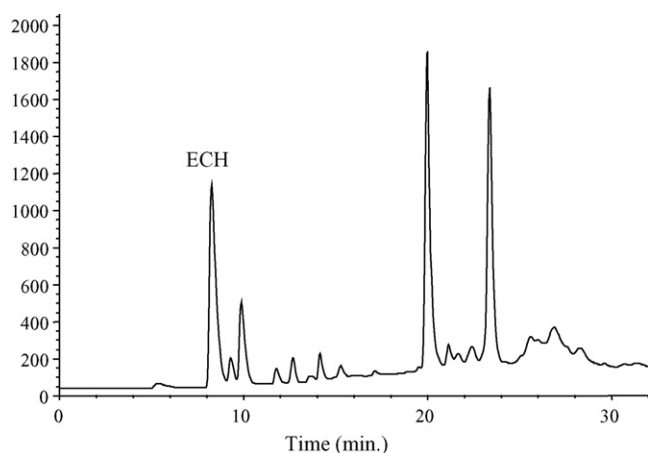


Fig. 2. Chromatogram of sewage sample obtained by HS-SPME-GC/ECD technique.

Therefore, the method of internal standard addition was used. Three samples were prepared for a quantitative analysis by adding the following amounts of ECH: 0.10, 0.20 and 0.30 $\mu\text{g l}^{-1}$, respectively. Each solution of sewage sample (three solutions containing sewage samples with the addition of various amounts of standard and one sample without standard added) was subjected to the developed HS-SPME-GC/ECD procedure.

Each point on the calibration curve was calculated from the mean values from six measurements (R.S.D. < 4.8%). The content of ECH was quantitatively determined from the calibration curve at the point of intersection with the x -axis. Moreover, the linear correlation coefficient (R^2) was determined (0.9921). The content of ECH in the sewage sample was 0.17 $\mu\text{g l}^{-1}$.

The developed method for the determination of ECH was applied to determine its content in the industrial sewage. Studies (HS-SPME-GC/FID ($n = 6$)) showed that concentrations of ECH in sewage supplied to the sewage-treatment plant was within the range from 600 to 1000 $\mu\text{g l}^{-1}$. After biological and chemical treatment, concentration of ECH decreased to the range from 10 to 15 $\mu\text{g l}^{-1}$. The final content of ECH in sewage after treatment in secondary settling tank was below 0.2 $\mu\text{g l}^{-1}$. The plotted calibration curves showed good linearity ($R^2 > 0.98$) and good precision for each point on the curve (R.S.D. < 5.9). Moreover, it was found that application of the method for analysis of sewage containing concentrations of ECH higher than 100 $\mu\text{g l}^{-1}$ required dilution of sample.

4. Summary

The paper presents the results of studies on the development of various methods for the ECH determination in water samples. The developed procedures were compared with respect to their abilities to determine ECH in water solutions at as low concentration levels as possible.

The most favourable limits of detection obtained by the developed analytical methods with the application of MS(SIM) and AED(Cl) detectors are presented in Table 3.

The less time-consuming analysis performed by a direct aqueous injection (DAI) onto the chromatographic column is

Table 3

Limits of ECH detection in water determined by various analytical procedures

Analytical procedure	LOD ($\mu\text{g l}^{-1}$)
DAI	100
HS	200
Stripping	0.8
LLE	1.4
SPE	0.5
SPME	0.001

characterized by high limits of detection ($\text{LOD}_{\text{ECH}} = 100 \mu\text{g l}^{-1}$) and moreover, it is limited to solutions free from suspended matter which is often found in environmental samples. Liquid–liquid extraction is a common method for concentration of samples analyzed by means of the chromatographic method because of its simplicity of performance (low labour-intensive and time-consuming) and reliability. However, it does not give satisfying results when applied to determine the trace amounts of ECH in water samples in spite of salting out.

Solid phase extraction (SPE) technique allows to concentrate the sample repeatedly and to obtain low limits of detection; however, its application for the analysis of sewage samples is limited due to sorbent clogging by matter suspended in the environmental samples.

For monitoring of drinking-water quality ($\text{LOD}_{\text{ECH}} < 0.1 \mu\text{g l}^{-1}$), the solid phase microextraction (SPME) seems to be the most applicable method.

The results of the studies on the selection of the method for the determination of micro trace amounts of ECH in samples containing high amounts of various organic compounds were presented. From among all the extraction methods studied, only transfer of analytes to gaseous phase allows a selective isolation of the analyte from a sewage sample. Headspace analysis with the application of solid phase microextraction (HS-SPME) together with the analysis with ECD detector is the most applicable technique for the determination of trace amounts of ECH in sewage samples.

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The application of ICP-MS and ICP-OES in determination of micronutrients in wood ashes used as soil conditioners

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Abstract

In the present paper, the elemental composition of wood ashes obtained by the combustion of wood in a fireplace was determined with the use of ICP-MS and ICP-OES techniques. Wood ashes may find a potential application as deacidifying agents and soil conditioners, since they contain calcium (in the form of CaCO_3 and CaO), potassium (in the form of K_2SO_4 and K_2CO_3) and significant levels of micronutrients. However, if applied to soil, it is important to assess the bioavailability of particular elements to plants. This process can be simulated by proper extraction procedures.

Various species of wood were combusted in a firestove in a single-family house. The ashes underwent multielemental analyses with ICP-MS Varian Ultra Mass 700 (Australia) and ICP-OES Vista-MPX from Varian (Australia) in order to determine the content of macro- and micronutrients as well as toxic elements. Ashes were also extracted with solutions of 0.1 M NaNO_3 and water in order to simulate the process of elemental transfer from ash (used as soil conditioner) to soil solution and consequently to plants. Also, the environmental impact of ash supplementation to soil was assessed in these experiments. Soil was supplemented with 0–20% of ash. After elution, the eluent underwent multielemental analysis by ICP-MS and ICP-OES techniques to determine the content of macronutrients (P, K, Mg), micronutrients (Fe, Mn, Co, Mo, Zn, Cu and Ti) and toxic elements (Hg, Pb, As and Cd).

It was shown that fireplace ashes can be applied for deacidification of homestead gardens. Ash may be described as a valuable soil conditioner with N:P:K formula 0:1:3. It is concluded therefore that in order to achieve full fertilization, additional supplementation with nitrogen fertilizer would be necessary.

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Keywords: Wood ashes; Macronutrients; Micronutrients; Toxic elements; Soil conditioners; Soil deacidification; ICP-MS; ICP-OES

1. Introduction

The combustion of wood has the potential to become more common way of heating houses. The use of biofuels conforms to recent political tendencies. In Kyoto protocol, 39 industrialized countries (USA, European Union, Russia, East European countries, Japan, Canada, Switzerland) have assigned CO_2 -emission limit. The combustion of wood enables to balance CO_2 emission with consumption. Therefore, in the future, forests will be exploited for wood more extensively [1] and this would lead to a depletion of nutrients in forest soils. One potential way to prevent this would be to recycle the nutrients taken up by burnt wood back to the forest [2]. This would

also be consistent with the policy of sustainable development [3].

Ash from the combustion of wood was sent in the past to landfills, however this method of disposal became expensive. Therefore, the possibility of applying wood ash in agriculture and forestry is being intensively investigated. There are several problems that need to be resolved in order to utilize this by-product in practice. First of all, bioavailability of the nutrients to plants should be assessed. Also, the environmental impact of wood ash supplementation to soil should be investigated, in particular the content and availability of toxic elements and the effect on microbial communities should be studied [4].

Wood ash contains all the nutrients that were taken up by trees from soil, except nitrogen and sulfur that volatilized during the combustion process. Therefore, when used as soil amendment, nitrogen fertilizers should also be applied. When introduced to soil, wood ash acts also as a liming agent by increasing soil pH.

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Table 1
The operating parameters of determination of elements by ICP-OES

Method parameters	Al	Ca, Mg, Na, K	P value
RF power (kW)	0.95	0.80	1.10
Plasma flow (l min ⁻¹)	15.0	12.0	16.5
Nebulizer Flow (l min ⁻¹)	0.69	0.76	0.79
Viewing height (mm)	12	15	15
Pump rate (rpm)	15	15	15
Rinse time (s)	10	10	10
Auxiliary flow (l min ⁻¹)	1.5	0.75	1.5
Replicates	3	3	3
Replicate read time (s)	10	5	5
Instrument stabilization (s)	15	15	15
Sample uptake delay (s)	30	25	23
Element	Emission line (nm)		
Al	396.152		
Ca	396.847		
K	766.491		
Na	589.592		
P	213.618		

Table 2
The operating parameters of determination of elements by ICP-MS

Method parameters	Value
Power (kW)	1.40
Plasma flow (l/min)	16.0
Nebulizer flow (l/min)	0.95
Sampling depth (mm)	5.5
Extraction lens (V)	–450
First lens (V)	–260
Second lens (V)	–11.2
Third lens (V)	0.4
Fourth lens (V)	–60
Photon stop (V)	–10.0
Entrance plate (V)	0.4
Exit plate (V)	0
Pump rate (rpm)	20
Rinse time (s)	10
Auxiliary flow (l/min)	1.25
Replicates	3
Replicate read time (s)	10
Instrument stabilization (s)	10
Sample delay uptake (s)	50

For this reason, it would be advantageous to apply wood ash to soil in the area of acid rain deposition [4].

The composition of wood ash varies and depends on both: tree species and combustion conditions [1]. Wood ash is considered as a rich source of potassium, calcium, magnesium and phosphorus, as well as micronutrients. The average reported composition is: Ca 180 g kg⁻¹, K 27.9 g kg⁻¹, Mg 9.7 g kg⁻¹, P 4.2 g kg⁻¹, N 0.6 g kg⁻¹. It was found that the availability of

potassium was the same as in potassium fertilizers, but phosphorus was lower when compared with phosphorus fertilizers. In the literature [3,11], there is inconsistency in the reports on the availability of magnesium. It is thought that bioavailability of micronutrients (Fe, Mn, Zn and Cu) is low due to increased pH. Wood ash is also a good source of boron [4]. It was found that the level of organic contaminants (PAHs, PCBs, chlorobenzenes and chlorophenols) was low [5,6].

Table 3
The comparison of recommended and information (*) values obtained for Polish Certified Reference Material for multielement trace analysis Fine Flye Ash (CTA-FFA-1)

Element	Certified concentration		Obtained result		Recovery (%)
	Mean	Uncertainty	Mean	Uncertainty	
Al (%)	14.87	0.39	12.64	5.06	85
As (mg kg ⁻¹)	53.6	2.7	46.1	16.1	86
Be (μg kg ⁻¹)	27*	–	28	14	105
Ca (%)	2.29*	–	2.24	0.90	98
Cd (mg kg ⁻¹)	2.8*	–	2.6	0.9	93
Ce (mg kg ⁻¹)	120	7	116	41	97
Cr (mg kg ⁻¹)	156	8	161	56	103
Cu (mg kg ⁻¹)	158	9	153	54	97
Fe (%)	4.89	0.14	4.94	1.98	101
Ga (mg kg ⁻¹)	49*	–	48	17	97
K (%)	2.20*	–	2.18	0.87	99
La (mg kg ⁻¹)	60.7	4.0	58.9	20.6	97
Mg (%)	1.55*	–	1.52	0.61	98
Mn (mg kg ⁻¹)	1066	41	1034	362	97
Na (%)	2.19	0.08	2.28	0.91	104
Ni (mg kg ⁻¹)	99.0	5.8	105.9	37.1	107
P (%)	0.0725	0.0074	0.07	0.02	96
Pb (mg kg ⁻¹)	369	46	362	127	98
Rb (mg kg ⁻¹)	185	5	176	62	95
Sb (mg kg ⁻¹)	17.6	2.5	17.1	6.0	97
Th (mg kg ⁻¹)	29.40	0.70	27.64	9.67	94
Ti (mg kg ⁻¹)	0.58*	–	0.56	0.19	96
U (mg kg ⁻¹)	15.10	0.80	14.04	4.92	93
V (mg kg ⁻¹)	260	10	255	89	98
Zn (mg kg ⁻¹)	569	58	563	197	99

Literature reports that the supplementation of wood ash to soil should be moderate [9]. If applied according to limestone needs, wood ash would be considered a valuable soil amendment, not causing additional soil pollution [4]. It is also advantageous to use wood ash as amendment to acidic forest soil [7,8] usually applied in the dosage 1–7 Mg ha⁻¹ [9].

Hallenbarter et al. [3] studied the combined effect of wood ash and liquid fertilization on nutritional status and growth of Norway spruce in a forest. The authors observed increased growth, but no major shifts in the levels and ratios of nutrients were detected. Arvidsson et al. [9] examined effects of the application of wood ash on ground vegetation of Norway spruce in the dosage 3 Mg ha⁻¹. No visible negative response to the ash application was recorded. Voundi Nkana et al. [10] studied chemical effects of wood ash on growth of rye grass in tropical acid soils. Plants grown on ash-amended soil showed higher biomass production than plants grown on lime and control treatments. Steenari et al. [11] studied the release of mineral nutrients and other species from soils amended with wood ash. Low leach-

ing rates of important plant nutrients were recorded (Mg, Fe and other metals) and rapid release of alkali metals (K, Na) was observed.

Mahmood et al. [12] investigated the effect of wood ash on microbial activity, plant growth and nutrients uptake by ectomycorrhizal spruce seedlings. The authors found that ash treatment had a highly significant positive effect on plant growth as well as on shoot and root concentrations of K, Ca and P irrespective on mycorrhizal status. Zimmermann and Frey [1] examined the effect of wood ash on soil and microbial respiration in soils fertilized with wood ash in the dosage 8 Mg ha⁻¹. Higher biomass and bioactivity of soil microorganisms were observed after supplementation with ash due to increase of pH as well as the level of nutrients, therefore increased mineralization of organic matter was detected. Perkiomaki and Fritze [13] studied the effects of wood ash on boreal forest humus microbial community. The authors found that the supplementation with wood ash resulted in increase of microbial activity. Similar results were obtained by Yrjälä et al. [14], who studied

Table 4
The elemental composition of ashes from various tree species and soils

Element	Literature data Etiegni et al. [17]	Huang et al. [18]	Oak	Oak II	Birch	Apple tree	Ash	Coniferous tree	Soil
Ag (mg kg ⁻¹)			4.19	3.91	6.53	3.65	4.56	4.84	13.4
Al (%)	2.36	1.30	0.85	0.20	0.23	0.15	1.76	0.72	0.64
As (mg kg ⁻¹)			22.0	21.3	19.1	19.8	23.6	23.2	63.9
B (mg kg ⁻¹)	8	127	345	222	511	257	521	274	24.3
Be (μg kg ⁻¹)			535	76	383	255	473	601.	469
Bi (μg kg ⁻¹)			1.37	0.05	60.3	≤0.0005	≤0.0005	177	4.21
Co (mg kg ⁻¹)			7.29	7.450	13.34	4.79	7.230	15.23	4.897
Ca (%)	31.74	10.94	31.1	36.1	29.4	35.1	24.3	19.8	2.32
Cd (mg kg ⁻¹)	21	3	7.8	4.2	23.3	1.7	3.2	16.0	0.27
Ce (mg kg ⁻¹)			1.8	1.9	2.4	0.91	2.9	4.9	6.5
Cr (mg kg ⁻¹)	86	14	75.9	6.07	16.3	5.79	5.69	82.8	25.3
Cu (mg kg ⁻¹)	145	78	111	153	143	96.8	176	123	24.2
Fe (%)	1.95	0.33	0.58	0.56	0.53	0.56	0.48	2.36	0.970
Ga (mg kg ⁻¹)			7.75	73.6	84.8	66.3	87.9	84.6	77.4
Ge (mg kg ⁻¹)			1.60	18.5	24.1	24.5	20.7	43.4	30.2
Hg (μg kg ⁻¹)			6.30	≤0.0001	0.01	≤0.0001	≤0.0001	≤0.0001	95.29
K (%)	4.13	2.86	4.85	8.22	6.25	5.16	12.26	3.43	0.454
La (mg kg ⁻¹)			1.11	5.16	2.31	0.666	1.99	10.4	3.20
Mo (mg kg ⁻¹)			2.81	0.334	0.960	1.28	0.419	1.95	1.74
Mg (%)	2.25	1.62	0.96	3.33	2.51	5.16	2.77	1.35	0.251
Mn (%)	0.669	0.347	0.999	1.23	1.69	0.191	0.236	0.775	0.047
Na (%)	0.34	0.16	0.21	0.15	0.16	0.19	0.24	0.86	0.122
Nb (μg kg ⁻¹)			521	464	419	362	316	8107	1397
Ni (mg kg ⁻¹)	47	12	87.7	63.4	37.8	47.0	102	53.1	10.8
P (%)	1.40	0.69	0.85	1.52	1.97	1.15	2.19	2.11	0.169
Pb (mg kg ⁻¹)	130	66	32.1	87.1	103	25.2	42.6	690	15.2
Pd (μg kg ⁻¹)			653	494	448	580	719	538	567
Rb (mg kg ⁻¹)			47	103	141	37.2	158	177	22.3
Rh (μg kg ⁻¹)			20.4	11.6	≤0.0006	29.2	12.0	11.5	≤0.0006
Sb (mg kg ⁻¹)			2.63	1.13	0.516	1.02	0.49	14.1	4.63
Sn (mg kg ⁻¹)			1.76	0.316	0.351	0.346	0.230	2.32	1.37
Th (μg kg ⁻¹)			≤0.0006	≤0.0006	≤0.0006	≤0.0006	≤0.0006	≤0.0006	0.0015
Ti (mg kg ⁻¹)			259	302	294	237	256	2941	675
Tl (μg kg ⁻¹)			75.6	17.2	0.05	0.00	0.06	119	15.2
U (μg kg ⁻¹)			28.8	13.5	15.5	6.45	18.0	40.0	47.5
V (mg kg ⁻¹)			0.00	2.71	≤0.03	≤0.03	≤0.03	20.3	5.12
Zn (mg kg ⁻¹)	700	794	228	401	4903	756	577	5020	117
Zr (mg kg ⁻¹)			1.08	1.66	1.21	0.770	0.769	7.54	6.53

Table 5

The comparison of the composition of wood ash and soil (a), extraction efficiency (b) and bioavailability (c) of elements from ash, soil and soil supplemented with ash

(a)				
Macronutrients and micronutrients the level of which was higher in wood ash when comparing with soil Macronutrients: K (15 times higher content in ash than in soil), Ca (13 times), Mg (11 times), P (10 times), Na (2.5 times) Micronutrients: Cd, Pb, Bi, Ni, Tl.		Elements the level of which was similar in ash and in soil Cr, Nb, La, Ti, Al, Pd, Ga Fe, Be, V, Sb, Ge, Sn, Mo	Toxic elements the level of which was lower in ash when comparing with soil Hg (91 times), Zr (3 times), As (3 times), Ag (3 times), Ce (2.5 times), U (2 times)	
(b)				
The extraction efficiency of the following elements increased after addition of wood ash to soil K, Sb, Mo, Rb, Ge, B, V, Ga, Na, Cr, U	The extraction efficiency of the following elements reached maximum at intermediate level of ash supplementation (5–10%) Al, Cd, Zr, Ni, Nb, Co, Zn, Mn, Pb, La, Ce, Be, Rh	The extraction of the following elements decreased with increase of ash supplementation Ca, Mg, P, Ti	The extraction efficiency of the following elements did not change after addition of wood ash to soil As, Cu, Fe, Ti, Bi, Sn	
(c)				
Availability of the following, elements increased with the increase of the level of ash supplementation Co, Zr, U, Sb, V, Cu, Ce, La, Ge, Ti, Nb, Fe, Sn, Ga, As, Pb, Cd, Pd, Ni, Mn, Zn	Availability of the following, elements decreased with the increase of the level of ash supplementation Rb, Ti, Cr, Al, Ca, P, Mg, Bi	Availability of the following, elements did not changed with the increase of the level of ash supplementation Mo, K, B	The following elements were available from soil (extraction efficiency was higher than 5%) Mo, K, Sb, V, Ti, Rb, Cr	The following elements were available from ash (the level of supplementation 5%) Mo, K, Rb

changes in *Archaea* from coniferous forest humus after ash treatment.

The aim of this study was to investigate the applicability of wood ash as a soil conditioner and deacidifying agent. The composition of wood ash and bioavailability to plants of macro-, micro- and toxic elements from soil supplemented with ash (as the function of the level of ash supplementation to soil that was in the range 0–20%) to plants were estimated. Also, the environmental impact was simulated under laboratory conditions. In the present study, the level of 39 elements was tested in the studied ash material as well as in leached extracts. The concentration of many elements was very low and therefore could be detected only with the use of the very sensitive methods of ICP-MS and also ICP-OES.

2. Materials and methods

The investigation into the micronutrient content of ash, soil and soil supplemented with ash and their potential availability to plants was assessed directly with the use of multielemental analysis and also after extraction with 0.1 M NaNO₃ (bioavailability test) and water (environmental impact test). Previously [15], several extraction solutions were investigated (1 mol/l MgCl₂, 0.1 mol/l NaNO₃, 0.1 mol/l K₂P₂O₇, 2% (w/v) CH₃COOH, 2% (w/v) HCOOH, 2% (w/v) ammonium citrate, 2% (w/v) citric acid, 0.05 mol/l Na₂EDTA, 0.1 mol/l EDTA, 0.05 mol/l CaCl₂, mixture of HCl and HNO₃) in order to select the extraction system that would enable to simulate the bioavailability of elements to plants. The extracting agent that showed the highest value of correlation coefficients between the composition of

soil extract and plant was 0.1 mol/l NaNO₃ (average correlation coefficient: 0.649). For this reason, in the present study this solution was used to estimate bioavailability of elements from soil, soil supplemented with wood ash and wood ash to plants.

2.1. Wood ash and soil

Wood ash was obtained through the combustion of oak wood in a single family house in a fireplace (Supra, France) having the following characteristics: power 15 kW, the rate of wood combustion was typical (0.06–0.07 m³ wood day^{−1}). After the combustion, the ash was collected, grinded and mixed. Soil used in the experiment was standard commercially available garden

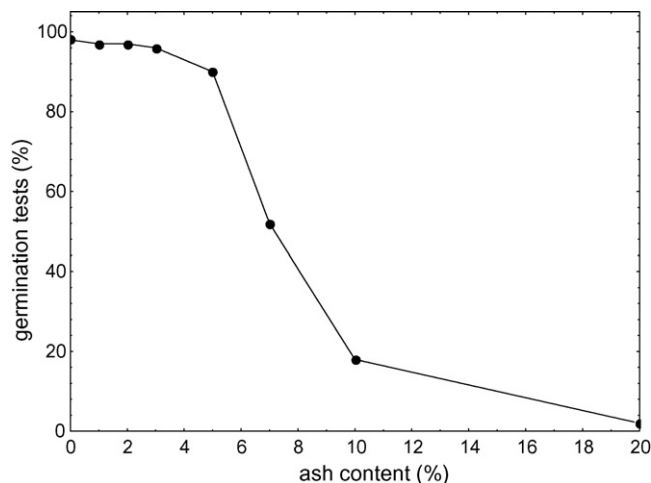


Fig. 1. Germination tests on soil supplemented with ash.

soil that was not enriched with neither organic nor mineral fertilizers. The ash was introduced to the soil in quantities ranging from 0 to 20%. The mixture underwent microwave digestion and afterwards the content of macronutrients, micronutrients and toxic elements was determined. The effect of ash supplementation on plants was also studied by germination tests that were performed on Petri dishes with the use of radish seeds. Germination efficiency was calculated as the fraction of seeds that germinated. Also, the composition of wood ashes originating from other tree species (birch, apple tree, ash tree and coniferous tree) was determined.

2.2. Elution

The ash and the mixture of ash and soil underwent the process of extraction with 0.1 M NaNO_3 solution and water. The composition of the eluents obtained after extraction (4 h) of 10 g of ashes in 100 ml of extractant was determined with the use of both: ICP-OES and ICP-MS. The eluents were analyzed in order to collect data on elemental solubilities in solutions considered to be commonly used extraction solutions and to determine the level of bioavailability to plants. High correlations between

the concentration of the majority of elements in the solution of 0.1 M NaNO_3 after extraction and in plants have been previously reported [15].

2.3. Analytical methods

The samples of ash and soil underwent microwave digestion by samples decomposition in Teflon bombs with the use of Milestone MLS-1200 MEGA microwave digestion system. For digestion of 0.1 g ash and 0.5 g soil, a mixture of ultra pure concentrated acids (2.5 ml of nitric acid and 7.5 ml of hydrochloric acid from Merck) was used. Afterwards, the samples were diluted with demineralized water to the volume 75 ml. The extracts were analyzed directly, without further digestion.

The digested samples of ash, soil mixed with ash, as well as solutions of the extractants after elution underwent multielemental analyses with the use of plasma spectrometry: macronutrients and Al were analyzed with the use of ICP-OES Varian Vista MPX (Australia) (Ca, K, Mg, Na, P and Al). The concentration of micronutrients and toxic elements (except Hg) was determined with ICP-MS Varian Ultra Mass 700 (Australia). Mercury was analyzed with Mercury analyzer AMA 254 (the

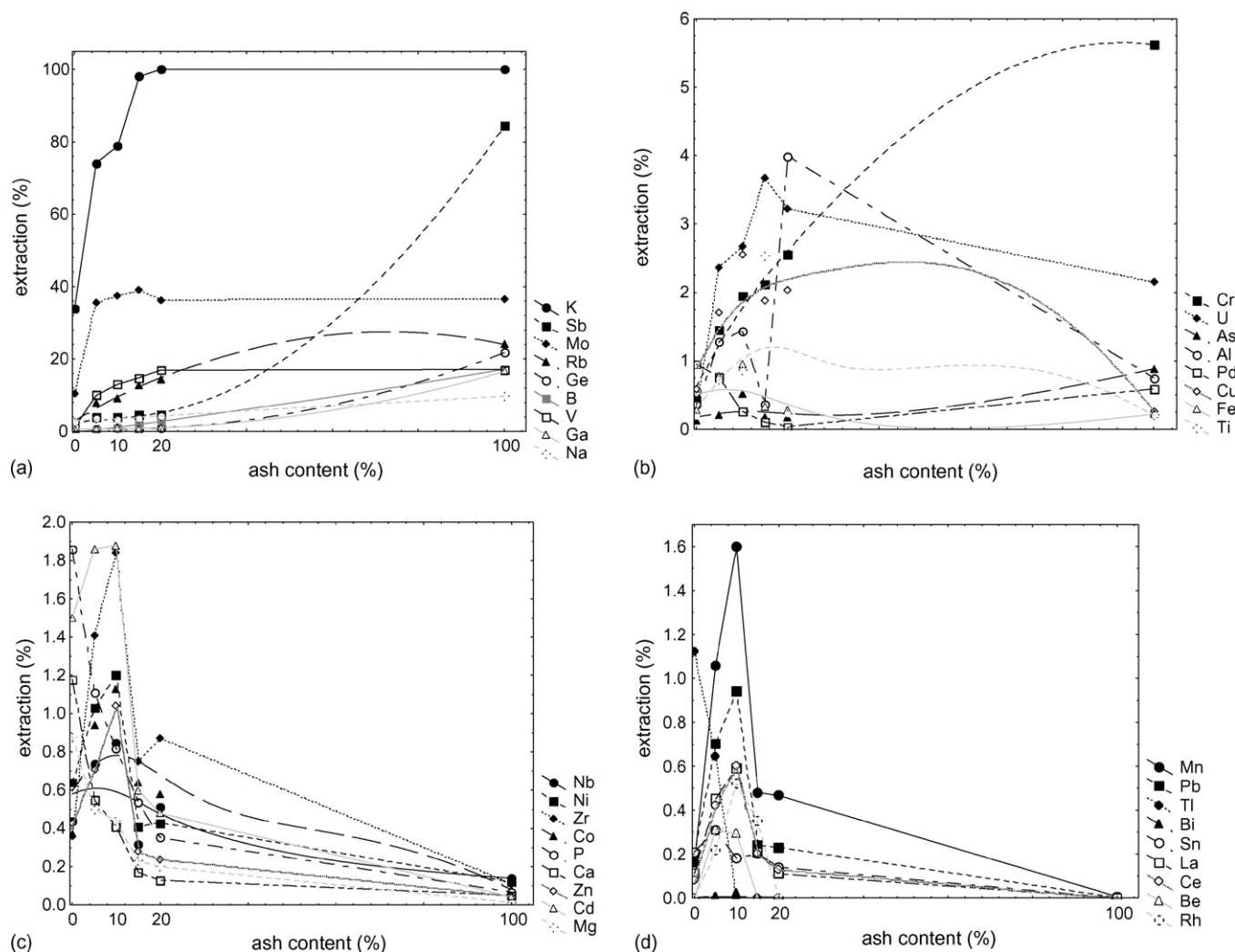


Fig. 2. Elution of elements from soil supplemented with ash with water.

operating parameters: sample mass: 0.2 g, drying time: ash and soil, 10 s, extract, 50 s, decomposition, 200 s, waiting: 60 s), atomic absorption spectrometer (Czech Republic). The operating parameters for ICP-OES are shown in Table 1 and ICP-MS in Table 2. The instruments were calibrated with the use of multielement standard solutions prepared from single elements standard solutions (Ultra Scientific ICP standards 1000 and 10,000 $\mu\text{g ml}^{-1}$). The analytical process was validated by the use of an ash matrix standard. Polish Certified Reference Material for multielement trace analysis Fine Flye Ash (CTA-FFA-1) from Institute of Nuclear Chemistry and Technology (Poland) was analyzed and the comparison of obtained results and certified concentrations is shown in Table 3. Uncertainty and recovery were calculated according to international standards [16].

3. Results and discussion

Many authors report [1,4] that the composition of wood ash varies in dependent on not only tree species, but also on local conditions (i.e. soil content) on which a tree was growing. In the present study, the analysis of wood ash from various tree species (oak, birch, apple tree, ash tree and coniferous tree) showed large

differences (Table 4). Also, differences in the composition of wood ash originating from the same tree species were observed. The average composition of wood ash was compared with the composition of the soil to which it was introduced. The comparison of ash and soil content is presented in Table 5a. It was found that wood ash is a source of macro- and micronutrients. The level of toxic elements was found to be similar. This data support the theory that wood ash if supplemented to soil is a rich source of macro- and micronutrients. It contains also some toxic elements, however since wood ash affects soil pH it would probably decrease bioavailability of elements (supplied by ash and also present in soil) to plants.

When introducing wood ash to soil, it is important to estimate the appropriate dosage. High dosages of ash might be dangerous to plants and the microbial community, due to pH changes. In the present study, the garden soil was supplemented with ash in the quantity 0–20%. We examined the effect of ash level supplementation on germination efficiency of radish seeds (Fig. 1). We also studied the effect of ash content in soil on elemental transfer to the aqueous environment in order to assess the potential transfer of toxic elements to ground and surface waters, as well as to determine the loss of fertilizer components: macro-

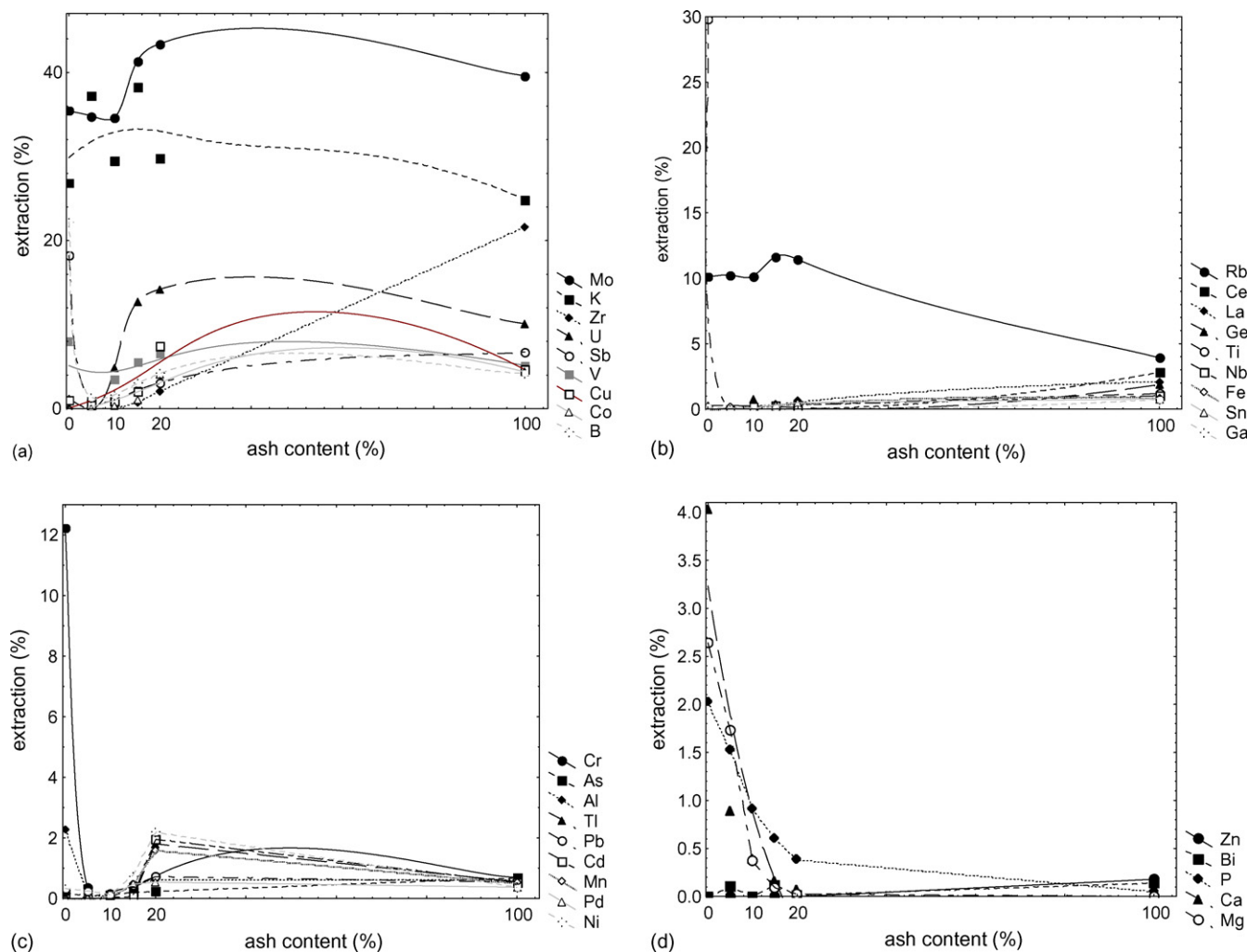


Fig. 3. Elution of elements from soil supplemented with ash with 0.1 M NaNO_3 .

and microelements (Fig. 2). Also the bioavailability of elements to plants was investigated (Fig. 3).

The level of ash supplementation had a substantial effect on seeds germination. It was found that ash supplementation at the level 1–5% did not have strong adverse effect on germination of radish. Introduction of 20% ash resulted in almost total growth inhibition (Fig. 1).

The environmental impact of ash supplementation was studied by extraction with water (Fig. 2). Extraction of ash, soil and soil supplemented with ash was performed. We found that the level of ash supplementation had an effect on elements transfer to water (Fig. 2). The results of experiments on the extraction efficiency are presented in Table 5b. Bioavailability of elements to plants was examined through the extraction with 0.1 M NaNO₃. Different results were obtained when comparing with the extraction with water. Availability of elements is discussed in Table 5c.

4. Conclusions

In order to investigate the theoretical bioavailability of elements from soil and soil supplemented with ash, extraction procedures were also utilized. The environmental impact of elemental transfer to ground and surface water was also investigated. The level of 39 elements was determined in wood ash, soil and extracts using the analytical techniques of both ICP-OES and ICP-MS.

The experimental results showed that the supplementation of wood ash to soil resulted in increase of the levels of macroelements, microelements and toxic elements, the solubility in water and availability to plants was low. Wood ash was found to be a rich source of macronutrients: the concentration of K, Ca, Mg and P was 10–15 times higher than in soil. Also, wood ash supplied micronutrients. Over 15 times higher levels of Mn, Zn and B were detected. The level of some toxic elements was higher (Cd, Pb), but the concentration of other (Hg, As, U) was lower when compared with soil. Although ash was the source of various elements it did not mean that they were readily available to plants and environment. We found that only potassium showed high solubilities in water, unlike the remaining macronutrients (Ca, Mg and P), the solubility of which significantly decreased. The solubility of micronutrients supplied by the ash: Mn and Zn increased at intermediate levels of ash supplementation and decreased at higher levels. The effect on boron solubility was reverse. The solubility of toxic elements supplied by ash (Cd and Pb) was very low: in the case of Cd

less than 2% and in the case of Pb less than 1%. Bioavailability tests showed that the level of ash supplementation did not affect the availability of K. Bioavailability of the remaining macronutrients (Ca, Mg and P) was greatly reduced. The availability of the majority of micronutrients and toxic elements decreased. This showed that ash acted as a fertilizer that released nutrients slowly. This also showed reduced environmental impact, since nutrients were not eluted to ground and surface waters. By taking into consideration germination efficiency of plants, the dosage of ash should not exceed 5%. Therefore, we concluded that the safe level of ash supplementation, when taking into consideration germination efficiency, bioavailability to plants and environmental safety would be on the level lower than 5%.

Summarizing, our data showed that it is possible to use ash obtained by heating a single family house (annual ash production 150 kg ash year⁻¹).

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Structure–activity correlations for illicit amphetamines using ANN and constitutional descriptors

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Abstract

The goal of this study was to develop an expert system capable to identify the potential biological activity of new substances having a molecular structure similar to illicit amphetamines. For this purpose we have designed two types of artificial neural network (ANN) systems, which have been trained to classify amphetamines according to their toxicological activity (stimulant amphetamines or hallucinogenic amphetamines) and distinguish them from nonamphetamines. Such a system is essential for testing new molecular structures for epidemiological, clinical, and forensic purposes.

The first type of artificial neural network is a “spectral” neural network, which has as input variables the most important 100 absorption intensities from a total of 260 measured for each normalized infrared spectrum 10 cm^{-1} apart. The spectral data consists of a database built with the GC–FT-IR spectra of the most popular drugs of abuse (mainly central stimulants, hallucinogens, sympathomimetic amines, narcotics and other potent analgesics), precursors and derivatized counterparts.

All samples were also characterized by their constitutional descriptors (CDs). For each sample, a number of 45 CDs were computed and introduced as input variables for a second type of ANN, which uses a structural database. The efficiency of this “structural” artificial neural network (CD-ANN) has been improved by optimizing the training set and increasing the number of input variables (CDs). A comparative analysis of the spectral and the structural networks is presented.

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Keywords: Amphetamines; GC–FT-IR; Artificial neural networks; Drugs of abuse; Constitutional descriptors

1. Introduction

The interest in using neural networks modeling has substantially increased in the recent years, along with an ever-increasing array of practical applications in chemistry and spectroscopy [1–5]. The ANN is a computer-based system derived from a simplified concept of the brain in which a number of nodes, called processing elements or neurons, are interconnected in a network like structure. The nodes operate in parallel and can be trained to map a set of input patterns into a set of output patterns. Each processing element has the basic functionality of a biological neuron: to receive signals, to sum the signals, to transform the signals with an activation

function, and to produce a signal that is passed into other elements.

The most common architecture of an ANN is the multi-layer feed-forward network, in which the nodes are organized in three types of layers: a layer of input nodes, one or more layers of hidden nodes and a layer of output nodes. Each node in one layer is connected to all the nodes of the next one. The activity of each hidden node is determined by the activities of the input nodes and the weights associated with the connections between the input and the hidden nodes.

The advantage of ANN is that it is possible to “train” the network. It learns by modifying the values of the weights in a well-defined manner, described by the learning rules. The general type of learning is supervised learning, which requires knowledge of the desired responses to input signals and incorporates an external teacher (algorithm to apply the learning rule).

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The aim is to minimize the errors between the desired and computed output values.

There are many chemical applications in which neural networks are used, although they could probably be solved equally well by using pattern recognition methods such as clustering methods, principal component analysis or partial least squares. In many cases, these methods should be able to deliver results that are as good as those obtained with a neural network. However, the use of an ANN system has some important advantages, due to the fact that the mathematical form of the relationship between the input and output data does not need to be provided. In addition, the ANNs are able to represent in a very simple way the non-linear relationships between the input and output patterns.

Amphetamines, generally referred to as phenylethylamines (see Fig. 1), are the class of compounds with the largest number of individual substances on the illicit drug market [6]. They are abused for their stimulant and/or hallucinogenic effect. In attempts to circumvent the law, clandestine laboratories are often changing the molecular structure of these compounds, without altering the psychotropic effect. Novel analogues emerge frequently and toxicologists are often confronted with structure and biological activity elucidation challenges. As amphetamines are relatively volatile substances, gas chromatography–mass spectrometry (GC–MS), and more recently gas chromatography–Fourier transform infrared spectroscopy (GC–FT-IR) became the most powerful techniques applied for their identification.

In previous papers [7–10] we have presented ANN systems that successfully perform an automated classification of illicit amphetamines using their GC–FT-IR spectra. In this study we are presenting an evaluation of the performances of two types of neural networks: a spectral neural network (ANN) and a structural neural network (CD-ANN). Both networks are specialized in the classification of amphetamines, the first on basis of their digitized GC–FT-IR spectra and the latter on basis of their constitutional descriptors.

2. Experimental

2.1. Experimental set-up

The experimental conditions in which the GC–FT-IR data was recorded and the mathematical processing of these spectra that was performed in order to build the spectral database are described in previous papers [7–10]. In order to compute the constitutional descriptors, the molecular structures of 146 molecules included in the spectral database were represented in 3D coordinates, using the *HyperChem* software (Version 6.03). The remaining 13 substances represent the levo or dextro isomers of the molecules included in the database. These molecules could not be included in the CD database because, although they do not have the same biological activity as their counterpart, they cannot be discriminated by these variables, as both forms are characterized by the same values of the constitutional descriptors. The geometries of these molecules were fully optimized using the molecular mechanics method of Allinger (the MM+ option). Molecular mechanic calculations treat atoms as Newtonian particles interacting through a potential energy function. Potential energy depends on bond length, bond angle, torsion angle, and non-bonded interactions (van der Waals force, electrostatic interactions, and hydrogen bond). Non-bonded electrostatic interactions were calculated using partial atomic charges.

The files containing these molecules have been used as input for the *Dragon* software (Version 1.11), which has been used to compute a number of 45 constitutional descriptors. All calculated descriptors are summarized in Table 1. The molecular weight (MW) and the sum of atomic van der Waals volumes (S_v), atomic Sanderson electro-negativities (S_e), atomic polarizabilities (S_p), Kier–Hall electro-topological states (S_s) were also calculated and used as global descriptors, based on the atomic properties accounting for the steric and electronic features of the molecule.

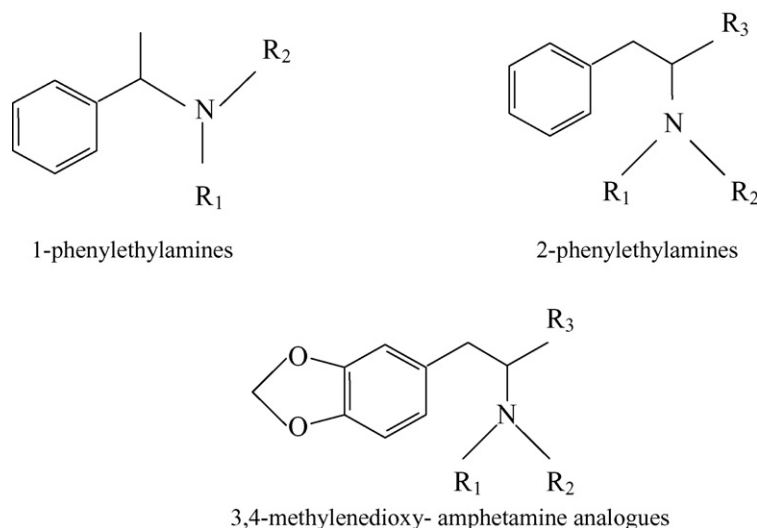


Fig. 1. Molecular skeletons of the main illicit amphetamines.

Table 1
List of computed constitutional descriptors

Description	Symbols
Average molecular weight	AMW
Molecular weight	MW
Sum of atomic van der Waals volumes	S_v
Sum of atomic Sanderson electro-negativities	S_e
Sum of atomic polarizabilities	S_p
Sum of Kier–Hall electro-topological states	S_s
Mean atomic van der Waals volume	M_v
Mean atomic Sanderson electro-negativity	M_e
Mean atomic polarizability	M_p
Mean electro-topological state	M_s
Number of molecule atoms	n_{AT}
Number of non-H atoms	n_{SK}
Number of bonds	n_{BT}
Number of non-H bonds	n_{BO}
Number of multiple bonds	n_{BM}
Number of double bonds	n_{DB}
Number of triple bonds	n_{TB}
Number of aromatic bonds	n_{AB}
Number of heavy atoms	n_{HM}
Atom-type counts	$n_H, n_C, n_N, n_O, n_F, n_{Cl}, n_{Br}, n_I, n_X$
Functional group counts	$n_{OH}, n_{NH}, n_{NH_2}, n_{CO}, n_{NO}, n_{NO_2}$
Number of donor atoms for H bonds	n_{HD}
Number of acceptor atoms for H bonds	n_{HA}
Counts of different size rings	$n_{CIC}, n_{CIR}, n_{R04}, n_{R05}, n_{R06}, n_{R07}, n_{R08}, n_{R09}, n_{R10}$

2.2. Development of the ANN systems

The database was used for the development of a spectral neural network aiming the automatic class identity assignment of amphetamines. The network has a single hidden layer (besides the input and the output layer). The output layer has three nodes, and distinguishes between stimulant amphetamines (class code M), hallucinogenic amphetamines (class code T) and nonamphetamines (class code N). The inputs were sample GC–FT-IR spectra, each containing the maximum number of the spectral data (260 variables). These data represent the absorption intensities measured in the normalized GC–FT-IR spectra, i.e. 260 absorption intensities measured 10 cm^{-1} apart. The number of neurons for the hidden layer and the weight of the connections were optimized at 12 hidden neurons and 3480 weight connections.

Due to the large number of input nodes and, implicitly, of the weight connections, the training process of this neural network takes a very long time. In order to remove this disadvantage and the ratio between the size of the training set and the number of input variables, we have optimized the system by reducing the number of input variables to only 100 absorption intensities. These variables are the most important direct spectral data, as determined taking into account their modeling and discrimination power.

Classification experiments were carried out on 159 spectra of 15 stimulant amphetamine analogues, 8 hallucinogenic amphetamine analogues and 136 nonamphetamines represent-

ing forensic substances such as drugs of abuse (mainly central stimulants, hallucinogens, sympathomimetic amines, narcotics and other potent analgesics), precursors, or derivatized counterparts. The samples represent reference standards and compounds synthesized at the laboratory.

The ANN system reached an optimum for an architecture consisting of 13 hidden neurons and 1339 weight connections. The network was trained using a backpropagation algorithm. As a result of the optimization process, the learning rate and the momentum were fixed to $\alpha = 0.70$ and $\mu = 0.80$, respectively. We have used the sigmoid function as transfer function.

The training set consisted of 29 samples, 7 stimulant amphetamines, 5 hallucinogenic amphetamines and 17 nonamphetamines. The remaining 130 samples were included in validation set. The stimulants and hallucinogens included in the training set are the following: *N*-ethylamphetamine, amphetamine, methamphetamine, *N*-*n*-propylamphetamine, β -phenylethylamine, α -phenylethylamine and *N*-methyl- α -phenylethylamine, and 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxy-*N*-ethylamphetamine, *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine and 1-(3,4-methylenedioxyphenyl)-2-butanamine. The stimulant and hallucinogenic amphetamines of the training set have been selected in the order of their similarity to the parent compounds (amphetamine for the stimulants and 3,4-methylenedioxyamphetamine for the hallucinogens). The order was set by calculating the Euclidian distance between the spectrum of the parent compound and the spectrum of each analogue. The nonamphetamines of the training set were selected randomly from the IR database: bemegride, β -butyrolactone, cadaverine and its HFB-derivate, codeine and its pentafluoropropionic (PFP)-derivate, caffeine, γ -butyrolactone, the trimethylsilyl (TMS)-derivate of γ -hydroxy butyric acid, the TMS-derivate of γ -hydroxy valeric acid, γ -valerolactone, nicotamide, piracetam, putrescine, dextromoramide, nicotine and prolintane.

2.3. Development of the CD-ANN systems

The main objective of this work was to obtain a structural ANN having as input variables the constitutional descriptors with a good performance in the validation process by the optimization the training set and the input variables, as compared with the spectral network. We have built four structural ANNs specialized in the automated classification of illicit amphetamines.

All structural networks were built with three layers, i.e. an input, a hidden and an output layer. The output layer has three nodes and distinguishes between stimulant amphetamines (class code M), hallucinogenic amphetamines (class code T) and nonamphetamines (class code N). On the other hand, they were built with a different number of input nodes (variables). These networks have been obtained using the same software, learning algorithm and transfer function as mentioned above for the spectral ANN.

Classification experiments were carried out on 146 molecules, out of which 14 are stimulant amphetamines, 7 are hallucinogenic amphetamines and 125 are nonamphetamines

(counterparts). The training set for the first two structural ANNs consists of 28 samples: 6 stimulant amphetamines, 5 hallucinogenic amphetamines and 17 nonamphetamines. The remaining 118 samples were included in validation set. The stimulants of the training set are: benzphetamine, amphetamine, methamphetamine, β -phenylethylamine, α -phenylethylamine and *N*-methyl- α -phenylethylamine. The hallucinogens are 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxy-*N*-ethylamphetamine, *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine and 1-(3,4-methylenedioxyphenyl)-2-butanamine. The nonamphetamines of the training set are the same as those used for the training set of the spectral ANN.

These 28 samples forming the training set were characterized by the 45 constitutional descriptors listed in Table 1. Among them, seven descriptors (*n*TB, *n*Cl, *n*Br, *n*I, *n*NO, *n*NO₂ and *n*R07) are zero for all the molecules from our training set and thus have no discrimination power. As a result, the number of input variables was narrowed down to the remaining 38 CDs. The set of constitutional descriptors for each sample in the database was first normalized in comparison with the molecular weight (MW), which has the largest values. Only these maximum values are obtained for MW in all cases, and thus, after normalization, the molecular weight becomes equal to 1 for all samples. As a result, this descriptor had to be eliminated as well, as it has no discrimination power, although the rest of the normalized variables were kept as such. These normalized and discriminating 37 descriptors were used as input variables for the first structural network (37MW-ANN system).

As MW has relatively large values in comparison with the other constitutional descriptors (more than five times larger), the descriptors were normalized (0–0.2) in order to reduce the variation in the data. The second structural database did not contain MW as input variable. In order to improve the variation of the normalized descriptors, we have built a second structural database. The MW descriptor was no longer included among the input variables. The remaining 37 CDs were normalized in comparison with the largest value computed for each of them. In this case, the normalized descriptors vary smoothly from 0 to 1, as these descriptors have absolute values in nearly the same range. The descriptors normalized in this way were used to build a second structural network (37CD-ANN).

The number of neurons for the hidden layer and the connection weights for both 37MW-ANN and 37CD-ANN were optimized to an architecture with 13 hidden neurons and 520 weight connections. The learning rate and the momentum were fixed after optimization at $\alpha=0.70$ and $\mu=0.80$, respectively.

In order to increase the efficiency of the system, the training set was optimized. As the descriptors computed for M7 (amphetamine) have nearly the same values as those computed for M102 (*N*-methyl- α -phenylethylamine), we have replaced M7 with M45 (fenproporex). This substance presents the advantage that it can be described by an additional constitutional descriptor, *n*TB (number of triple bonds). We have also added two new negatives (nonamphetamines), i.e. N64 (4-iodo-2,5-dimethoxyamphetamine) and N66 (ketamine). Both can be char-

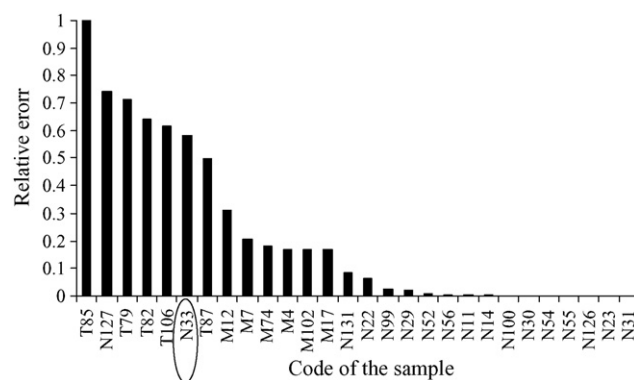


Fig. 2. The relative errors of the samples forming the training set of the 37CD-ANN system.

acterized by new descriptors: *n*I (number of I atoms) for N64 and *n*Cl (number of Cl atoms) for N66. The resulting new system, 40CD-ANN, has a larger training set (29 samples) and a larger number (40) of input variables.

The negatives included in the database have very different molecular structures and thus it is very difficult for the neural network to model this class of substances. Our experiments have shown that the nonamphetamines from the training set are well defined if the learning errors of the negatives are smaller than those of the positives. Slightly larger learning errors do not seem to affect the efficiency of the ANN in recognizing amphetamines as positives, and the type of their biological activity (stimulant or hallucinogen). The negative effect of the larger learning errors is probably compensated by the similarity of the molecular structures of the positives (they have a common molecular skeleton). In the case of the 37CD-ANN, the largest learning errors are encountered for N127 (prolintane) and N33 (dextromoramide) (see Fig. 2). Consequently, we have analyzed if N33 and/or N127 should be eliminated from the training set of 40CD-ANN, using a trial-and-error process.

The results obtained without N33 are illustrated in Fig. 3, and those obtained without N127 are presented in Fig. 4. In both cases, smaller learning errors are obtained for the negatives. However, the effect is stronger when N33 is eliminated. In this

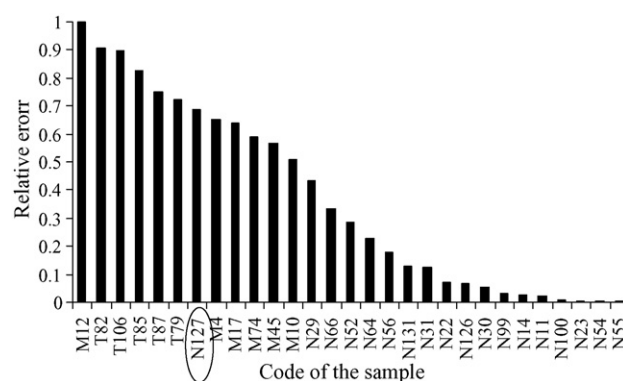


Fig. 3. The relative errors of the samples forming the training set of the 40CD-ANN system in the case when N33 (dextromoramide) is eliminated from the training set.

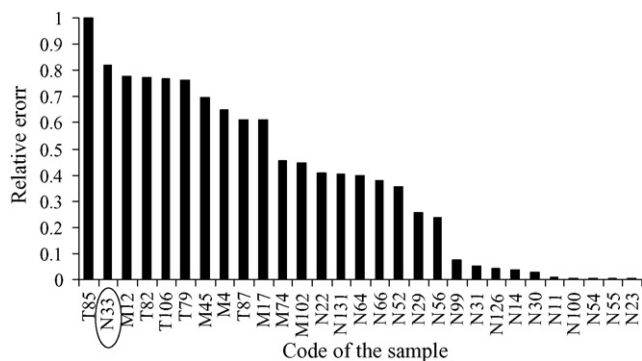


Fig. 4. The relative errors of the samples forming the training set of the 40CD-ANN system in the case when N127 (prolintane) is eliminated from the training set.

case, N127 is characterized by a relative error of 0.68, while when N127 is eliminated, N33 persists in having one of the largest relative learning errors (second largest relative learning error, i.e. 0.82). In addition, the training set used for the data displayed in Fig. 3 seems to be more homogenous (from the point of view of the learning errors) than the training set corresponding to the data displayed in Fig. 4, as the relative errors are decreasing more smoothly when N33 is not included in the training set.

Thus we have decided that N33 should be eliminated. The 40CD-ANN, built with the new training set, was optimized to an architecture consisting of 559 weight connections, with a learning rate $\alpha = 0.60$ and a momentum $\mu = 0.70$. Unfortunately, in these new conditions, the 40CD-ANN system shows larger difficulties in recognizing the true negatives. The elimination of one negative from the training set has lowered significantly the rate of true negatives, showing that the new training set has not the modeling and/or the discrimination power necessary to characterize the nonamphetamines appropriately. In consequence, we have added several negatives to the training set. They have been selected among those molecular structures that could be described by additional input variables (constitutional descriptors): N50 (flunitrazepam), characterized by two new descriptors ($n\text{NO}_2$ – number of NO_2 groups and R07 – number of seven membered rings), N155 (3,4-methylenedioxy-*N*-hydroxyamphetamine) described by $n\text{NO}$ (number of NO groups), and N152 (4-bromo-2,5-dimethoxyamphetamine) described by $n\text{Br}$ (number of Br atoms). We have thus obtained a 44CD-ANN.

Adding the three negative samples to the training set has still not brought a significant improvement. So the next step was to remove N127 from the training set, as it was still introducing large learning errors. The final training set of the 44CD-ANN consisted of 31 samples and 44 constitutional descriptors as input variables. The network has reached an optimum for 658 weight connections, a learning rate $\alpha = 0.70$ and a momentum $\mu = 0.80$.

3. Results and discussion

All networks have been programmed to stop the training process when the average error of the training drops below the

Table 2

The results of the validation process

	37MW-ANN	37CD-ANN	40CD-ANN	44CD-ANN	ANN
TP (%)	100	100	100	100	100
TN (%)	75.49	72.32	84.82	78.26	85.07
FP (%)	24.51	27.68	15.18	21.74	14.93
FN (%)	0.0	0.0	0.0	0.0	0.0
C (%)	84.25	91.09	91.09	93.15	98.74
CC (%)	79.67	76.69	87.22	81.62	87.26

target error, which was set up at 0.01. All the networks have been validated using all the samples from the database. The method was full cross-validation, as the number of samples in the database is relatively small. In order to evaluate the classification performed by each system, several figures of merit were calculated: the rate of true positives (TP), of true negatives (TN), of false positives (FP), of false negatives (FN), of classification (C), and of correctly classified samples (CC). Their values are presented in Table 2 and offer a complete image of the validation results.

The correct classification of positive samples is especially important in forensic practice, where the identification of the drugs of abuse must not fail. A first and very encouraging observation is that all networks have a TP rate of 100%, meaning all positives (amphetamines) are recognized as such.

By comparing the results obtained for the first two networks (37MW-ANN and 37CD-ANN), we can also see that increasing the variation of the input variables from 0–0.2 to 0–1 has resulted in a higher classification rate (from 84.25% in the case of 37MW-ANN to 91.09% for 37CD-ANN). At first sight, we could say that an increased variation has also a disadvantage, as the correct classification rate drops from 79.67% for 37MW-ANN to 76.69% for 37CD-ANN. In fact, this drop is only relative. The percentage of correctly classified samples is calculated taking into account the total number of samples that could be classified, which is smaller in the case of 37MW-ANN than in the case of 37CD-ANN. As a result, the correct conclusion is that 37CD-ANN performs better than 37MW-ANN. This was the reason why 37CD-ANN, and not 37MW-ANN, has been chosen for further optimization.

In order to analyze the validation results for 37CD-ANN, 40CD-ANN and 44CD-ANN, we have compared the classification rate (C rate) and the correct classification rate (CC rate). Fig. 5 shows that the best results are obtained for 40CD-ANN. The change made in the nature of the samples included in the training set and the addition of the new descriptors as input variables has significantly increased the CC rate (from 76.69% for 37CD-ANN to 87.22% for 40CD-ANN), while the C rate has remained the same (91.09%).

The latter figure of merit has been improved by the next optimization steps. Increasing the number of negative samples in the training set and increasing the number of input variables has led to a better C rate in the case of 44CD-ANN (93.15%) than in the case of the 40CD-ANN (91.09%). However, this gain has been obtained at the expense of the CC rate, which has dropped from 87.22% for 40CD-ANN to 81.62% for

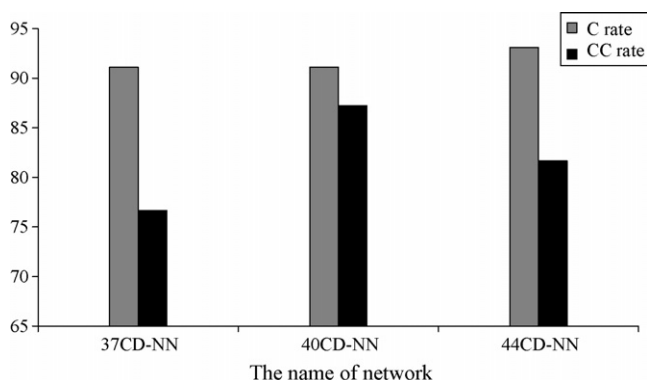


Fig. 5. The classification (C) rate and the correct classification (CC) rate for 37CD-ANN, 40CD-ANN and 44CD-ANN.

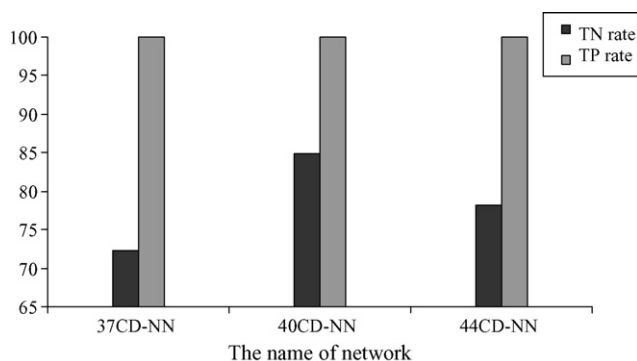


Fig. 6. The true negative (TN) rate and the true positive (TP) rate for 37CD-ANN, 40CD-ANN and 44CD-ANN.

44CD-ANN. The increase of the CC rate from 37CD-ANN to 40CD-ANN and then its decrease from 40CD-ANN to 44CD-ANN is reflected in a similar behavior of the TN rate (see Fig. 6).

4. Conclusion

The recognition of illicit amphetamines, according to their biological activities, from their GC–FT-IR spectra, with an artificial neural network makes it possible to identify the class identity and the toxicity of an unknown sample and thus eliminate further costs related to additional analysis performed in order to establish its individual identity. Establishing this qualitative structure–activity relationship for a compound is possible only if we have a sample of the given substance, so we can record its GC–FT-IR spectrum and then perform the chemometrical analysis.

Amphetamines do have analogues with legal use. When a new compound is to be synthesized, it is very useful to know beforehand if it will have a biological activity similar to the legal amphetamines or its toxicity is similar to the illicit amphetamines and thus the compound will not have extensive use. The costs related to the synthesis, clinical and toxicological tests can be avoided if the molecular structure is first analyzed with the structural system presented in this paper. If the new molecular structure is classified by the structural artificial neu-

ral network as positive, then we know that the compound most probably has the same biological activity as the illegal stimulant or hallucinogenic amphetamines, and thus we can eliminate that structure from the research and/or production objectives. If it is classified as “nonamphetamine”, then we can conclude that although it has a molecular structure similar to the illegal amphetamines, it is not likely to have the same toxicity as this class of forbidden compounds. The chances to be useful for legal pharmaceutical purposes are then significant and further research and testing effort is justified.

As we have shown, the structural artificial neural network built with constitutional descriptors has very encouraging results, although not yet as good as those obtained with the spectral network. A first disadvantage of the structural network is that, as opposed to the spectral neural network, it cannot distinguish among isomers such as amphetamine and dextroamphetamine, isomers that are both stimulants, but which have significantly different intensities of biological activity.

A second aspect that could not yet be improved is the level of the C rate for 37CD-ANN, 40CD-ANN and 44CD-ANN, which remains at relatively low values. A possible explanation may be that the input variables (i.e. the constitutional descriptors) do not bring enough information into the system. If we compare the validation results with those obtained for the spectral ANN (which had normalized GC–FT-IR absorption intensities as input variables), we see that the figures of merit are nearly the same as those obtained for the best structural network, i.e. 40CD-ANN, with the exception of the C rate. The spectral ANN has a C rate of 98.74%, which is much better than that of the 40CD-ANN (91.09%). Practically, the spectral ANN is able to classify a larger number of substances and identifies as such a larger number of negative samples than the best structural 40CD-ANN.

In conclusion, the GC–FT-IR absorption intensities show a stronger modeling and discrimination power than the constitutional descriptors. We intend to continue the research for finding a better performing structural artificial neural network by using a structural database consisting of several types of descriptors. Adding to the present structural database relevant geometrical descriptors, molecular walk counts, Galvez topological charge indices, charge descriptors, Randic molecular profiles, 3D-MorSE descriptors, GETAWAY descriptors, topological descriptors, BCUT descriptors, 2D-autocorrelations, aromaticity descriptors, geometrical descriptors, or WHIM descriptors may lead towards a structural network with at least the same efficiency as the spectral ANN.

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Validation of mercury determination by solid sampling Zeeman atomic absorption spectrometry and a specially designed furnace

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Abstract

Certified reference materials (CRMs) of different origin were used to validate the direct determination of total mercury by solid sampling Zeeman atomic absorption spectrometry (SS-ZAAS) and a specially designed furnace. The temperature program provides only for one step. Atomisation of mercury and pyrolysis of the matrix is performed at a constant temperature in the range of 900–1000 °C. Calibration points achieved by CRMs and aqueous solutions are covered by one calibration line, indicating the absence of matrix effects. Relatively high amounts of chlorine, known for causing problems in mercury determination do not influence analytical results. The excellent accuracy of the method results in a very good agreement with the certified values. The precision of SS-ZAAS measurements in a range from 0.5 to 50 ng Hg does not exceed 3% R.S.D. A limit of quantification of 0.008 $\mu\text{g g}^{-1}$ Hg was achieved.

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Keywords: Solid sampling Zeeman atomic absorption spectrometry; Method validation; Mercury; Specially designed furnace; Matrix interferences

1. Introduction

The Minamata disaster in 1956 showed the drastic effects of mercury on human health. Humans and animals exposed to high mercury concentrations have suffered disruption of the nervous system, brain damage, kidney malfunction, stomach disruption, DNA alteration and negative reproductive effects.

Mercury occurs in the environment from natural sources and as a result of atmospheric deposition and pollution caused by man's activity. It easily accumulates in the aquatic food chain, including fish and seafood, largely as methyl mercury, which is the form of toxicological concern. Codex guideline levels and European Commission Regulation amendment 221/2002 (OJEC 2002) maximum levels are set at 1 mg kg⁻¹ for large predatory fish and 0.5 mg kg⁻¹ for all other fish [1–4]. Fast, sensitive, accurate and precise analytical methods are required for the control of mercury in samples of environmental and biological origin.

The detection of mercury at trace levels is a complex analytical task because of its specific physical and chemical properties.

The most sensitive mercury resonance line lies in the vacuum UV region and is therefore not suitable for conventional spectrometers. The less sensitive wavelength at 253.7 nm is the only available alternative. Additionally chemical separation of mercury from the sample matrix is difficult due to its high volatility. Already at normal drying temperatures mercury losses have been observed [5].

A plenty of studies were published in recent decades dealing with a variety of acid mixtures for the digestion process while others were concentrating on different noble metals for trapping mercury vapours prior to determination by cold vapour atomic absorption spectrometry (CV-AAS) [6–9]. All of them were more or less prone to analyte losses and/or contamination. The inevitable dilution of the sample is another drawback.

Direct determination of mercury by graphite furnace AAS was described by few authors and didn't gain much attention because of the inferior sensitivity compared to CV-AAS [10,11].

To present all methodical attempts would go beyond the scope of this study, but the most rigorous approach to improve sensitivity and avoid contamination and losses, the direct mercury analysis in solid samples cannot be ignored. Numerous approaches using different types of atomisers including also the

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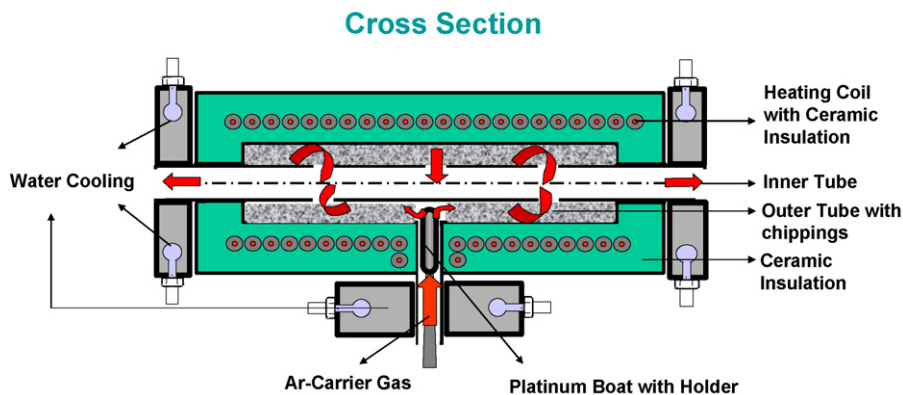


Fig. 1. Schematic cross-section of the specially designed furnace.

graphite furnace with the addition of various chemical modifiers have been reported [1,12–15].

In recent publications a differential atomic absorption spectrometer and a two-section catalytic pyrolyser is described. The solid sample in a container is directly placed in the first section of the pyrolyser and vapourised in an air stream at 800 °C. All mercury compounds are decomposed and the organic compounds are catalytically oxidised in the second section. Mercury vapour and remaining smoke are transferred into a multi-path analytical cell (optical length 9.6 m). The efficient pyrolysis allows for sample weights of more than 100 mg achieving a detection limit of 0.5 ng l⁻¹, while Zeeman background correction guarantees interference-free measurements [16–18].

SS-ZAAS in combination with specially designed atomisers seems to be a very promising solution for the direct detection of mercury in environmental and biological samples [19–23].

In this study we used a modified Hadeishi furnace [24] in combination to a direct ZAAS. Total mercury was determined in reference materials of various origins to validate this method. Precision and accuracy were calculated from the results obtained. The linear range and the limit of quantification were determined as well.

2. Experimental

2.1. Instrumentation

A Grün SM20 ZAAS (Grün Analytische Meß-Systeme GmbH, Germany) equipped with a modified two-chamber Hadeishi furnace (Fig. 1) was used for the determination of mercury. This furnace prototype is not commercially available and was developed at the Institute for Reference Materials and Measurements.

Samples were weighed on a micro-balance (Sartorius 4503 MP6, Germany) into platinum transport boats (250 µl volume). Platinum boats are commercially available and have all the necessary physical and chemical properties. One boat at a time is transferred to an automatic introduction system which inserts the sample boat in the furnace and takes it back when the pyrolysis is complete.

If direct ZAAS (magnetic field at the light source) is applied, the dimensions of the furnace can vary in a wide range. The absorption cell (22.5 cm length) used in this study is about 10 times longer than recent graphite furnace atomisers and results in a corresponding increase of the sensitivity according to Lambert-Beer-law.

The tube of the furnace made of nickel–chromium alloy is heated at a constant temperature in the range of 900–1000 °C to achieve highest sensitivity and a rapid release of Hg from the matrix [24]. These tubes showed a superior durability (>2000 h) compared to pure nickel tubes. The special design of the furnace makes mercury sorption on cold ducts impossible and improves analysis reproducibility and correctness. Water cooling at the sample introduction zone prevents losses of mercury and at the ends of the inner tube it protects optical and electronic devices of the spectrometer against excessive heat.

In the constantly heated furnace the atomisation runs under isothermal conditions. The nickel–chromium surface of the tube including the chippings which are pressed tightly into the outer tube is large and together with the applied temperature it completes the catalytic reduction of mercury and matrix compounds.

A constant magnetic field is applied to the light source—a mercury gas discharge lamp. The orientation of this magnetic field of 1.0 T is transverse to the optical axis. The experimental conditions for the SS-ZAAS determination of mercury are listed in Table 1.

2.2. Reference samples and solutions

The solid CRMs sewage sludge BCR CRM-146 ($9.49 \pm 0.76 \mu\text{g g}^{-1}$ Hg); Tuna fish BCR CRM-464 ($5.24 \pm 0.10 \mu\text{g g}^{-1}$

Table 1
Experimental conditions for the SSZAAS determination of Hg

Wavelength	253.7 nm
Spectral bandpass	0.25 nm
Lamp type	ENL
Lamp current	4.0 mA
Measurement mode	Peak height
Measurement time	4.0 s
Magnetic field conditions	Permanent, 1.0 T
Argon flow rate	30 l h ⁻¹

Hg) and City Waste BCR CRM-176 ($31.4 \pm 1.1 \mu\text{g g}^{-1}$ Hg) were used for calibration. The different points on the calibration curve were achieved by variation of the CRMs weight.

Aqueous calibration solutions were prepared from a $1000 \mu\text{g ml}^{-1}$ Hg stock standard solution (Merck, Germany). Polypropylene ware was used throughout.

3. Results and discussion

3.1. Matrix effects

Three experimental signal shapes of a blank, a liquid standard solution (0.2 ng Hg) and a certified reference material (human hair BCR 397 $12.3 \pm 0.5 \mu\text{g g}^{-1}$ Hg) are shown in Fig. 2. The signal shape of the human hair sample is representative for all organic materials measured and the background is not affecting the signal if the sample weight does not exceed 10 mg of BCR 397. All signals were of the ideal single peak form, no double peaks were observed, which is a prerequisite for using peak height evaluation. Peak height measurements of the Grün SM 20 showed sufficient precision to be used for data evaluation.

Despite different matrices of three solid CRMs Sewage Sludge BCR-146, Tuna fish BCR-464, City Waste BCR-176 and 10 μl aliquots of calibration solutions which correspond to 0.5; 1.0; 2.5; 5.0; 10.0; 25.0 and 50.0 ng Hg, all points are covered by the same curve (Fig. 3), indicating the absence of matrix effects.

Some of the certified reference materials used in this study are of marine origin and contain various amounts of chlorine and iodine. Findings presented in Table 2 show that relatively high amounts of chlorine do not affect the analytical results.

The linear range surmounts 50 ng Hg and a quantification limit of $0.008 \mu\text{g g}^{-1}$ Hg was achieved by measuring Salmon BCR-725 (up to 35 mg sample weight).

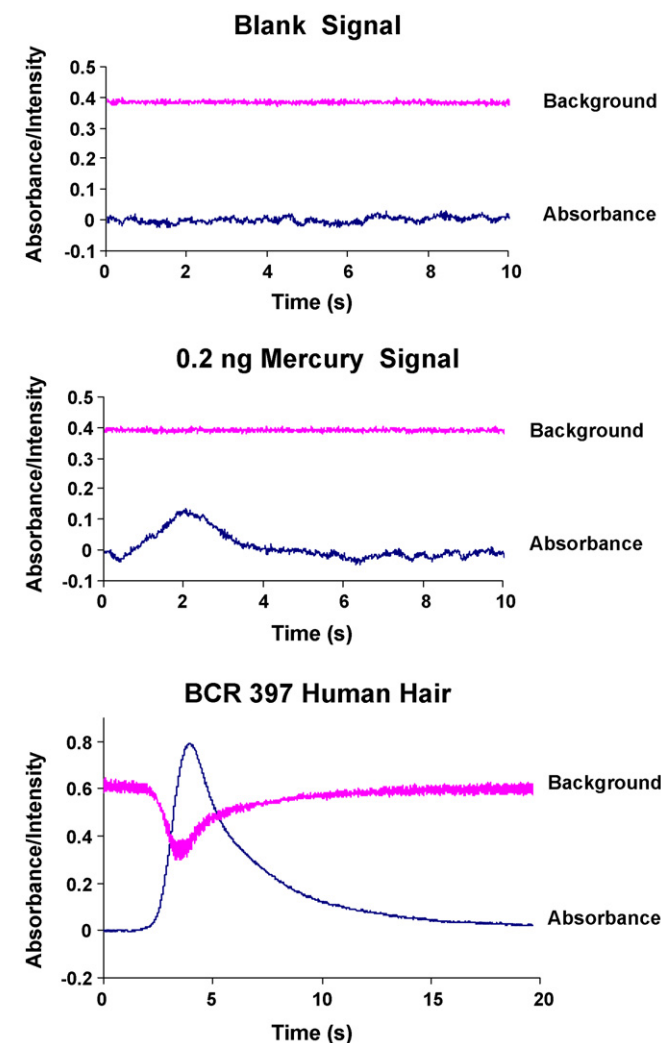


Fig. 2. Mercury signals obtained with the specially designed furnace.

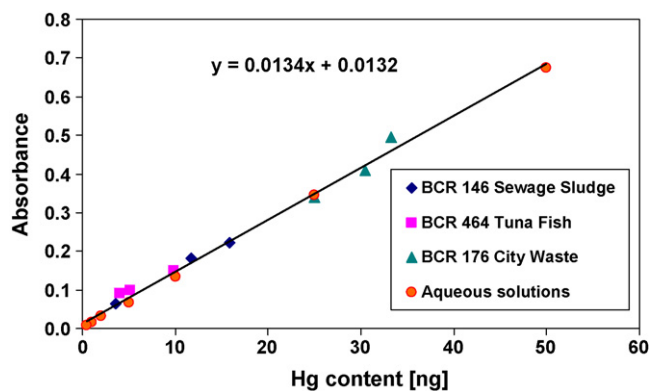


Fig. 3. Calibration curve for Hg using solid CRMs and liquid standards—sewage sludge BCR-146 ($9.49 \pm 0.76 \mu\text{g g}^{-1}$ Hg); Tuna fish BCR-464 ($5.24 \pm 0.10 \mu\text{g g}^{-1}$ Hg); city waste BCR-176 ($31.4 \pm 1.1 \mu\text{g g}^{-1}$ Hg); aqueous standard solutions.

Table 2
Mercury determination of chlorine containing samples

Sample	Concentration ($\mu\text{g g}^{-1}$ Hg)		
	Cert. value	Exp. value	CI (%)
Aquatic plant BCR-60	0.343 ± 0.04	0.32 ± 0.06	(1.0)
Aquatic plant BCR-61	0.230 ± 0.02	0.25 ± 0.03	(0.23)
Olive leaves BCR-62	0.284 ± 0.02	0.29 ± 0.04	(0.07)
Mussel tissue BCR-278	0.188 ± 0.007	0.19 ± 0.04	(3.2)
Dogfish muscle NRCC DORM-1	0.798 ± 0.074	0.779 ± 0.044	1.13
Lobster Hepat. NRCC TORT-1	0.33 ± 0.05	0.34 ± 0.04	5.58
Lobster Hepat. NRCC TORT-2	0.27 ± 0.06	0.28 ± 0.04	Not cert.

Indicative values: in parenthesis.

Table 3
Calculation of precision

Hg (ng)	Absorbance	n	Precision (%)	Gain
50	0.0421 ± 0.0008	50	1.84	1
25	0.0215 ± 0.0004	20	1.86	1
10	0.1342 ± 0.0015	20	1.15	5
5	0.0673 ± 0.0010	20	1.49	5
2	0.0325 ± 0.0009	20	2.77	5
1	0.0173 ± 0.0004	20	2.37	5
0.5	0.0168 ± 0.0004	20	2.38	6

Table 4
Calculation of accuracy

Sample	Concentration (µg g ⁻¹ Hg)		Accuracy (%)
	Cert. value	Exp. value	
Tuna fish BCR-464	5.24	5.34	1.91
Light sandy soil BCR-142	0.1040	0.1043	2.88
Light sandy soil BCR-142R	0.067	0.063	5.97
Aquatic plant BCR-60	0.343	0.344	0.29
Aquatic plant BCR-61	0.230	0.240	4.35
Olive leaves BCR-62	0.284	0.289	1.76
Milk powder BCR-151	0.101	0.103	1.98
Pig kidney BCR-186	1.970	1.962	0.41
Mussel tissue BCR-278	0.188	0.190	1.06
Spinach NIST-1570	0.031	0.032	3.23
Pepperbush NIES-1	0.056	0.053	5.36
Human hair NIES-5	4.41	4.43	0.45
Fly ash BCR-38	2.10	2.12	0.95
Bovine liver NIST-1577a	0.016	0.017	6.25
Orchard leaves NIST-1571	0.155	0.158	1.93
Plankton BCR-414	0.276	0.265	3.99
Bovine liver BCR-185	0.044	0.042	4.54
Human hair BCR-397	12.3	12.2	0.81
Dogfish muscle NRCC DORM-1	0.798	0.779	2.38

3.2. Precision and accuracy

The precision of SS-ZAAS mercury measurements was calculated over a range from 0.5 to 50 ng Hg by analysing aqueous solutions. The relative standard deviation in all cases does not exceed 3% R.S.D. The results are presented in Table 3.

The accuracy of SS-ZAAS mercury measurements was calculated by analysing CRMs of different origin according to the formula:

$$\text{accuracy (\%)} = \frac{|\text{exp. value} - \text{cert. value}|}{\text{cert. value}} \times 100$$

where exp. value is mean experimental value of minimum five replicates and cert. value is value from certificate.

An excellent agreement between the experimental and certified values was obtained, which is a proof for the good accuracy of the method. The results are presented in Table 4.

4. Conclusions

The validation of total mercury determination by direct SS-ZAAS and a specially designed furnace without any chemical sample preparation was successfully performed by analysing environmental and biological CRMs.

Experimental Hg signal shapes derived from liquid and solid samples were of the ideal single peak form, which allows peak height evaluation.

A calibration curve was achieved by using aqueous solutions and solid CRMs, all points were covered by one line, indicating that SS-ZAAS mercury measurements were matrix independent. Even relatively high amounts of chlorine were not affecting analytical results.

The high accuracy of the method is evident when comparing certified and experimental values. The precision of the method does not exceed 3% R.S.D. over a range from 0.5 to 50 ng Hg.

The limit of quantification was calculated to be 0.008 µg g⁻¹ Hg and allowed to measure very low Hg contents without extraction or accumulation procedures.

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Separation and determination of L-tyrosine and its metabolites by capillary zone electrophoresis with a wall-jet amperometric detection

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Abstract

A method of capillary electrophoresis with wall-jet amperometric detection (AD) has been developed for separation and determination of L-tyrosine (Tyr) and its metabolites, such as Tyramine (TA), *p*-hydroxyphenylpyruvic (pHPP), homogentisic acid (HGA) and some dipeptides containing Tyr, such as Tyr-Gly-Gly (YGG), Tyr-Arg (YR) and Tyr-D-Arg (Y-D-R). A carbon disk electrode was used as the working electrode and the optimal detection potential was 1.00 V (versus Ag/AgCl). At 18 kV of applied voltage, the seven compounds were completely separated within 20 min in 110×10^{-3} mol/L Na_2HPO_4 – NaH_2PO_4 buffer (pH 7.10) containing 3×10^{-3} mol/L β -cyclodextrin (β -CD). Good linear relationship was obtained for all analytes and the detection limits of seven analytes were in the range of 0.95–4.25 ng/mL. The proposed method has been applied to examine the metabolic process of L-tyrosine in rabbit's urine.

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Keywords: Capillary electrophoresis; β -Cyclodextrin; Amperometric detection; Pharmacokinetics; L-Tyrosine

1. Introduction

L-tyrosine is one important amino acid to constitute the molecules of proteins. The most L-tyrosine in the human body is metabolized from phenylalanine, and some is from food directly [1]. In humans' liver Tyr is converted to pHPP firstly by transaminase of tyrosine, and the pyridoxal phosphate is needed as coenzyme in this course. pHPP is then transferred to HGA in the presence of hydroxidase of *p*-hydroxyphenylpyruvic acid, the reaction needs antiscorbutic acid as coenzyme and consumes oxygen. Under the catalysis of oxidase of homogentisic acid, HGA is then oxidized to *cis*-butylene diacyl acetyl acetic acid, which is further converted into fumaryl acetyl acetic acid via the isomerase. At last *cis*-butylene diacyl acetyl acetic acid is hydrolyzed to form the fumaric acid and acetyl acetic acid in the presence of corresponding hydrolyzing enzyme. In addition Tyr can be converted to TA in the presence of tyrosine decarboxylase.

The study of Tyr and its metabolism has important biological and clinical significance. The level of Tyr and its metabolites

keep relatively stabilization in a normal human body. However for a few individuals suffering from a congenital metabolic defect known as tyrosinemia, which is an autosomal recessive disorder caused by a deficiency of fumarylacetoacetate hydrolyase in the tyrosine degradation pathway [2]. This inborn error of metabolism can cause dramatic elevation of Tyr and pHPP levels in blood and urine [3,4] and cause severe liver disease and renal tubular dysfunction. Because of their biological and clinical significance, chemical reactions of Tyr and its related derivatives have been studied quite extensively, and the electrochemical studies were the most popular [5–11], especially for Tyr [5–7]. HPLC with electrochemical detection has been also used for separation and determination for these compounds [12–16]. In addition, flow injection with chemiluminescent (CL) [17] and electrochemiluminescent (ECL) [18] detection have been used for determination of these compounds.

As a newly arisen analytical technique, capillary electrophoresis (CE) has some distinctive advantages, such as higher column efficiency and low sample volume requirement [19], which makes it more and more important in the separation of multitudinous samples. Compared with ultra-violet (UV) detector, amperometric detector (AD) is more sensitive and selective, and it is much cheaper than laser-induced fluorescence (LIF)

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detector, which make AD very popular for analysis of electrochemically active compounds [19–22]. Recently, a microchip capillary electrophoresis with amperometric detection was also employed to detect aromatic amino acids [23]. Owing to the electroactivity of Tyr and its metabolites and derivatives, such as TA, pHPP, HGA, YR, YGG and Y-D-R, capillary electrophoresis with amperometric detection has been used to separate and detect Tyr and its related derivatives simultaneously as well as to study the pharmacokinetics of Tyr in rabbit urine, which was benefit to the application of CE in the study of metabolism of medicines.

2. Experimental

2.1. Apparatus

The laboratory-built CE–AD system has been described previously [22,23]. A ± 30 kV high-voltage dc power supply (Shanghai Institute of Atomic Science Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet of the capillary was held at a positive potential and outlet was connected to ground. The separation capillary was an untreated fused silica capillary with $65\text{ cm} \times 25\text{ }\mu\text{m}$

i.d. $\times 370\text{ }\mu\text{m}$ o.d. (Hebei Yongnian Optic Fiber Factory, China). A laboratory-built pre-aligned electrochemical cell, consisting of a three-electrode system (a $500\text{ }\mu\text{m}$ diameter carbon disc working electrode, a platinum auxiliary electrode and a Ag/AgCl reference electrode), was used in combination with a BAS LC-4C amperometric detector (Bioanalytical System, West Lafayette, IN, USA). The use of the pre-aligned electrochemical cell avoids the complicated operation of aiming working electrode, saves experiment time and improves the reproducibility of experiments. The electropherograms were monitored using a Chromatogram workstation (Model HW-2000, Qianpu Software Company, Shanghai, China).

A PHS-3C meter (Shanghai Dapu Instrument Company, Shanghai, China) was used to measure the pH of the running buffer. A CHI660 electrochemical system (CH instrument, TX, USA) was chosen to perform cyclic voltammograms.

2.2. Chemicals

Tyr, TA, pHPP, HGA, YR, YGG, Y-D-R and β -CD were supplied by Sigma (St. Louis, MO, USA). Other chemicals were of analytical reagent grade and obtained from Shanghai Company of Chemical Reagent (Shanghai, China). All the water used

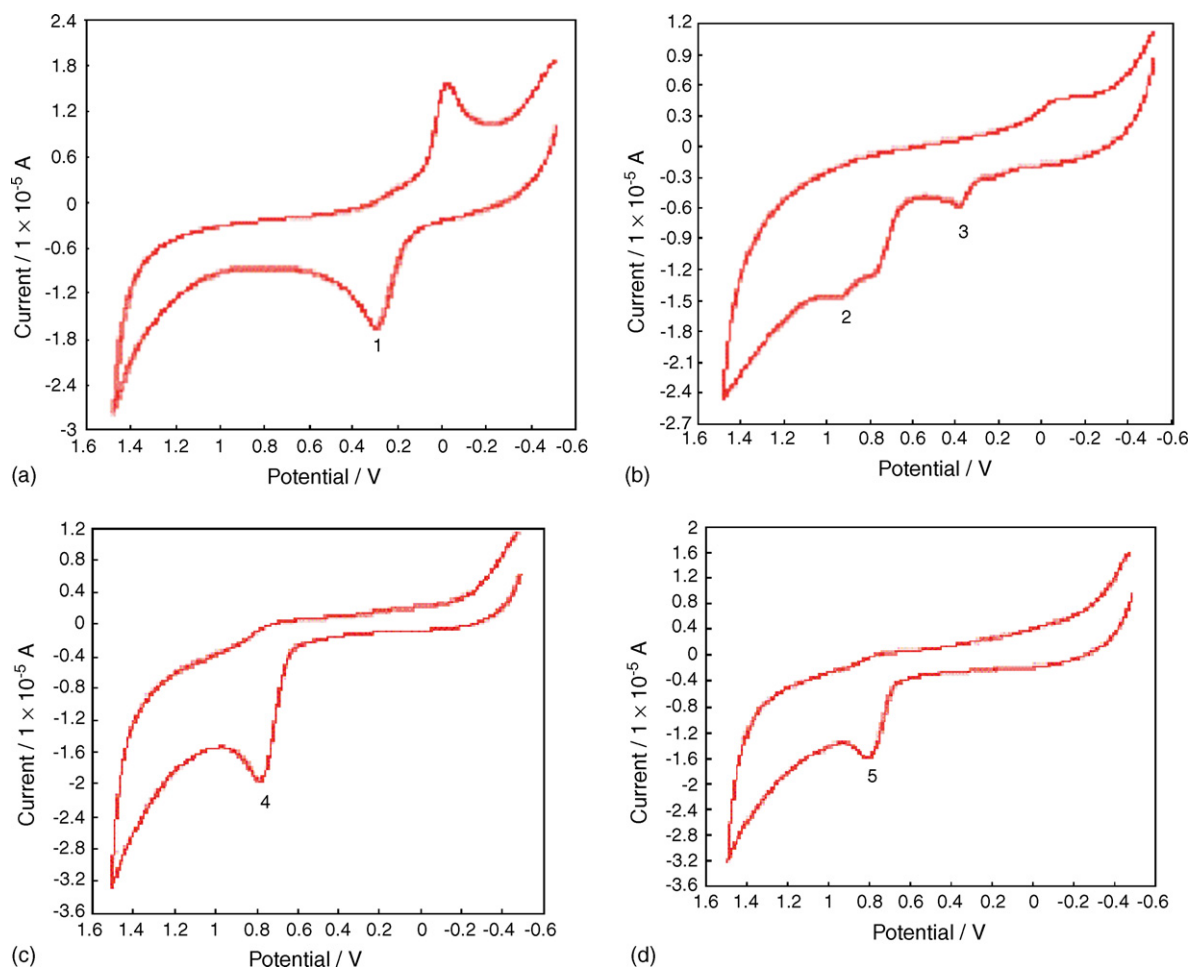


Fig. 1. Cyclic voltammeters of Tyr and its related derivatives. Experiment conditions: the concentration of the seven compound namely HGA, pHPP, TA, YGG, Tyr, Y-D-R and YR are 2.0×10^{-4} mol/L. Buffer is Na_2HPO_4 (110 mmol/L)– NaH_2PO_4 (110 mmol/L) with pH 7.10. The cyclic voltammeter's scan speed is 100 mV/s. The 'fig.' mark the place where is oxidation peaks: (a) HGA, (b) pHPP, (c) TA, (d) YGG, (e) Tyr, (f) Y-D-R, and (g) YR.

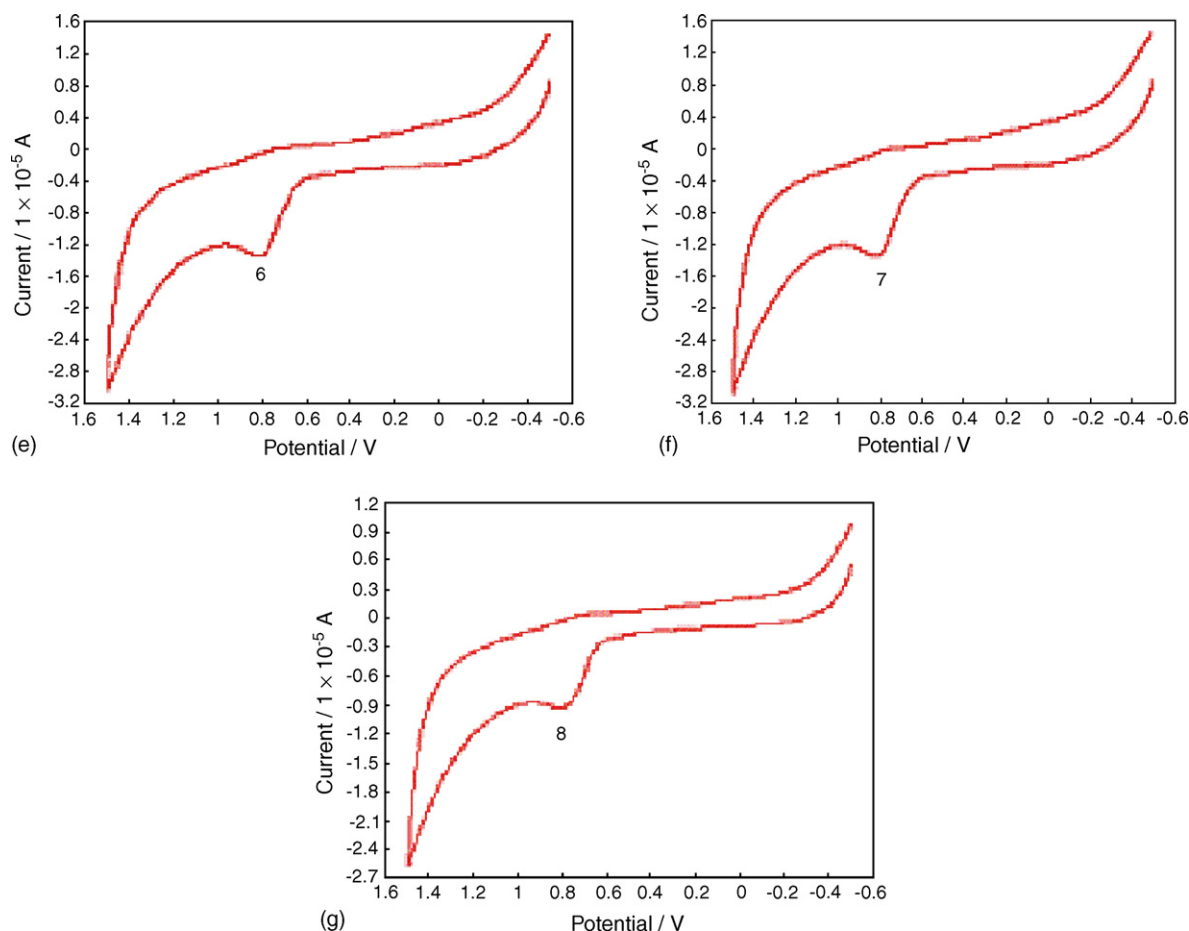


Fig. 1. (Continued).

was Milli-Q purified water (Millipore, Bedford, USA). Tyr, TA, HGA, YR, YGG and Y-D-R were dissolved, respectively, with water to obtain stock solutions of 5.0×10^{-3} mol/L, and pHPP was dissolved with methanol in order to remain its keto–enol tautomerization. β -CD was dissolved with water to obtain stock solutions of 10×10^{-3} mol/L. All of the stock solutions were stored at 4°C before use, and diluted to the desired concentration with the running buffer.

2.3. Sample preparation

Urine samples were obtained from domestic rabbits offered by Experimental Animal Center of Fujian Medical University. Before feeding L-tyrosine, all rabbits were forbidden to eat anything for 12 h. After that, they were feed with L-tyrosine dose in accordance with their weight (4 mg/kg). The rabbits were then put in the metabolism cages, the urine samples were collected at intervals of time. Urine samples were centrifuged for 10 min at 3000 rpm, 0.2 mL of clear liquid was diluted to 2.0 mL with buffer (pH 7.10), filtered by passing a $0.22 \mu\text{m}$ polypropylene filter film and ultrasonicated before use.

2.4. Procedures

Prior to use, the surface of the carbon disk electrode was smoothly polished with emery paper and 0.05 mm alumina pow-

der, then ultrasonicated in de-ionized water, and finally carefully positioned opposite the capillary outlet. Each new capillary was prerinsed with 0.1 mol/L NaOH and water both for 3 h in a washing system. Before each run, the capillary was sequentially rinsed with 0.1 mol/L NaOH for 5 min, water for 5 min and running buffer for 10 min in the CE–AD system in order to obtain repeatable results. Sample was injected electrokinetically at 18 kV for 10 s. Electrophoresis was performed at a separation voltage of 18 kV. The running buffer consists of 110×10^{-3} mol/L Na_2HPO_4 – NaH_2PO_4 and 3×10^{-3} mol/L β -CD (pH 7.1). Before use, all solutions were filtered through a $0.22 \mu\text{m}$ polypropylene filter film, and were then thoroughly degassed under ultra sonication. In addition, all experiments were performed in room temperature (25°C).

3. Results and discussion

3.1. The electrochemical behavior of Tyr and its derivatives

3.1.1. Cyclic voltammetry

The electrochemical behavior of Tyr and its derivatives at the glassy carbon electrode in 110×10^{-3} mol/L Na_2HPO_4 – NaH_2PO_4 buffer (pH 7.10) was investigated by cyclic voltammetry (CV). The results were showed in Fig. 1. From Fig. 1, we can see that all of the seven compounds are electroactive.

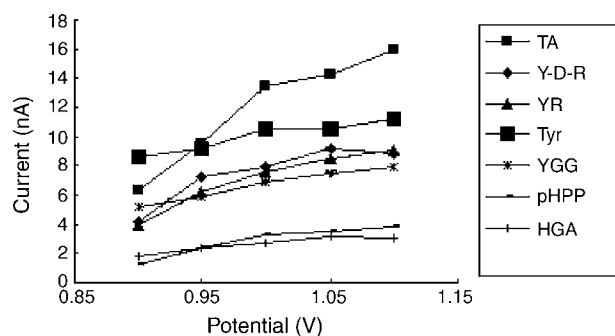


Fig. 2. Hydrodynamic voltammetry of Tyr and its related derivatives. Running solution: 110 mmol/L Na_2HPO_4 –110 mmol/L NaH_2PO_4 buffer (pH 7.10) including 3 mmol/L β -CD; separation voltage: 18 kV; injection time: 10 s; concentration: seven ingredients namely TA, Y-D-R, YR, Tyr, YGG, pHPP and HGA are all of 1.0×10^{-5} mol/L.

The potential of oxidation peak of HGA is at about +0.25 V, and the CV of HGA shows that the electrode reaction of HGA is reversible (see Fig. 1a). It can be known from the structure that HGA is a quinol, it is easier to be oxidized than Tyr and its other derivatives, so the potentials of oxidation peak of HGA is much lower, while the other compounds can be oxidized at potentials about +0.8 V. Tyr, TA, YGG, YR and Y-D-R have the similar electrochemical character, their oxidations are irreversible. pHPP exists the tautomerization of ketonic and enolic in phosphate medium [10], so we can observed two oxidation peak, the ethylene of enolic isomer is oxidated at the low potential, while the ketonic isomer is oxidated at a higher potential.

3.1.2. Effect of the potential applied to the working electrode

The potential applied to the working electrode would affected strongly the electrochemical response of the analytes. In order to find an optimum detection potential, the hydrodynamic voltammetric experiment in 110×10^{-3} mol/L Na_2HPO_4 – NaH_2PO_4 buffer (pH 7.10) was carried out. As showed in Fig. 2, when the applied potential was lower than +0.9 V (versus Ag/AgCl, 3 mol/L KCl), the peak currents of them were relatively small. In the potential range of +0.9–1.1 V, the oxidation currents of them were increased greatly with the applied potential. However, when the applied potential exceeded +1.0 V, the baseline current was obviously increased. In considering both peak current and baseline current, +1.00 V was selected as the optimum working potential, where the baseline was not too high and the S/N ratio was relatively high, and the working electrode could maintain good stability and reproducibility as well.

3.2. Optimization of the separation conditions

3.2.1. Separation by capillary zone electrophoresis (CZE) in the absence of (β -CD)

Three buffers, namely phosphate, borate-sodium hydroxide and Britton-Robinson have been selected as the running buffer for testing their effects on the separation of compounds mentioned above. The experimental results showed that under the same electrophoretic conditions (pH 7.1, concentration of buffer

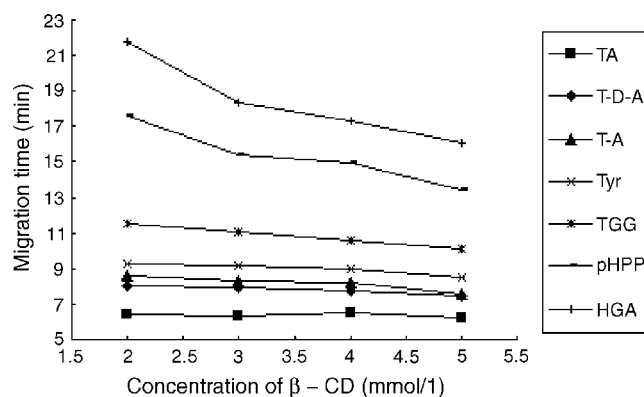


Fig. 3. Effect of the concentration of β -CD on the migration time of the analytes. Running solution: 110 mmol/L Na_2HPO_4 –110 mmol/L NaH_2PO_4 buffer (pH 7.10) including different concentration of β -CD; separation voltage: 18 kV; injection time: 10 s; detection potential: +1.000 V; concentration: seven ingredients namely TA, Y-D-R, YR, Tyr, YGG, pHPP and HGA are all of 1.0×10^{-5} mol/L.

was 110×10^{-3} mol/L and applied voltage was 18 kV), phosphate gave not only the best separation but also the highest sensitivity. So, the phosphate buffer was chosen as the running solution in the subsequent experiments. However, adjusting pH and concentration of the buffer solution, the baseline separation of the seven compounds was still hard to be achieved, especially for pHPP and HGA.

3.2.2. Choice of buffer component

The major problem in the separation of above seven analytes was the separation of pHPP and HGA, and the peak shape of pHPP (behaved as front-peak) was also a problem. In order to improve the separation efficiency, some organic solvents or chiral selectors are usually used as the additive to optimize the analysis process. Several additives such as methanol, acetonitrile, sodium dodecyl sulphate (SDS), sodium cholate, and β -cyclodextrin (β -CD) were examined for their effects on the separation of the analytes in this experiment. It was found that they made no effect on the separation of pHPP and HGA and the peak shape of pHPP except β -CD. Addition of β -CD not only changed the migrating order of pHPP and HGA, shorten the migration time and obtained good separation, but also improved the shape of pHPP. So we think β -CD is a proper and good additive for the separation of our interested analytes. This phenomenon might be due to the interaction between β -CD and the analytes to form the host–guest inclusion complexes, thus changed the migrating behavior of the analytes. Many examples of this kind of interaction between β -CD and the analytes have been reported [24–26]. The effect of the concentrations of β -CD on the migration time of the analytes was tested in the range of 2 – 5×10^{-3} mol/L, and the results are shown in Fig. 3. It can be found that only at the concentration of 3×10^{-3} mol/L, the peaks with symmetry shape could be obtained. When β -CD concentration was below 3 mmol/L, it was hard to separate pHPP and HGA, and when β -CD concentration was higher than 3×10^{-3} mol/L, the peak was broaden and incomplete separation of most compounds were observed. As the results shown in Fig. 4, with the increase of β -CD concentration after

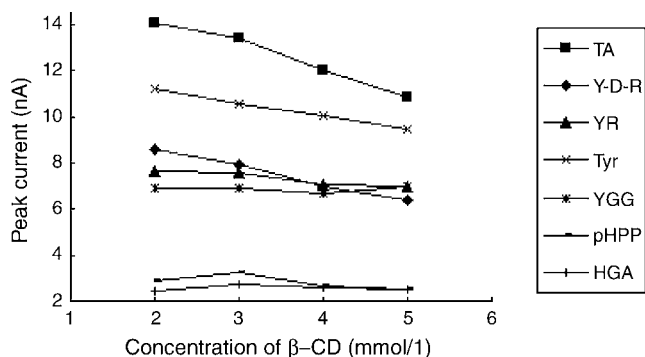


Fig. 4. Effect of the concentration of β -CD on the peak current of the analytes. All conditions as in Fig. 3.

3×10^{-3} mol/L, the peak currents of the most compounds were decreased. Therefore, 3×10^{-3} mol/L of β -CD was chosen as the optimal additive.

3.2.3. Effects of the pH

As we know, the pH of the running buffer strongly affects the resolution and selectivity, so it is important for us to select the most suitable pH of the running buffer for the best separation and determination of the above mentioned compounds. The dependence of the migration times of the seven analytes on pH was studied in detail with phosphate buffer in the pH range of 6.0–8.0. The experiment showed that the suitable pH value should be strictly limited in a narrow range. When pH is below 7.0, both Y-D-R and YR could not get baseline separation, while when pH is higher than 7.4, Y-D-R, YR and Tyr could not get baseline separation. Therefore, pH 7.10 was chosen as the optimum pH in the subsequent experiments.

3.2.4. Effects of the concentration of running buffer

The effect of concentration of the running solution on the separation of the analytes was examined at same pH (7.1) and applied voltage (18 kV), and the results are showed in Fig. 5. The result showed that the migration time of analytes increased

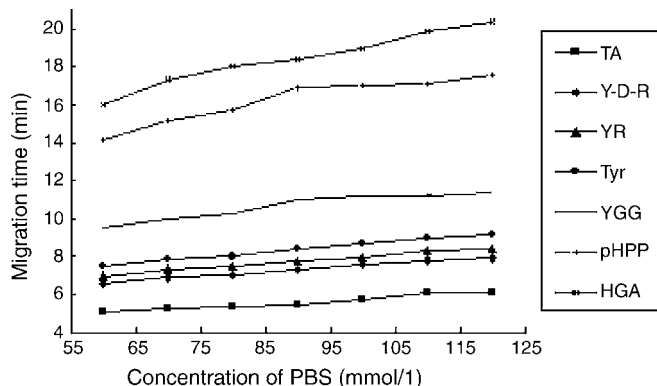


Fig. 5. Effect of the concentration of running buffer on the migration time of the analytes. Running solution: different concentrations of buffer (pH 7.10) including 3 mmol/L β -CD; separation voltage: 18 kV; injection time: 10 s; detection potential: +1.000 V; concentration: seven ingredients namely TA, Y-D-R, YR, Tyr, YGG, pHPP and HGA are all of 1.0×10^{-5} mol/L.

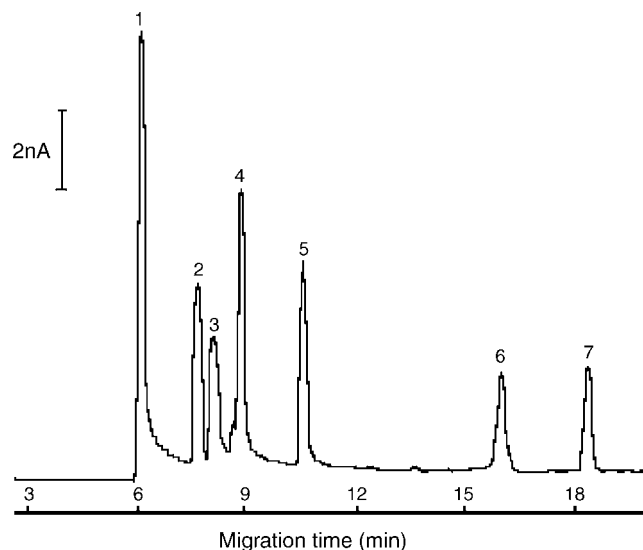


Fig. 6. Electrophoretogram of 1.0×10^{-5} mol/L standard solution of Tyr and its metabolites. Running solution: 110 mmol/L Na_2HPO_4 –110 mmol/L NaH_2PO_4 buffer (pH 7.10) including 3 mmol/L β -CD; separation voltage: 18 kV; injection time: 10 s; detection potential: +1.000 V; concentration: seven ingredients namely TA, Y-D-R, YR, Tyr, YGG, pHPP and HGA are all of 1.0×10^{-5} mol/L. (1) TA; (2) Y-D-R; (3) YR; (4) Tyr; (5) YGG; (6) pHPP; (7) HGA.

with the concentration of the running buffer in the range of 60 – 120×10^{-3} mol/L; Y-D-R, YR and Tyr could not be separated completely when PBS concentration was lower than 100×10^{-3} mol/L. In addition, with the increase of buffer concentration, the electric current in the capillary was also increased, which would cause Joule heat, so the higher electric current in the capillary would lead to peak broadening. In order to obtain a better resolution and shorter analysis time, 110×10^{-3} mol/L of PBS was selected.

Finally, 110×10^{-3} mol/L of Na_2HPO_4 – NaH_2PO_4 buffer (pH 7.10) containing 3×10^{-3} mol/L β -CD was select as running solution.

3.2.5. Effect of separation voltage and injection time

The effect of the separation voltage on the migration was investigated in the range of 10–20 kV, the result showed that the higher voltage was favorable to shorten the migration time and prompt the response of the wall-jet electrode. However, too high voltage would lead to peak broadening because of Joule heat. In order to save analysis time and obtain higher separation efficiency, 18 kV was selected as the separation voltage.

The effect of injection time in the range of 2–15 s at 18 kV was examined in order to obtain the best sensitivity. The results showed that peak height would be increased with the injection time. However, when injection time was longer than 10 s, the peak current was not increased much and the peak exhibited a significant broadening. Therefore, 10 s was selected as the sampling time.

Under the selected optimum conditions, the electropherogram for the mixture of Tyr and its related derivatives is shown in Fig. 6. It is obvious that the seven analytes are separated completely within 20 min.

Table 1

The regression equations, correlation coefficient and the detection limits

Compounds	Regression equation ^a	Correlation coefficient	Linear range (μg/mL)	Detection limits (ng/mL)
TA	$Y = 9.426X + 0.8401$	0.9949	6.85×10^{-3} –1.37	0.95
Y-D-R	$Y = 2.291X + 0.447$	0.9940	2.36×10^{-2} –3.37	3.84
YR	$Y = 0.750X + 0.3606$	0.9933	2.36×10^{-2} –3.37	3.57
Tyr	$Y = 2.227X + 0.4726$	0.9986	9.05×10^{-3} –1.81	1.54
YGG	$Y = 2.237X + 0.3482$	0.9962	1.77×10^{-2} –2.95	2.66
pHPP	$Y = 1.608X + 0.6145$	0.9985	1.80×10^{-2} –1.80	4.25
HGA	$Y = 1.476X + 0.2756$	0.9992	1.68×10^{-2} –1.68	3.33

^a “Y” is the peak current (nA) and “X” is the concentration of analytes (μg/mL).

Table 2

Recoveries of L-tyrosine ($n = 5$)

Analyte	Added amount (μg/ml)	Founded amount (μg/ml)	Recovery (%)	R.S.D. (%)
L-tyrosine	10.00	9.82	98.2	3.17
	50.00	52.20	104.4	2.12
	100.00	101.80	101.8	2.31

3.3. Linearity and detection limit

In order to determine the linearity of the seven analytes, a series of concentrations of mixed solutions, were tested under the optimized condition. The detection limits were calculated on the basis of a S/N ratio of 3 and the results were listed in Table 1.

3.4. Application in pharmacokinetics

According to the previously described procedure, rabbit urine samples collected at intervals of time were pretreated and the active ingredients were separated and determined. After giving the oral dose to the domestic rabbit, L-tyrosine was excreted in urine. In order to study the metabolic process of L-tyrosine in rabbit urine, some pharmacokinetics parameters have been calculated. When the collecting time was 360 min, the cumulated quantity of L-tyrosine was 4.560 mg, which take 45.60% of the total amount.

To validate the method, recovery experiments for Tyr were performed under the condition mentioned in Table 2. The analytical date demonstrated that the method is sensitive, reproducible and precise for determination of Tyr.

4. Conclusions

A simple, rapid and sensitive method was developed for separation and determination of L-tyrosine and its six related derivatives by capillary zone electrophoresis with end column amperometric detection. Under the optimal condition, complete separation, low detection limits (0.95–4.25 ng/mL) and good reproducibility were obtained for the seven analytes. The method was applied to the study of pharmacokinetics of L-tyrosine in rabbit urine, and 45.6% of total amount of L-tyrosine was excreted in 360 min. The results demonstrate that CE–AD is a powerful technique for the analysis of tyrosine and its related

derivatives. We hope the method would be helpful to study the medicine metabolism with high-performance capillary electrophoresis.

Acknowledgements

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Preface

Colloquium Spectroscopicum Internationale XXXIV, Antwerp, Belgium, 4–9 September 2005

The 34th Colloquium Spectroscopicum Internationale (CSI-XXXIV) was organized in Antwerp, Belgium, on 4–9 September 2005, hosted by the University of Antwerp. A special issue with proceedings of the contributions predominantly dealing with fundamental aspects of atomic spectroscopy as well as some applications of atomic spectroscopy will soon appear in *Spectrochimica Acta Part B*. In the present issue of *Talanta*, a number of papers dealing with applications of spectroscopic methods are brought together.

Since the first CSI conference, organized in Paris, France, in 1949, this prestigious series of biannual conferences has established itself as the premier forum for presentation and discussion of new developments in all branches of analytical spectroscopy. The most recent CSI conferences took place in Granada, Spain (2003), Pretoria, South Africa (2001) and Ankara, Turkey (1999). The next one will be organized in Xiamen, China, on 24–28 September 2007, while in 2009, the event will take place in Budapest, Hungary. While many conference series decay after 20–30 years, the CSI series is still vigorous, probably because it is a truly international series of conferences. In Antwerp, as in the previous conferences, there were participants from about 40 countries and from all continents. Moreover, the conference alternates, in principle, between Europe and other parts of the world.

Following the tradition of the preceding CSI conferences, emphasis was placed on new developments and applications of spectroscopy in all branches of analytical chemistry. At this conference there were almost 300 participants, and more than 300 abstracts were received for oral and poster contributions. The topics included new research of various spectroscopic techniques and methodologies (such as atomic plasma spectrometry, molecular spectroscopy, organic and inorganic mass spectrometry, X-ray spectrometry, hybrid techniques, laser spectroscopy, imaging techniques, quality control and chemometrics), as well as applications of spectroscopy in, among others, micro, surface and interface analysis, speciation, proteomics, environmental and geochemical analysis, archaeometry and cultural heritage, etc. The programme included 5 invited plenary lectures (by Y. Baba, M. Blades, D. Günther, G.M. Hieftje and R. Niessner), 8 invited keynote lectures (by A. de Juan Capdevilla, M. Höhn, J. Kolar, A. Laskin, N. Omenetto, C. Gruening, K. Shimizu and

F. Vanhaecke), 120 oral contributions in 20 parallel sessions introduced by the invited speakers, and 3 poster sessions. Two interesting vendor sessions have been arranged as well.

In the opening ceremony, the CSI-XXXIV Award was given to Prof. Yoichi Gohshi, whose professional career has been of impressive relevance both to X-ray spectrometry and to analytical science in Japan. Prof. Gohshi retired recently from the National Institute for Environmental Studies in Tsukuba, Japan, and earlier from the University of Tokyo. This award was sponsored by Wiley, publisher of scientific books and of the journal *X-Ray Spectrometry*, for which Prof. Gohshi served for many years as editor for Japan. The award implied first of all much honour, but also a certificate nicely made by Wiley, and finally an envelope with a check. The laudation for the award was given by Prof. Jun Kawai from Kyoto University, one of the famous students of Prof. Gohshi. After this the awardee highlighted some of his research over the decades.

Furthermore, at the conference dinner, three poster prizes, sponsored by Elsevier, were awarded to:

- Kouichi Tsuji (Osaka City University, Japan), for the poster “Depth unlimited X-ray fluorescence analysis for soft materials using needle-type collimators” (co-authors: A. Matsuda, K. Nakano and A. Okhrimovskyy);
- David De Muynck (Ghent University, Belgium), for the poster “Lead poisoning in the Roman Empire: isotopic study using single collector – and multi-collector – ICPMS” (co-authors: K. Van Hoecke, G. Quitté, L. Balcaen, E. Smits, F.A. de Wolff and F. Vanhaecke);
- Gerardo Gamez (Indiana University, US), for the poster “Fundamental characterization of a millisecond-pulsed glow discharge via laser-scattering techniques” (co-authors: G.M. Hieftje and A. Bogaerts).

Besides the interesting scientific contributions, the participants of the CSI-XXXIV conference did find opportunities to sample some of Antwerp’s cultural and historical treasures and some of its gastronomy and lifestyle. The city of Antwerp, in addition of hosting one of the most important harbours, the second petrochemical complex and the most important diamond centre in the world, has indeed a unique late medieval historical

centre, the home of Rubens and numerous fine museums, including the Royal Museum of Fine Arts where, in fact, CSI-XXXIV was opened with a guided visit and a welcome reception. The conference itself was held in the beautiful and medieval “Elzenveld” conference centre of the city of Antwerp. The complex has been a hospital since 1238. It was given to the city of Antwerp by Napoleon, and used by a religious order until 1989. Then, it was turned into a conference centre. Most of the buildings are original. The CSI participants enjoyed the quiet environment and beautiful gardens of the Elzenveld complex, which were especially attractive in the nice summer weather.

The guest editors of this special issue thank the participants of CSI-XXXIV for their contributions, and especially those who submitted an article to this issue, as well as Jean-Michel Kauffmann for managing this special issue. Furthermore, thanks go to the International Scientific Board and the Organising Committee, the sponsors, and last but not least, the conference secretary,

Mr. Luc Van’t dack, who did so much to put (and keep) the conference on the right tracks.

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25 April 2006

Differentiation of young red wines based on cultivar and geographical origin with application of chemometrics of principal polyphenolic constituents

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Abstract

Nineteen major polyphenolic phytochemicals including hydroxycinnamate derivatives, flavanols, flavonols, and anthocyanins, were determined in 40 experimental red wines employing HPLC-DAD. All wines analysed were young (non-aged), produced, and stored under identical conditions, in an effort to minimize the effect of oak wood and vinification technology. The data obtained from this examination composed the matrix for the implementation of chemometrics, which aimed at differentiating the wine samples on the basis of cultivar and geographical region of origin. Discriminant analysis performed at a 95% significance level revealed a very satisfactory categorization of the samples both in terms of cultivar and region of origin, thus illustrating the validity of major phenolic phytochemicals for studies pertaining to wine quality and authenticity.

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Keywords: Anthocyanins; Chemometrics; Differentiation; Discriminant analysis; Flavanols; Flavonols; Hydroxycinnamates; Polyphenols; Red wines

1. Introduction

For the wine industry and market sector, it is particularly essential that the intended value traits created via genetics (variety), origin of production (typicity), and unique inputs or processing method (vinification technology) are preserved. In other words, it must be ensured that a product's label is accurate and not misleading, since consumers distinguish peculiar commodities from a mass of other similar ones, on the belief that they bear a superior quality. However, wine is a product that can be easily adulterated, and for this reason wine authenticity is guaranteed by strict guidelines laid down by responsible national authorities, and includes sensory evaluation, chemical analyses, and examination of the records kept by wine producers.

The application of sophisticated statistical techniques, the so-called “chemometrics”, which take into consideration a large amount of analytical data related with wine composition, has been proven a versatile and valuable tool for assessing wine authenticity [1], and for this reason several inorganic elements

[2–5] and organic wine constituents [6,7], as well as data from sensory studies [8] have been the basis for discrimination of wines according to vinification technology, or classification according to region and variety.

The polyphenolic constituents of red wines present an undisputed interest because they are potent bioactive agents, and may contribute to preventing degenerative processes, detrimental to human health [9–11]. In particular, degenerative diseases such as cancers, cardiovascular disorders, and osteoporosis, are associated with oxidative damage to DNA, proteins, and lipids. Antioxidants present in wines can help limit this damage by acting directly on reactive oxygen species or by stimulating endogenous defence systems. The phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components [12]. In this regard, several studies have been dealing with the generation of analytical data concerning the polyphenolic composition. However, recent studies have also stress the significance of these components in the classification of wines [13].

The scope of this study was the utilization of several major polyphenolic components as critical parameters for categorizing young, non-aged, and varietal wines according to region of

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origin and cultivar, thus eliminating the impact of oak wood, as well as the reactions that might take place during ageing in an oak barrel. Furthermore, vinification and storage of all samples were carried out under identical conditions, to ascertain minimal influence deriving from implementation of techniques or conditions that could substantially alter the polyphenolic composition, thereby resulting in misleading conclusions.

2. Materials and methods

2.1. Chemicals

Procyanidins B1 and B2 and dephinidin, cyaniding, paeonidin, petunidin, and malvidin 3-*O*-glucosides were from Extrasynthese (Genay, France). Caffeic acid, *p*-coumaric acid, rutin, myricetin, quercetin, kaempferol, catechin, and epicatechin were from Sigma Chemical Co. (St. Louis, MO, USA). Chemical structures of all phenolic phytochemicals used may be seen in Fig. 1.

2.2. Wine samples

All 40 samples examined were young (non-aged) wines from the 2004 vintage, produced, and stored under identical conditions. Briefly, grapes were harvested at technological maturity, based on indices of sugar content and acidity established by the Vine & Wine Institute, destemmed, and crushed. Musts were inoculated with selected yeast strain (*S. cerevisiae* var *cerevisiae*) and fermented under controlled temperature (26–30 °C) to dryness (reducing sugar content <4 g L⁻¹), with a pomace contact period of 7 days. Following this, wines were racked, added SO₂ (30 mg L⁻¹), bottled, and stored at 15 ± 2 °C in the dark.

2.3. HPLC-DAD analysis

Equipment consisting of a HP 1050 chromatography apparatus coupled to a HP 1100 diode array detector was employed. Analyses were performed on a Lichrospher, 5 μm, 250 mm × 4 mm, at a flow rate of 1 mL min⁻¹, using a 20-

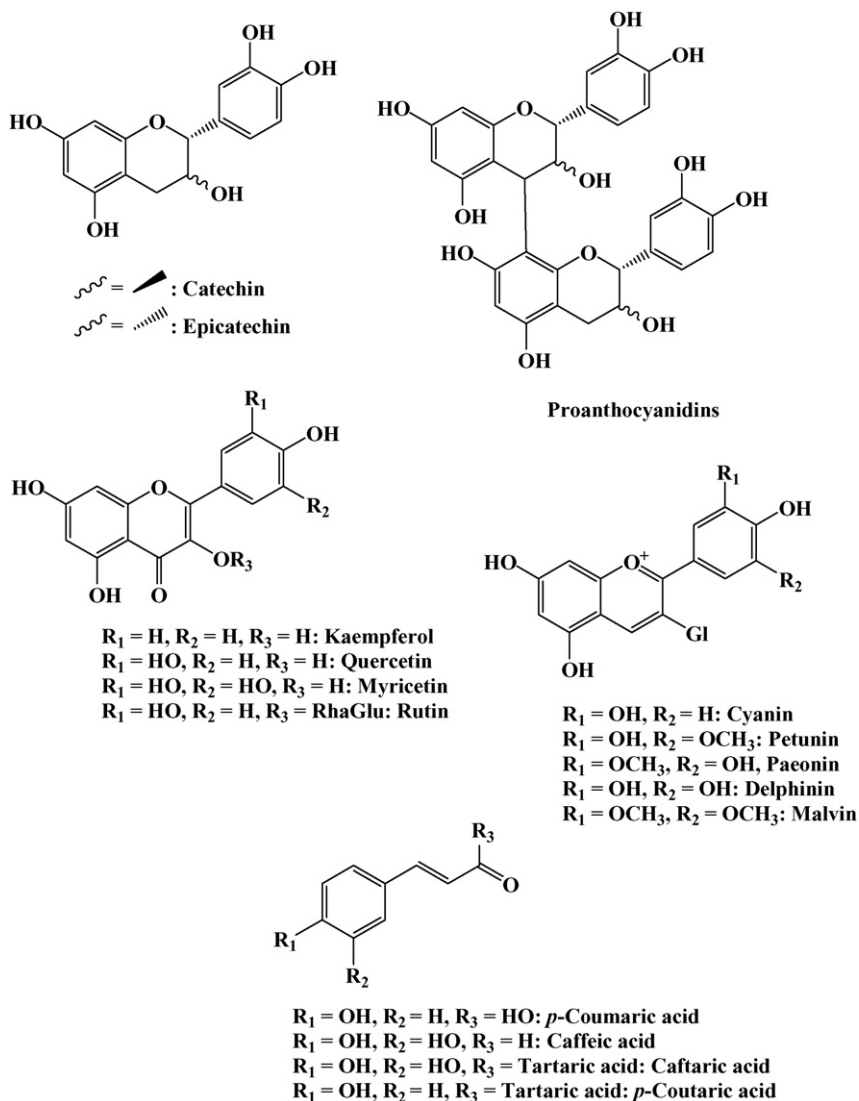


Fig. 1. Chemical structures of all polyphenols considered in this study.

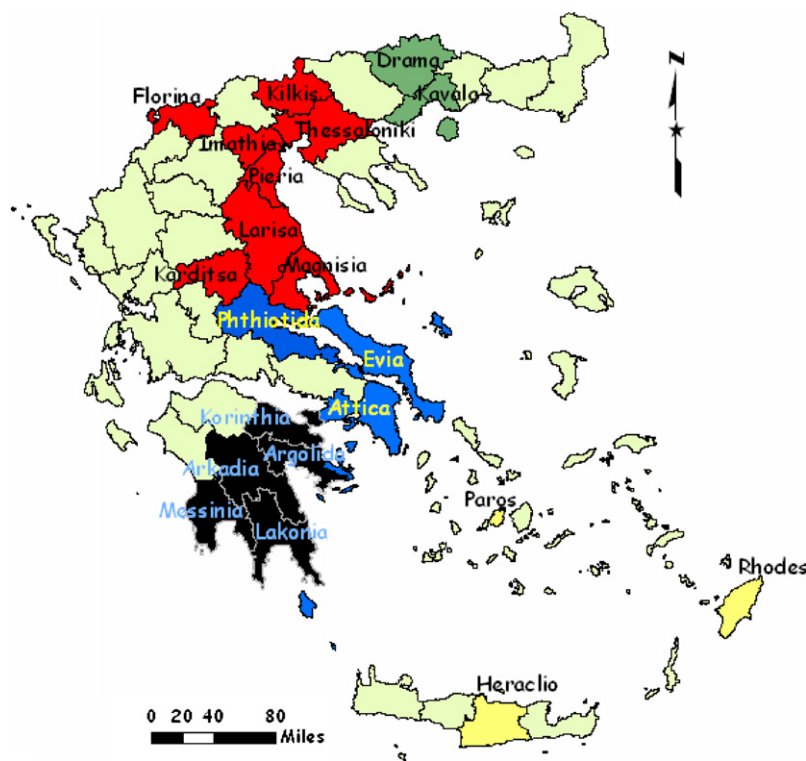


Fig. 2. Map illustrating the viticultural areas used for geographical origin-based differentiation. Varietal composition of each viticultural area may be seen in Table 1.

μL injection volume. Detection was accomplished at 280, 320, 360, and 520 nm for flavanols, hydroxycinnamates, flavonols, and anthocyanins, respectively. Details concerning eluents and elution programme are analytically described elsewhere [14]. Identification was based on comparing retention times of the peaks detected with those of original compounds, and on UV–vis on-line spectral data. Quantification was carried out using external standard method and results were expressed as mg L^{-1} . The external standards used are those corresponding to compounds seen in Fig. 1, with the exception of malvin coumarate, caftaric, and coumaric acids, which were quantified as malvin, caffeic, and coumaric acid, respectively.

2.4. Sample collection and experimental design

The grapes used for the production of wine samples were from the six most cultivated red varieties (*V. vinifera* sp.) and originated from major viticultural areas of Greece that cover the entire Hellenic vineyard (Table 1), with defined soil composition, cultural practices, and climatic conditions. Samples were grouped geographically according to the vicinity of the region of origin with emphasis to peculiar microclimatic characteristics, which could potentially have a prominent impact of the polyphenolic composition (Fig. 2). These specific areas were assigned as A1 (prefectures of Imathia, Pieria, Kilkis, Thessaloniki, Florina, Magnesia, Larisa, and Karditsa), A2 (prefectures of Attica, Phthiotida, and Evia), A3 (prefectures of Kavala and Drama), A4 (prefectures of Arkadia, Messinia, Lakonia, Argolida, and Korinthos), and A5 (the isles of Rhodes, Paros, and Crete).

2.5. Statistics and data processing

HPLC analyses were performed in duplicate and data are given as average values $\pm$ standard deviation (S.D.). The average values of the obtained data set were subjected to discriminant analysis (DA). Samples were distributed to a predetermined number of groups, resulting in a number of discriminant functions equal to the number of groups minus one. The maximization of the ratio variance between groups-to-variance of samples within the same group is a fundamental principle during the calculation of the functions. SPSS 10 was used to calculate the discriminant functions and plot the data for the discriminant analysis.

3. Results

The examination was based on the determination of characteristic, predominant polyphenols, which could be very well separated and quantified, employing routine HPLC analysis with conventional diode array detection (Figs. 3 and 4). The analysis of 19 representative components showed that their concentration may vary widely, even for samples of the same cultivar. In general, wines made from the Hellenic native cultivar Mandilaria were particularly rich in flavanols (average value: 243.1 mg L^{-1}) and hydroxycinnamates (99 mg L^{-1}) (Tables 2 and 3), whereas Syrah samples contained relatively high amounts of flavonols (60.1 mg L^{-1}) and anthocyanins (975.4 mg L^{-1}) (Tables 4 and 5). On average, the corresponding levels for flavanols, hydroxycinnamates, flavonols, and antho-

Table 1
Origin of grapes used for the production of experimental wines

Cultivar	Code	Sample no.	Area	Location
Merlot	M003	1	Drama	Eastern Macedonia
	M007	2	Imathia	Western Macedonia
	M001	3	Phthiotida	Stereia Ellada
	M013	4	Kavala	Eastern Macedonia
	M021	5	Arkadia	Peloponnese
Cabernet Sauvignon	M004	1	Phthiotida	Stereia Ellada
	M033	2	Messinia	Peloponnese
	M034	3	Messinia	Peloponnese
	M006	4	Evia	Stereia Ellada
	M009	5	Larisa	Thessaly
	M051	6	Arkadia	Peloponnese
	M016017	7	Thessaloniki	Central Macedonia
Syrah	MAR029	1	Karditsa	Thessaly
	MAR005	2	Attica	Stereia Ellada
	OIN022	3	Lakonia	Peloponnese
	MAR024	4	Karditsa	Thessaly
	OIN031	5	Florina	Western Macedonia
	OIN032	6	Pieria	Central Macedonia
	OIN033	7	Kilkis	Central Macedonia
	MAR038	8	Drama	Eastern Macedonia
	OIN040	9	Mantinia	Peloponnese
Agiorgitiko	ASP040	1	Attica	Stereia Ellada
	ET-AG	2	Attica	Stereia Ellada
	EYX	3	Attica	Stereia Ellada
	TEI	4	Korinthia	Peloponnese
	M041	5	Argolida	Peloponnese
	LAK	6	Lakonia	Peloponnese
Xinomavro	OIN052	1	Florina	Western Macedonia
	M030	2	Magnisia	Thessaly
	OIN041	3	Imathia	Western Macedonia
	OIN047	4	Imathia	Western Macedonia
	M059	6	Kilkis	Central Macedonia
	M048	7	Pieria	Central Macedonia
	MAR046	8	Imathia	Western Macedonia
Mandilaria	M028	1	Paros	Aegean Isles
	M018	2	Heraklio	Crete
	M019	3	Heraklio	Crete
	M027	4	Rhodes	Aegean Isles
	OIN028	5	Attica	Stereia Ellada
	M057	6	Rhodes	Aegean Isles

cyanins were 140.4, 68, 45, and 550.2 mg L⁻¹, representing 17.5, 8.5, 5.5, and 68.5% of the total polyphenol concentration, respectively.

Cultivar-based discriminant analysis was carried out using the sets of data given in Tables 2–5 as the data matrix. The analysis on the sample groups using all variables (phenolics) resulted in five discriminant functions (DF). The first, assigned as DF1, accounted for 52% of total variability, while DF2 and DF3 for 22.1 and 17.3%, respectively, the sum of which covered 91.3% of the total variance explained (Table 6). All variables were significant at 95% significance level. The scores for the first three discriminant functions were plotted as a 3D scatter diagram, yielding an explicit separation of samples into different clusters (Fig. 5). The main discriminating axis was DF1, which was mainly correlated with malvin acetate, caftaric acid, and pro-

Table 2
Composition of major flavanols and flavanol dimers (procyanidins) in the experimental wines tested

Sample	Procyanidin B1	Catechin	Procyanidin B2	Epicatechin	Total
Merlot					
1	0	26.4 ± 2.5	4.6 ± 0.5	9.9 ± 0.2	40.9
2	31.2 ± 5.6	34.6 ± 2.7	0	11.7 ± 0.2	77.5
3	68.4 ± 2.0	80.7 ± 0.2	19.1 ± 2.7	39.2 ± 0.9	207.4
4	37.2 ± 1.4	36.9 ± 0.1	0	19.1 ± 1.0	93.2
5	29.9 ± 4.8	33.5 ± 6.8	2.9 ± 0.4	12.9 ± 2.3	79.2
Average	33.3	42.4	5.3	18.6	99.6
Cabernet Sauvignon					
1	21.4 ± 0.2	18.2 ± 0.1	8.5 ± 0.0	8.6 ± 0.7	56.7
2	27.9 ± 2.5	49.1 ± 2.6	12.1 ± 0.1	16.4 ± 4.7	105.5
3	30.7 ± 0.8	50.9 ± 1.1	19.2 ± 2.5	22.4 ± 1.1	123.2
4	46.4 ± 0.1	58.9 ± 6.4	30.2 ± 1.1	23.4 ± 1.3	158.9
5	14.9 ± 1.1	35.7 ± 4.0	9.2 ± 2.3	18.3 ± 0.7	78.1
6	14.3 ± 0.3	29.6 ± 5.6	0	24.6 ± 1.0	68.6
7	10.0 ± 4.2	41.4 ± 2.0	19.3 ± 1.4	22.0 ± 5.3	92.7
Average	23.7	40.5	14.1	19.4	97.7
Syrah					
1	31.7 ± 0.4	43.5 ± 2.7	0	18.0 ± 3.1	93.2
2	30.6 ± 0.7	39.3 ± 4.7	14.5 ± 0.5	24.1 ± 2.7	108.5
3	0	0	0	0	0
4	0	51.4 ± 0.4	26.9 ± 1.6	37.1 ± 1.2	115.4
5	20.1 ± 3.8	0	0	31.5 ± 0.5	51.6
6	0	32.1 ± 0.0	0	0	32.1
7	47.7 ± 5.9	75.0 ± 9.6	0	59.9 ± 0.3	182.7
8	24.4 ± 1.6	25.1 ± 0.3	10.0 ± 0.1	23.5 ± 1.7	82.9
9	40.7 ± 3.1	108.7 ± 2.5	59.4 ± 0.3	101.8 ± 6.4	310.6
Average	21.7	41.7	12.3	32.9	108.6
Agiorgitiko					
1	44.5 ± 1.3	47.3 ± 1.5	0	14.9 ± 0.1	106.6
2	73.7 ± 9.4	74.6 ± 2.5	19.8 ± 1.7	13.3 ± 0.9	181.4
3	52.5 ± 6.9	63.2 ± 0.5	6.6 ± 0.2	15.2 ± 2.8	137.6
4	22.5 ± 1.8	70.4 ± 7.2	0	25.1 ± 0.7	118.0
5	40.0 ± 0.5	15.3 ± 1.6	0	11.3 ± 1.4	66.6
6	28.0 ± 4.2	34.3 ± 6.5	3.4 ± 0.8	10.8 ± 2.4	76.6
Average	43.5	50.9	5.0	15.1	114.5
Xinomavro					
1	60.2 ± 1.7	84.5 ± 2.4	50.6 ± 4.1	58.1 ± 4.2	253.4
2	46.1 ± 0.7	43.9 ± 1.9	32.5 ± 0.1	48.0 ± 3.7	170.4
3	49.3 ± 1.1	56.2 ± 2.1	25.5 ± 0.7	33.6 ± 4.8	164.5
4	37.6 ± 5.2	42.3 ± 2.7	22.7 ± 0.4	37.3 ± 1.9	140.0
5	61.4 ± 5.9	89.1 ± 2.7	41.2 ± 6.7	51.5 ± 3.7	243.1
6	43.4 ± 3.1	53.1 ± 2.3	34.2 ± 0.9	27.6 ± 0.2	158.4
7	34.5 ± 0.1	52.2 ± 0.6	27.1 ± 5.4	9.5 ± 0.6	123.4
Average	47.5	60.2	33.4	37.9	179.0
Mandilaria					
1	41.8 ± 0.9	98.3 ± 0.2	26.2 ± 3.3	44.6 ± 0.3	210.8
2	100.3 ± 6.4	96.5 ± 6.1	43.7 ± 2.4	36.4 ± 3.4	276.8
3	70.5 ± 0.2	84.0 ± 0.8	28.3 ± 4.6	33.7 ± 0.7	216.4
4	66.6 ± 0.7	87.9 ± 5.3	27.2 ± 4.0	22.1 ± 0.5	203.8
5	113.8 ± 14.9	99.1 ± 3.8	40.4 ± 0.4	33.0 ± 3.0	266.0
6	96.7 ± 1.9	104.6 ± 7.6	29.8 ± 0.1	32.8 ± 0.9	263.9
Average	81.6	95.1	32.6	33.8	243.1

Values are expressed as mg L⁻¹ and represent means of duplicate determination ± standard deviation.

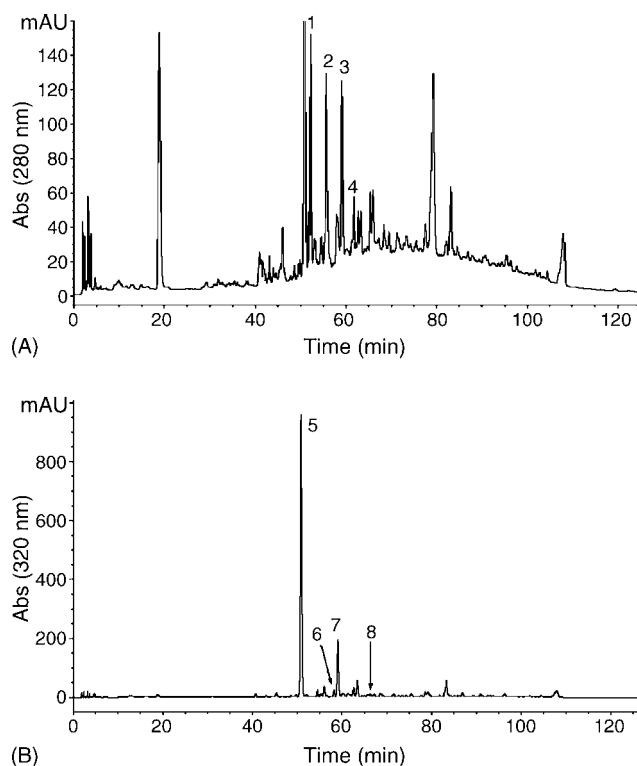


Fig. 3. Characteristic chromatograms showing major flavanols (A) and hydroxycinnamates (B) determined. Corresponding detection wavelengths were 280 and 320 nm. Peak assignment: 1, procyanidin B1; 2, catechin; 3, procyanidin B2; 4, epicatechin; 5, caftaric acid; 6, caffeic acid; 7, coumaric acid; 8, *p*-coumaric acid.

cyanidin B1, as shown in Table 7. Mandilaria, Xinomavro, and Syrah samples formed well-defined clusters according to DF1. Syrah, Cabernet Sauvignon, and Merlot samples differentiated according to DF2. This function was strongly influenced by cyanin, procyanidin B2, coumaric acid, epicatechin, and delphinine. Finally, Mandilaria and Agiorgitiko, as well as Xinomavro and Agiorgitiko were discriminated according to DF3, which was affected mainly by procyanidin B2, caffeic acid, malvin, and malvin coumarate. It is worth mentioning that the contribution of all flavonols to differentiation was statistically insignificant.

In the same framework described for cultivar-based classification, discriminant analysis with regard to geographical origin resulted in four DFs. DF1 accounted for 50.8% of total variability, while DF2 and DF3 for 26.3 and 15.2%, respectively (92.3% of total variance explained; Table 8). All variables were significant at 95% level. The scores for the first three discriminant functions composed the 3D scatter diagram (Fig. 6). The main axis of differentiation (DF1) was strongly influenced by flavanols (procyanidin B1, B2, and catechin) and the major anthocyanins (delphinin, cyanin, malvin, and malvin acetate) as well as caftaric acid. Geographical areas A1, A4, and A5 formed distinct clusters according to DF1. DF2 was associated with the flavanols (procyanidin B1, catechin, and epicatechin) and flavonols (rutin, quercetin, and kaempferol) as well as caftaric acid. Samples from areas A1 and A2 differentiated according to this function. Finally DF3, responsible for the separation of areas A3 and A4, was mainly affected by hydroxycinnamates (caftaric

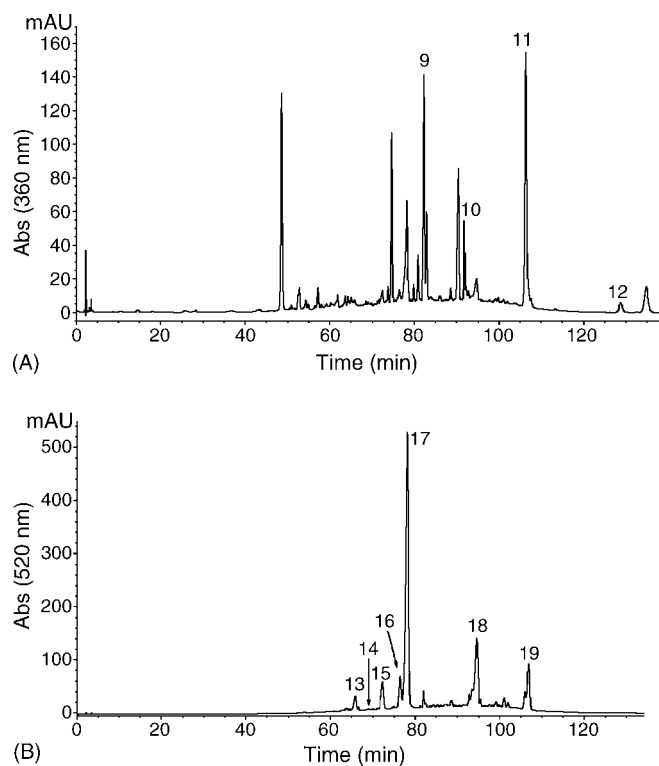


Fig. 4. Typical flavonol (A) and anthocyanin (B) profiles of the wines analysed. Peak assignment: 9, rutin; 10, myricetin; 11, quercetin; 12, kaempferol; 13–17, glucosides of delphinidin, cyaniding, petunidin, paeonidin, and malvidin, respectively; 18 and 19, acetate and *p*-coumarate derivatives of malvidin glucoside.

acid, coumaric acid, caffeic acid, and coumaric acid), flavonols (quercetin, myricetin, and kaempferol) as well as procyanidin B1, B2, catechin, and malvin coumarate. The correlations of the variables with the discriminant functions are analytically given in Table 9.

4. Discussion

Polyphenol biosynthesis is strictly controlled by the genes of the corresponding enzymes involved in the relevant biosynthetic pathways. Thus, the polyphenolic profile of a given cultivar reflects to a great extent its genetic potential. In a similar fashion, environmental stimuli, i.e. sun exposure, play critical roles in regulating activities of enzymes implicated in polyphenol biosynthesis, and this effect could as well be reflected on the polyphenolic profile. In fact, all phenolics exhibited notable variations even in samples made from the same cultivar, evidencing an important impact of climatic conditions occurring in different geographical areas. It was thus hypothesised that the differences arising from these two key parameters might be revealed after appropriate statistical analysis, which would take into consideration major polyphenolic components as credible indices. In addition to these criteria, all samples were vinified under identical conditions to minimize technological influence, but most significantly they were non-aged. This prerequisite was met bearing in mind that the concentration of certain phenolics may change appreciably as a result of oxidation, co-pigmentation,

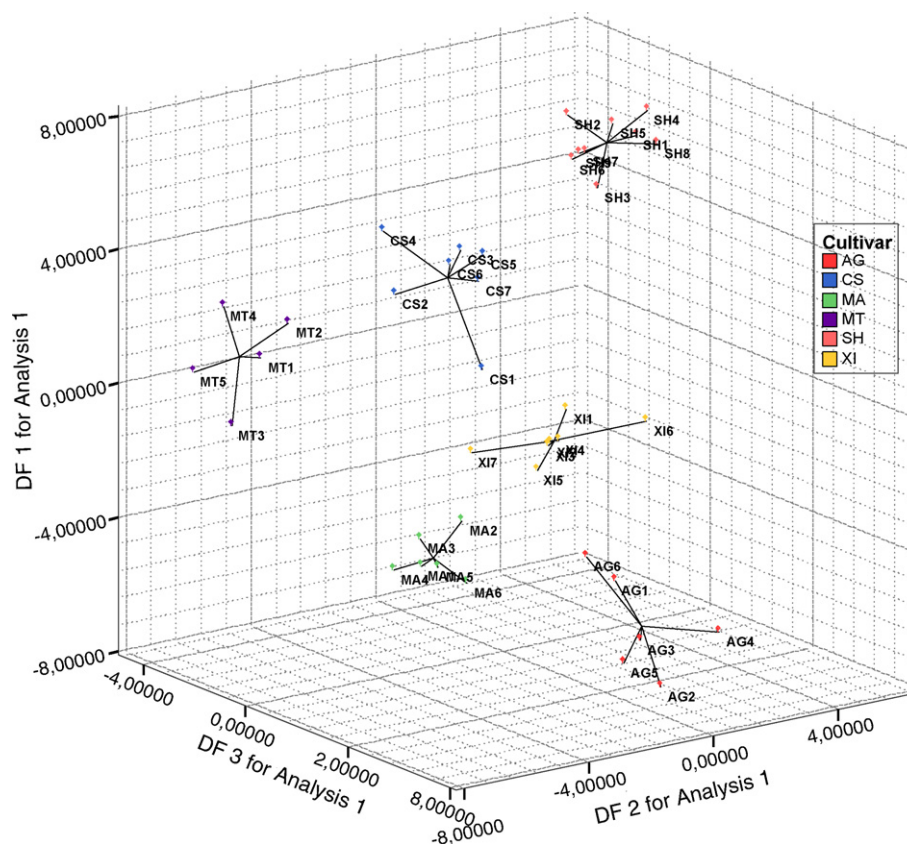


Fig. 5. Three-dimensional plot showing cultivar-based differentiation of samples. Assignments: AG, Agiorgitiko; CS, Cabernet Sauvignon; MA, Mandilaria; MT, Merlot; SH, Syrah; XI, Xinomavro.

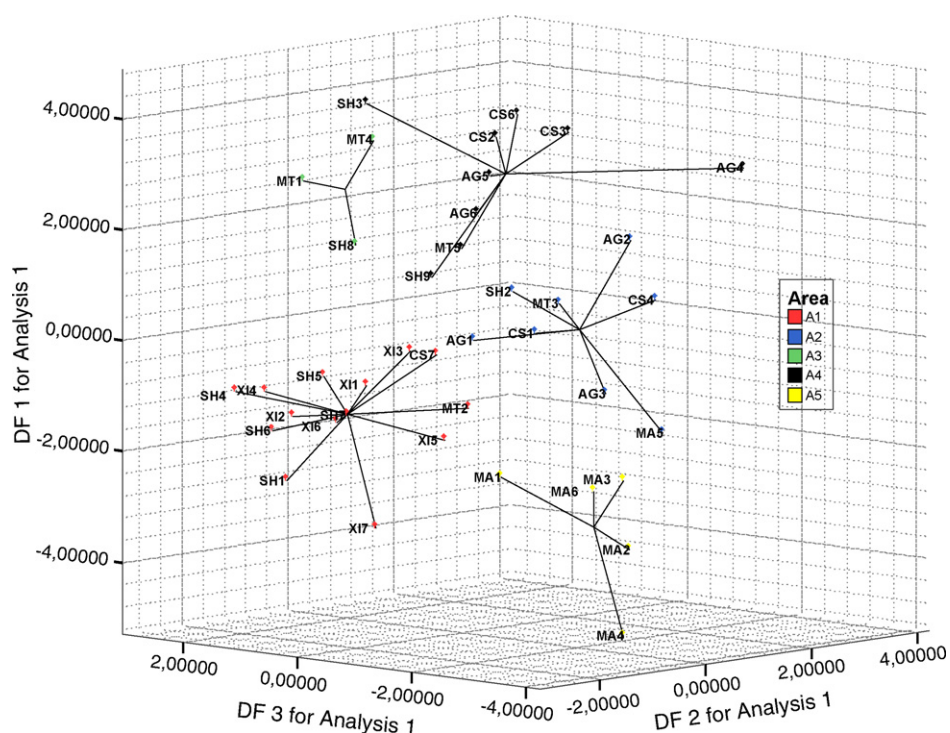


Fig. 6. Three-dimensional plot showing geographical area-based differentiation of samples.

Table 3
Composition of major hydroxycinnamates in the experimental wines tested

Sample	Caftaric acid	Coutaric acid	Caffeic acid	Coumaric acid	Total
Merlot					
1	24.9 ± 0.0	6.5 ± 0.8	0	0.9 ± 0.1	32.2
2	18.7 ± 0.3	3.8 ± 0.6	2.4 ± 0.3	3.0 ± 0.5	27.8
3	18.7 ± 2.8	4.5 ± 0.3	0.9 ± 0.2	1.6 ± 0.5	25.7
4	25.2 ± 0.1	5.2 ± 0.2	1.9 ± 0.1	1.4 ± 0.0	33.7
5	34.0 ± 0.0	9.4 ± 0.2	1.6 ± 0.1	1.9 ± 0.3	46.8
Average	24.3	5.9	1.4	1.8	33.3
Cabernet Sauvignon					
1	35.7 ± 0.5	12.3 ± 1.6	0.5 ± 0.0	0	48.5
2	19.7 ± 0.2	6.0 ± 0.9	5.5 ± 0.1	2.6 ± 0.3	33.8
3	29.6 ± 0.4	12.4 ± 0.1	1.2 ± 0.0	0	43.2
4	42.1 ± 1.1	12.1 ± 0.4	0.7 ± 0.0	0	54.9
5	23.1 ± 0.7	7.1 ± 0.0	2.7 ± 0.1	2.3 ± 0.0	35.3
6	4.8 ± 0.3	4.2 ± 0.1	6.9 ± 0.2	0.9 ± 0.2	16.8
7	4.3 ± 0.3	3.0 ± 0.3	4.0 ± 0.5	0.3 ± 0.1	11.6
Average	22.8	8.2	3.1	0.9	34.9
Syrah					
1	38.8 ± 1.9	1.9 ± 0.4	19.9 ± 0.3	3.1 ± 0.0	63.6
2	34.0 ± 0.5	18.2 ± 0.8	0.4 ± 0.0	5.2 ± 0.2	57.8
3	22.3 ± 0.1	12.7 ± 0.2	0	3.1 ± 0.0	38.1
4	41.2 ± 1.3	26.7 ± 2.2	2.3 ± 0.0	2.6 ± 0.2	72.8
5	38.1 ± 0.2	24.6 ± 4.2	0.6 ± 0.1	7.1 ± 0.1	70.4
6	45.5 ± 0.5	28.6 ± 0.8	0.9 ± 0.1	3.9 ± 0.6	78.8
7	27.2 ± 0.7	17.3 ± 0.8	0.4 ± 0.0	1.9 ± 0.2	46.7
8	22.5 ± 0.2	14.1 ± 0.8	0	3.9 ± 0.9	40.2
9	36.4 ± 0.4	10.7 ± 1.7	1.3 ± 0.0	1.7 ± 0.0	50.1
Average	38.0	17.2	2.9	3.6	61.7
Agiorgitiko					
1	53.7 ± 0.8	3.8 ± 0.1	17.7 ± 0.6	1.9 ± 0.2	77.1
2	85.8 ± 1.3	3.2 ± 0.5	31.2 ± 4.5	2.1 ± 0.2	122.3
3	69.6 ± 4.1	2.8 ± 0.8	30.9 ± 0.0	1.3 ± 0.2	104.5
4	90.4 ± 2.7	39.0 ± 5.5	4.9 ± 0.3	3.3 ± 0.9	137.5
5	53.9 ± 0.1	24.0 ± 0.2	1.7 ± 0.1	1.1 ± 0.2	90.7
6	33.6 ± 1.1	17.0 ± 1.6	0.6 ± 0.0	1.1 ± 0.0	52.3
Average	64.5	15.0	14.5	1.8	95.8
Xinomavro					
1	78.0 ± 1.4	3.3 ± 0.2	11.7 ± 0.3	0.8 ± 0.3	92.8
2	83.5 ± 3.5	12.4 ± 0.5	1.1 ± 0.0	0.6 ± 0.0	97.5
3	64.4 ± 0.0	12.5 ± 0.0	2.9 ± 0.0	1.6 ± 0.3	81.5
4	69.3 ± 0.2	10.4 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	82.2
5	54.6 ± 0.4	9.4 ± 1.2	1.6 ± 0.0	0.8 ± 0.1	66.5
6	65.9 ± 0.2	12.2 ± 2.4	3.1 ± 0.2	1.3 ± 0.1	82.5
7	65.9 ± 0.4	8.4 ± 0.0	1.8 ± 0.0	0.9 ± 0.1	77.1
Average	68.8	9.8	3.4	1.0	83.0
Mandilaria					
1	51.6 ± 5.0	10.4 ± 0.3	1.4 ± 0.1	1.0 ± 0.1	64.4
2	85.7 ± 1.7	15.7 ± 1.7	1.7 ± 0.0	0.7 ± 0.0	103.7
3	56.9 ± 1.4	12.7 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	71.7
4	122.3 ± 1.1	19.7 ± 0.1	1.9 ± 0.0	1.0 ± 0.1	144.9
5	63.7 ± 6.5	12.5 ± 1.5	1.2 ± 0.2	1.4 ± 0.3	78.9
6	106.7 ± 2.5	21.1 ± 0.9	1.6 ± 0.2	0.7 ± 0.0	130.1
Average	81.2	15.4	1.5	1.0	99.0

Values are expressed as mg L⁻¹ and represent means of duplicate determination ± standard deviation.

Table 4
Composition of major flavonols in the experimental wines tested

Sample	Rutin	Quercetin	Myricetin	Kaempferol	Total
Merlot					
1	17.7 ± 2.9	28.6 ± 0.9	5.6 ± 0.6	5.5 ± 0.1	57.3
2	27.5 ± 6.1	21.6 ± 0.2	0	2.5 ± 0.1	51.6
3	11.5 ± 0.4	15.5 ± 3.4	4.9 ± 0.3	1.1 ± 0.1	33.0
4	32.4 ± 0.3	53.1 ± 0.9	9.8 ± 0.1	3.4 ± 0.1	98.7
5	14.4 ± 0.4	30.6 ± 2.0	14.8 ± 0.4	2.4 ± 0.1	62.3
Average	20.7	29.9	7.0	3.0	60.6
Cabernet Sauvignon					
1	71.8 ± 3.8	19.9 ± 2.5	2.1 ± 0.7	3.1 ± 0.0	96.9
2	10.0 ± 0.8	15.3 ± 2.0	4.0 ± 0.1	2.4 ± 0.0	31.7
3	16.9 ± 3.3	7.2 ± 0.3	5.3 ± 0.7	0.8 ± 0.0	30.2
4	80.4 ± 2.7	13.2 ± 1.3	0	1.9 ± 0.4	95.5
5	7.3 ± 0.2	4.8 ± 0.1	4.1 ± 0.9	0.7 ± 0.0	16.9
6	15.5 ± 1.0	22.4 ± 0.2	13.7 ± 3.2	2.8 ± 0.7	54.4
7	14.1 ± 0.9	12.9 ± 0.8	9.6 ± 0.1	1.0 ± 0.3	37.6
Average	30.9	13.7	5.5	1.8	51.9
Syrah					
1	21.0 ± 4.0	42.2 ± 3.2	11.1 ± 1.1	2.8 ± 0.2	77.1
2	41.6 ± 6.2	6.4 ± 0.3	23.8 ± 0.9	0	71.9
3	9.6 ± 0.1	9.9 ± 0.2	4.1 ± 0.3	0.7 ± 0.1	24.3
4	26.4 ± 1.4	36.7 ± 2.1	19.1 ± 0.1	3.7 ± 0.1	85.9
5	10.8 ± 0.1	63.0 ± 2.0	18.0 ± 0.5	5.0 ± 0.1	96.8
6	12.1 ± 0.8	59.9 ± 0.7	13.4 ± 0.1	4.8 ± 0.0	90.2
7	9.7 ± 0.6	7.1 ± 0.8	12.8 ± 1.6	0.6 ± 0.1	30.2
8	6.5 ± 0.6	7.4 ± 0.7	13.6 ± 1.6	0.2 ± 0.1	27.7
9	16.5 ± 0.5	15.0 ± 1.9	4.2 ± 0.3	1.3 ± 0.0	37.0
Average	17.1	27.5	13.3	2.1	60.1
Agiorgitiko					
1	16.9 ± 0.2	9.6 ± 0.1	1.6 ± 0.1	1.8 ± 0.3	29.9
2	26.9 ± 4.2	8.9 ± 1.6	6.0 ± 0.4	0.2 ± 0.0	42.1
3	24.3 ± 0.2	21.9 ± 0.8	9.8 ± 0.4	3.2 ± 0.1	59.2
4	42.7 ± 2.9	2.4 ± 0.2	5.7 ± 0.0	0	50.8
5	29.0 ± 0.0	24.8 ± 0.3	2.2 ± 0.4	5.0 ± 0.0	61.0
6	9.5 ± 0.4	5.1 ± 0.2	1.3 ± 0.4	0.8 ± 0.0	16.6
Average	26.5	12.6	5.0	1.8	43.3
Xinomavro					
1	5.5 ± 0.1	11.7 ± 0.3	4.4 ± 0.2	2.6 ± 0.3	24.2
2	11.6 ± 0.8	20.8 ± 0.8	1.7 ± 0.1	6.0 ± 0.9	40.0
3	9.3 ± 0.1	12.8 ± 1.3	4.4 ± 0.0	2.2 ± 0.1	28.7
4	6.1 ± 0.8	16.4 ± 2.3	2.8 ± 0.2	3.9 ± 1.1	29.2
5	2.7 ± 0.1	3.1 ± 0.3	2.7 ± 0.0	0	8.4
6	8.4 ± 0.8	11.7 ± 1.3	6.0 ± 0.0	1.5 ± 0.0	27.6
7	4.5 ± 0.2	8.7 ± 0.1	2.8 ± 0.2	0.9 ± 0.2	16.8
Average	6.9	12.2	3.5	2.4	25.0
Mandilaria					
1	8.0 ± 0.9	8.7 ± 1.0	1.0 ± 0.2	0	17.8
2	27.3 ± 0.6	7.6 ± 0.5	1.1 ± 0.0	0.6 ± 0.0	36.8
3	19.4 ± 1.9	8.6 ± 0.5	1.0 ± 0.3	0	29.0
4	38.0 ± 0.1	10.7 ± 0.5	1.7 ± 0.1	0	50.4
5	13.6 ± 1.6	7.8 ± 0.9	1.3 ± 0.4	0	22.7
6	9.3 ± 1.8	8.0 ± 0.9	1.0 ± 0.0	0	18.3
Average	19.3	8.6	1.2	0.1	29.1

Values are expressed as mg L⁻¹ and represent means of duplicate determination ± standard deviation.

Table 5

Composition of principal anthocyanins in the experimental wines tested

Sample	Dp	Cy	Pt	Pn	Mv	MvAc	MvCoun	Total
Merlot								
1	7.6 ± 0.0	1.3 ± 0.0	14.4 ± 0.4	15.2 ± 0.7	135.0 ± 1.0	44.4 ± 0.4	17.1 ± 1.5	235.0
2	8.2 ± 0.9	0.3 ± 0.1	21.5 ± 2.3	10.1 ± 0.1	289.9 ± 19.7	90.6 ± 10.1	32.6 ± 3.0	453.2
3	10.5 ± 2.2	1.2 ± 0.7	22.6 ± 3.1	18.2 ± 4.4	271.9 ± 7.0	71.3 ± 1.4	29.0 ± 5.5	424.6
4	22.6 ± 0.9	2.2 ± 0.1	41.9 ± 0.1	24.7 ± 1.1	405.0 ± 3.5	113.0 ± 0.2	52.9 ± 0.5	662.5
5	37.7 ± 1.3	2.0 ± 0.0	58.7 ± 1.9	25.1 ± 0.9	455.6 ± 13.1	132.3 ± 3.7	46.2 ± 1.7	757.6
Average	17.3	1.4	31.8	18.7	311.5	90.3	35.6	506.6
Cabernet Sauvignon								
1	6.7 ± 0.2	0.2 ± 0.0	8.9 ± 0.6	5.6 ± 0.4	133.2 ± 1.6	55.7 ± 0.7	15.8 ± 0.0	226.2
2	6.0 ± 0.4	0.8 ± 0.0	11.3 ± 0.1	23.3 ± 2.1	197.4 ± 8.9	106.3 ± 10.9	21.9 ± 7.1	367.1
3	7.6 ± 0.9	1.3 ± 0.1	22.3 ± 0.7	40.6 ± 6.7	500.9 ± 21.2	246.6 ± 0.1	44.2 ± 0.5	863.4
4	20.0 ± 0.8	0.7 ± 0.2	28.0 ± 1.7	11.2 ± 0.5	307.8 ± 14.5	155.4 ± 8.0	32.4 ± 1.1	555.3
5	3.7 ± 0.1	0	9.6 ± 0.0	7.7 ± 0.8	294.0 ± 6.8	182.7 ± 3.4	28.4 ± 0.7	526.0
6	43.6 ± 3.8	0.7 ± 0.0	55.1 ± 4.6	18.4 ± 1.3	848.4 ± 21.7	399.8 ± 15.0	96.1 ± 2.8	1462.0
7	10.8 ± 0.2	0.2 ± 0.1	19.6 ± 0.8	6.9 ± 1.4	458.5 ± 9.8	219.6 ± 6.5	43.9 ± 0.6	759.6
Average	14.1	0.6	22.1	16.2	391.5	195.2	40.4	680.0
Syrah								
1	12.6 ± 1.5	0	35.9 ± 4.5	24.6 ± 4.0	531.7 ± 32.1	207.6 ± 6.4	95.2 ± 1.4	907.6
2	1.5 ± 0.8	0.4 ± 0.2	23.6 ± 1.6	11.6 ± 1.5	560.1 ± 1.6	235.0 ± 13.3	77.2 ± 2.3	909.4
3	1.7 ± 0.0	0	10.3 ± 0.1	5.1 ± 0.1	316.2 ± 4.8	183.7 ± 4.5	46.9 ± 2.8	563.9
4	32.5 ± 0.6	0.9 ± 0.1	66.3 ± 4.5	35.6 ± 0.8	581.4 ± 13.5	154.0 ± 12.6	99.1 ± 4.1	972.5
5	28.1 ± 0.7	0.3 ± 0.0	62.2 ± 1.2	26.8 ± 1.7	652.3 ± 10.1	183.2 ± 1.1	67.4 ± 0.5	1020.4
6	21.7 ± 2.2	0.6 ± 0.1	55.3 ± 0.6	41.0 ± 3.8	658.1 ± 12.7	182.2 ± 2.4	94.7 ± 0.6	1053.6
7	14.6 ± 0.8	0	42.8 ± 0.5	27.3 ± 1.8	579.7 ± 10.5	273.6 ± 3.5	122.5 ± 2.9	1060.5
8	4.7 ± 0.2	0	26.7 ± 1.0	12.0 ± 1.1	697.6 ± 4.5	292.2 ± 5.9	107.4 ± 0.1	1140.6
9	14.7 ± 0.6	0.6 ± 0.2	26.3 ± 0.2	15.7 ± 1.9	353.1 ± 8.8	164.0 ± 10.8	27.4 ± 1.5	601.8
Average	16.8	0.3	42.7	24.6	588.2	219.0	83.9	975.4
Agiorgitiko								
1	3.1 ± 0.0	0	9.6 ± 0.3	11.8 ± 0.5	360.7 ± 4.4	33.4 ± 2.0	61.7 ± 5.7	480.4
2	18.2 ± 2.7	1.1 ± 0.1	34.9 ± 4.2	19.0 ± 2.8	751.2 ± 10.3	56.3 ± 8.4	90.7 ± 13.4	971.4
3	5.8 ± 0.1	0	11.2 ± 0.5	6.2 ± 0.7	362.2 ± 10.1	23.3 ± 6.0	21.3 ± 2.8	430.0
4	15.1 ± 1.9	0.4 ± 0.0	28.4 ± 0.7	24.1 ± 2.8	503.3 ± 28.7	26.7 ± 1.1	49.3 ± 1.1	647.4
5	12.9 ± 0.2	0.5 ± 0.0	27.2 ± 0.6	17.0 ± 0.3	593.0 ± 10.6	46.4 ± 0.6	76.3 ± 0.1	773.2
6	0.7 ± 0.2	0	5.6 ± 0.0	2.7 ± 0.5	282.6 ± 2.0	38.1 ± 0.3	42.8 ± 0.4	372.5
Average	9.3	0.3	19.5	13.5	475.5	37.4	57.0	612.5
Xinomavro								
1	4.6 ± 0.2	0.7 ± 0.1	8.0 ± 0.1	9.7 ± 0.7	132.1 ± 2.2	16.5 ± 0.0	20.5 ± 0.1	192.1
2	1.6 ± 0.1	0.2 ± 0.0	3.7 ± 0.0	3.7 ± 0.1	84.3 ± 2.4	9.9 ± 0.2	16.1 ± 0.5	119.5
3	1.7 ± 0.2	0.2 ± 0.0	4.1 ± 0.1	2.3 ± 0.7	103.8 ± 3.3	16.7 ± 0.6	23.1 ± 1.9	152.0
4	2.3 ± 0.2	0.2 ± 0.0	6.2 ± 0.7	3.0 ± 0.7	150.1 ± 22.3	28.6 ± 0.7	40.4 ± 0.7	230.7
5	2.4 ± 0.5	0.3 ± 0.1	5.9 ± 0.0	7.7 ± 0.6	161.9 ± 0.3	23.0 ± 1.0	33.5 ± 0.6	234.6
6	2.4 ± 0.1	0.1 ± 0.0	6.3 ± 1.0	4.7 ± 0.0	188.4 ± 3.5	33.1 ± 1.7	49.6 ± 0.1	284.7
7	0.7 ± 0.0	0.2 ± 0.0	3.1 ± 0.2	3.6 ± 0.0	113.0 ± 0.9	21.3 ± 1.1	25.9 ± 0.3	167.8
Average	2.2	0.3	5.3	5.0	133.4	21.3	29.9	197.3
Mandilaria								
1	2.7 ± 0.1	0.4 ± 0.0	8.3 ± 0.9	6.2 ± 0.9	292.4 ± 29.5	23.2 ± 1.9	30.8 ± 2.8	363.9
2	5.3 ± 0.1	0.5 ± 0.0	13.5 ± 0.1	14.0 ± 0.1	219.9 ± 2.4	14.9 ± 0.7	32.5 ± 0.5	300.8
3	3.9 ± 0.1	0.4 ± 0.0	9.3 ± 0.1	11.5 ± 0.6	176.1 ± 6.0	9.4 ± 0.3	12.7 ± 0.3	223.2
4	15.2 ± 0.3	1.0 ± 0.2	25.9 ± 0.4	22.4 ± 0.5	282.3 ± 0.3	10.7 ± 0.2	31.9 ± 0.3	389.5
5	2.5 ± 0.3	0.4 ± 0.0	7.9 ± 1.1	5.5 ± 0.1	296.2 ± 8.0	39.0 ± 1.9	44.0 ± 6.0	396.0
6	7.4 ± 0.7	0.6 ± 0.1	15.2 ± 2.5	13.2 ± 2.1	210.8 ± 15.2	14.4 ± 3.2	40.3 ± 7.9	301.9
Average	6.2	0.6	13.4	12.1	246.3	18.6	32.0	329.1

Values are expressed as mg L⁻¹ and represent means of duplicate determination ± standard deviation.

hydrolysis and decomposition, a fact that could yield misleading results.

The discriminant analysis revealed that flavanols, especially procyanidin B1 and B2, exerted a profound influence on both cultivar and geographical origin-based differentiation. Quite on

the contrary, flavonols had a rather minor impact, assigned only to the geographical origin-based differentiation. Likewise, caffeic acid significantly affected differentiation both in terms of cultivar and origin, whereas the rest hydroxycinnamate derivatives were less important in this regard. Major anthocyanins

Table 6
Eigenvalues and percent variance explained for cultivar-based differentiation

Function	Eigenvalue	Variance (%)	Cumulative (%)	Canonical correlation
1	29.155 ^a	52.0	52.0	0.983
2	12.391 ^a	22.1	74.0	0.962
3	9.693 ^a	17.3	91.3	0.952
4	3.334 ^a	5.9	97.3	0.877
5	1.541 ^a	2.7	100.0	0.779

^a First five canonical discriminant functions used in the analysis.

including malvin and its acetate derivative appeared to play an important role in both differentiations, but the effect of paeonin and petunin was without particular value. It appears that major metabolites, such as flavanols, caftaric acid, malvin, and malvin acetate are predominant factors with respect to differentiation, a finding that stresses the importance of concentration. It can be supported that differences related with genetic (varietal), potential, and environmental influences are more profoundly associated with principal polyphenolic constituents, and in this context these substances may be considered as the most representative indices for a more reliable categorization. On the other hand, the influence of other phenolics, such as flavonols, should not be overlooked, especially in relation to region of origin, as their contribution might be of value for a more unambiguous differentiation.

As a conclusion, it must be emphasized that, in spite of the low number of samples, both categorizations yielded very distinct grouping of samples without overlapping, which clearly demonstrates the validity of the procedure for credible red wine differentiation. This outcome could be regarded as an additional

Table 7
Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions for cultivar-based differentiation

	Function				
	1	2	3	4	5
Malvin acetate	0.294 ^a	−0.010	0.083	−0.168	0.225
Caftaric acid	−0.242 ^a	0.194	−0.045	0.070	0.176
Cyanin	0.012	−0.234 ^a	−0.099	0.134	−0.039
Epicatechin	−0.001	0.115 ^a	−0.107	0.033	0.025
Delphinin	0.080	−0.093 ^a	0.040	0.053	0.093
Procyanidin B2	−0.082	0.148	−0.216 ^a	−0.153	0.021
Caffeic acid	−0.053	0.010	0.207 ^a	0.033	−0.080
Coumaric acid	0.129	0.094	0.096	0.361 ^a	0.194
Quercetin	0.092	−0.039	−0.012	0.222 ^a	−0.037
Myricetin	0.166	0.055	0.062	0.216 ^a	0.047
Kaempferol	0.046	−0.024	0.017	0.095	−0.362 ^a
Procyanidin B1	−0.184	0.028	−0.131	0.015	0.344 ^a
Catechin	−0.121	0.050	−0.122	−0.017	0.336 ^a
Malvin	0.121	−0.001	0.237	0.112	0.334 ^a
Coutaric acid	0.000	0.107	0.064	0.102	0.300 ^a
Paeonin	0.097	−0.053	0.053	0.106	0.245 ^a
Malvin coumarate	0.112	0.098	0.158	0.197	0.225 ^a
Petunin	0.133	−0.052	0.066	0.202	0.217 ^a
Rutin	0.018	−0.091	0.082	−0.125	0.152 ^a

Variables ordered by absolute size of correlation within function.

^a Largest absolute correlation between each variable and any discriminant function.

Table 8
Eigenvalues and percent variance explained for geographical origin-based differentiation

Function	Eigenvalue	Variance (%)	Cumulative (%)	Canonical correlation
1	5.340 ^a	50.8	50.8	0.918
2	2.758 ^a	26.3	77.1	0.857
3	1.591 ^a	15.2	92.3	0.784
4	0.812 ^a	7.7	100.0	0.669

^a First four canonical discriminant functions were used in the analysis.

Table 9
Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions for geographical origin-based differentiation

	Function			
	1	2	3	4
Procyanidin B2	−0.191 ^a	−0.003	−0.141	−0.023
Malvin acetate	0.182 ^a	−0.090	0.120	−0.079
Malvin	0.144 ^a	0.020	0.041	−0.085
Delphinin	0.112 ^a	−0.066	−0.036	−0.048
Rutin	0.012	0.369 ^a	0.001	−0.058
Procyanidin B1	−0.268	0.305 ^a	−0.269	0.104
Epicatechin	−0.087	−0.112 ^a	−0.082	−0.050
Paeonin	0.090	−0.102 ^a	−0.057	0.055
Petunin	0.084	−0.095 ^a	0.063	−0.026
Kaempferol	0.073	−0.194	0.357 ^a	−0.101
Catechin	−0.244	0.146	−0.348 ^a	0.118
Quercetin	0.022	−0.169	0.299 ^a	0.065
Caftaric acid	−0.265	0.059	−0.282 ^a	0.075
Myricetin	0.073	−0.074	0.278 ^a	−0.085
Coutaric acid	0.000	−0.116	−0.229 ^a	0.015
Malvin coumarate	0.060	−0.074	0.178 ^a	−0.078
Coumaric acid	0.040	−0.065	0.164 ^a	−0.103
Cyanin	0.142	0.076	−0.019	0.424 ^a
Caffeic acid	−0.029	0.214	0.111	−0.305 ^a

Variables ordered by absolute size of correlation within function.

^a Largest absolute correlation between each variable and any discriminant function.

criterion for studies pertaining to red wine quality control and authenticity, in addition to minor [15] and major components, including several polyphenolic classes [13,16] and/or other compositional parameters [17–19]. To the extent similar approaches are proven valid for a larger number of samples, the results may be directly applicable.

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Speciation analysis of plants in the determination of V(V) by ETAAS

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Abstract

Vanadium(IV) and vanadium(V) were easily separated from each other in the same plant sample and be determined independently by ETAAS (electrothermal atomic absorption spectrometry). This was achieved by treating the sample with 1 M $(\text{NH}_4)_2\text{HPO}_4$ which transfer only insoluble V(V) species into solution leaving V(IV) species in the solid part of the sample solution. V(IV) was then transferred into solution by ashing the precipitates and dissolving them in dilute acid. Statistical evaluations indicate that the sum of the concentrations of V(IV) and V(V) species is the same as the total concentration of vanadium determined by an independent method from the same plant sample at 95% level of confidence.

The maximum concentrations for V(V) and total vanadium in plants around the vanadium mine were found to be 24.3 and 350 $\mu\text{g g}^{-1}$, respectively.
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Keywords: Speciation; Plants; ETAAS; Leaching

1. Introduction

Vanadium speciation in environmental matrices is very important since its bio-availability, accumulation and toxicological properties depends on the chemical forms in which it occurs. The accurate determination of the chemical species of vanadium reveals the potential risk posed by it [1]. Though vanadium can exist in oxidation states from -1 to $+5$ in the environment, the two forms, vanadium(IV) and vanadium(V), are the only stable ones to co-exist in the natural environment. It is recognized that V(V) as vanadate is more toxic than V(IV) present as vanadyl ion [2,3]. Vanadium is an essential trace element, with specific physiological functions, however, numerous reports have warned about its carcinogenic and toxic effects from excessive exposure [4,5].

Vanadium is emitted into the environment from various sources like vanadium mine, vanadium refineries, iron and steel industries, and chemical industries [6,7]. The other sources of vanadium in soils are the parent rocks from which soils are formed where the highest concentrations are found in clays [8].

The concentration levels of vanadium in soils is approximately about 10 times higher than that in plants growing in it. Vanadium can accumulate in cells of both plants and animals, mostly bound to glutathione, catecholamines or other small peptides [9]. Vanadium(IV) and vanadium(V) in these cells are capable of reacting with phosphate groups and sugar alcohol groups of nucleotides to form complexes that inhibit or stimulate the activity of many DNA or RNA enzymes.

Several analytical techniques have been used to determine vanadium and its species at trace levels in various samples [7,10,11]. Electrothermal atomic absorption spectrometry (ETAAS) is mostly the technique of choice for trace element analysis due to its high sensitivity, low sample consumption, simplicity in operation, reduced matrix effects [12].

Most publications concerning the speciation of vanadium in environmental samples deal with liquid samples such as water [4,13–16], while selective determination of vanadium species in solids, till now, requires additional effort [8,17,18]. Finally, there is no official method of V(V) speciation in solid samples.

The aim of this work was to develop a method for the speciation of vanadium in plants so that the levels of this pollutant can easily be monitored in crops, grass, etc. during routine analysis. V(V) was successfully leached from ground plants samples by $(\text{NH}_4)_2\text{HPO}_4$. The developed method was applied in the

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determination of V(V) in plants taken from the vicinity of the vanadium mine.

2. Experimental

2.1. Instrumentation

A Perkin-Elmer Atomic Absorption Spectrophotometer model Analyst 600 with Zeeman background correction system was used for all measurements. The Analyst 600 spectrophotometer was equipped with AS-800 autosampler and the whole system has been controlled by means of AAWinlab control software running under Microsoft Windows program. All determinations were carried out at analytical line of V 318.4 nm, with a 0.7 nm band pass using hollow cathode lamp of vanadium (Perkin-Elmer), operating at 25 mA. The tubes of transversely heated graphite atomizer (THGA) with integrated L'vov's platforms (Perkin-Elmer, part N B050-4033) were used for atomization of vanadium.

2.2. Reagents and standard solutions

Standard stock solution containing 1000 mg l^{-1} V(V) as NH_4VO_3 (Merck) was used for the preparation, by appropriate dilution, of working standards. Ultra-pure water (resistivity $18.2 \text{ M}\Omega$) was obtained with Milli-Q water purification system (Millipore Corp., USA) and had been used for all dilutions and samples preparation. $(\text{NH}_4)_2\text{HPO}_4$ (Merck) was used to prepare reagent solutions for the leaching of V(V) from plants samples. A mixture of HNO_3 (Merk) and HCl (Merk) were used to dissolve the residue after ashing the sample. Argon (Afrox, South Africa) was used as a protective gas throughout.

3. Analytical procedure

3.1. Samples

Acacia plant leaves (*Acacia xathophloea*) and grass samples (*Chloris gayana* and *Digitaria eriantha*) were collected manually from soils in the vicinity of the vanadium mine in the North West Province of South Africa. Each sample consisted of materials from the same individual plant species, in accordance to sampling recommendations [19]. The samples were carefully washed with deionized water, air-dried and homogenized by grinding in a IKA A11 milling system to a grain size less than $200 \mu\text{m}$.

3.2. Leaching procedure for V(V) from plants

Approximately 0.25 g of the plant sample was weighed into a 100 ml glass beaker wherein 25.0 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ was added, and the mixture was boiled for 15 min. The contents of the beaker was cooled and transferred into a 50 ml plastic tube. The final volume of the sample was adjusted to 25 ml with deionized water. The sample solution was filtered through the $0.45 \mu\text{m}$ hydrophilic PVDF filter and transferred into 1 ml sampling cup

of the AAS autosampler to determine V(V) concentration. All other samples were prepared in the same manner.

3.3. The determination of V(IV) and total vanadium concentrations

For the determination of V(IV), a mixture of 25.0 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ and approximately 0.25 g ground plant sample were boiled for 15 min, allowed to cool and filtered through the Whatman No. 1 filter paper. The precipitates were washed several times with 1 M $(\text{NH}_4)_2\text{HPO}_4$ and after that with deionized water. They were then transferred on the filter paper to a platinum crucible and placed in a muffle furnace. The temperature of the furnace was slowly increased from room temperature to 650°C for over an hour. The samples were ashed until a white or gray ash residue was obtained. The resultant ash was allowed to cool, and 0.5 ml of a mixture of 1:1 concentrated HNO_3 and HCl was added to dissolve the product. The solution was transferred to a 25.0 ml volumetric flask and made to volume with deionized water.

The determination of total V was achieved by ashing approximately 0.25 g of plant samples as the precipitates above.

4. Results and discussion

4.1. Analytical results of V(IV) and V(V) determination by ETAAS

Vanadium was atomized at high temperature in a freshly coated pyrolytic graphite tube as recommended by Manning et al. [20] during its determination by ETAAS. A suitable temperature program that was used to atomize vanadium efficiently is summarized in Table 1.

The ETAAS determination of vanadium standards was achieved with the temperature program summarized above. The absorption signals of vanadium, obtained after the atomization of the same amount of V(IV) and V(V) from the corresponding standard solutions, do not differ in their appearance time nor their integrated absorbance (Fig. 1).

The calibration curve for both vanadium species is linear and is described by the following equations: $Y = 0.1060x + 0.0030$ for V(IV) and $Y = 0.1060x + 0.0020$ for V(V) determinations, where x is the analyte concentration ($\mu\text{g l}^{-1}$) and Y is the integrated absorbance, with correlation coefficients of $R^2 = 0.9982$ and 0.9940 ($n = 5$), respectively, for the concentration range of $0\text{--}2000 \mu\text{g l}^{-1}$. The results of these standards lead to the

Table 1
Thermal program for the determination of V using THGA-AAS

Step	Temperature ($^\circ\text{C}$)	Ramp (s)	Hold (s)	Internal gas flow (ml min^{-1})
1	120	1	10	250
2	250	5	10	250
3	1800	5	20	250
4	2600	0	5	0
5	2600	1	3	250

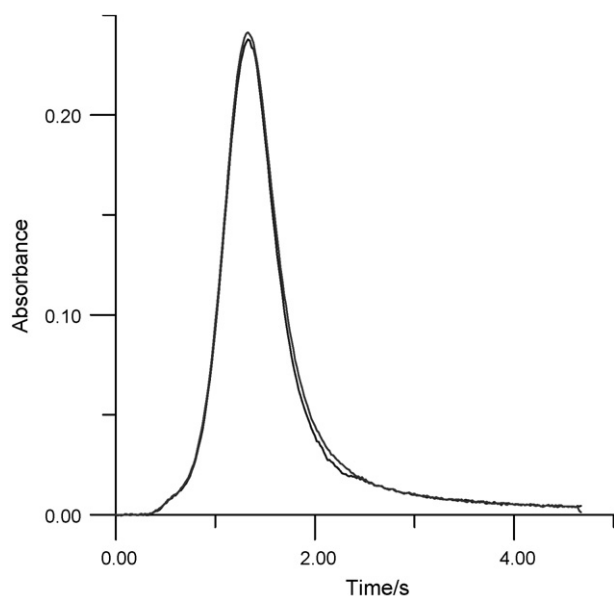


Fig. 1. Absorbance–time signals of V(IV) and V(V) from standard of the same solution.

conclusion that the calibration of vanadium can be performed using any vanadium standard irrespective of the oxidation state.

The limit of detection (LOD) for V(V) determination has been established using blank solutions of 1 M $(\text{NH}_4)_2\text{HPO}_4$. The LOD, calculated according to the equation: $\text{LOD} = X_{bl} + 3S_{bl}$ ($n = 25$), was found to be $0.2 \mu\text{g l}^{-1}$ for a $10 \mu\text{l}$ sample aliquot. With respect to the plant samples, assuming that 0.25 g of plant after digestion has been adjusted to 25.0 ml, the LOD for V(V) determination was estimated to be $0.02 \mu\text{g g}^{-1}$.

4.2. Optimization of the extraction of V(V) from plants

Several concentrations of $(\text{NH}_4)_2\text{HPO}_4$ were used to extract V(V) from ground plant samples to obtain the most appropriate concentration of the reagent solution that can transfer the total concentration of the analyte into solution. Precautions were made so as not to use extremely concentrated solution of $(\text{NH}_4)_2\text{HPO}_4$ to avoid introducing severe matrix into the graphite tube.

Fig. 2 shows a plot of $\log[\text{V(V)}]$ against $-\log[\text{PO}_4^{3-}]$ which indicate that 1 M $(\text{NH}_4)_2\text{HPO}_4$ was the least concentration of the phosphate reagent to extract the total concentration of V(V) in samples. The multiple extraction of V(V) by fresh portions of $(\text{NH}_4)_2\text{HPO}_4$ on the same solution conclude that the first and second extractions transfer all the species of interest into solution, and approximately 97% being achieved on the earlier extraction. The appropriate adjustment of the pH of 0.01 M $(\text{NH}_4)_2\text{HPO}_4$ to ~ 12 lead to the same extraction efficiency as that of 0.01 M Na_3PO_4 .

The efficiency of on the leaching of V(V) from plants was further optimized by boiling the mixture at different time intervals. The results of this investigation presented in Fig. 3, shows that 1 M $(\text{NH}_4)_2\text{HPO}_4$ leaches all V(V) species within 15 min.

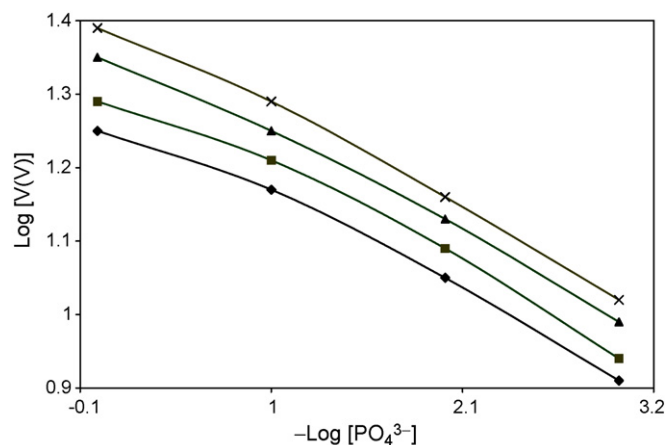


Fig. 2. Optimization of $[\text{PO}_4^{3-}]$ for V(V) extraction from plant samples: (▲) *Acacia xathophloea* 1, (×) *Acacia xathophloea* 2, (◆) *Chloris gayana* and (■) *Digitaria eriantha*.

4.3. Mechanism for the separation of V(IV) from V(V) in plant sample

Vanadium exists in a multitude of different oxidation states from -2 to $+5$ even though the forms naturally found in the environment are 3, 4, and 5. In nature, V(III) exists in very reducing environments or is complexed to organic ligands and it is immediately oxidized upon leaching to V(IV) [21]. Therefore, after the treatment of samples with $(\text{NH}_4)_2\text{HPO}_4$, all V(V) species goes into solution and the remaining vanadium species in the precipitates is only V(IV) such that the ashing of the precipitates lead to the determination of the total concentration of V(IV) in the plant sample.

4.4. Validation of the analytical methods

The validation of the method of extracting V(V) with 1 M $(\text{NH}_4)_2\text{HPO}_4$ using certified reference materials was impossible as such materials with certified values of vanadium species

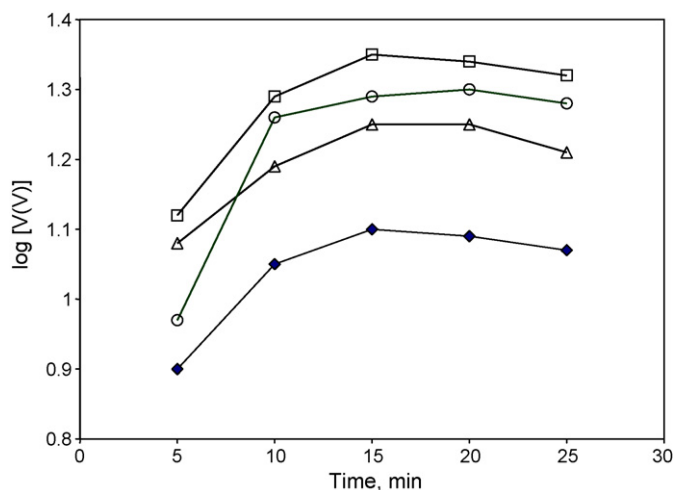


Fig. 3. Optimization of time for the extraction of V(V) from plant samples with 1 M $(\text{NH}_4)_2\text{HPO}_4$: (○) *Acacia xathophloea* 1, (□) *Acacia xathophloea* 2, (◆) *Chloris gayana* and (△) *Digitaria eriantha*.

Table 2
Results of V(V) in plants around the vanadium mine

Sample	[V(IV)] ^b ($\mu\text{g g}^{-1}$)	[V(V)] ^b ($\mu\text{g g}^{-1}$)	{Total [V]} ^b ($\mu\text{g g}^{-1}$)
<i>Acacia xathophloea</i> 1	270 $\pm$ 11	22.4 $\pm$ 0.5	300 $\pm$ 13
<i>Acacia xathophloea</i> 2	317 $\pm$ 14	24.3 $\pm$ 0.8	350 $\pm$ 10
<i>Chloris gayana</i>	170 $\pm$ 7	17.8 $\pm$ 0.3	190 $\pm$ 9
<i>Digitaria eriantha</i>	194 $\pm$ 10	19.3 $\pm$ 0.6	215 $\pm$ 8

^b Average of six samples at 95% level of confidence: $X \pm t(s/\sqrt{N})$.

are not yet available in the market. Therefore, the only available alternative was to compare the sum of [V(IV)] and [V] with total [V] obtained independently from the same plant sample. A comparison of the concentration of the sum of vanadium species should be equivalent to the total vanadium concentration [17]. The results shown in Table 2 and Fig. 4 confirm that it can be accepted at 95% level of confidence that 1 M (NH₄)₂HPO₄ leaches V(V) successfully from finely ground plant samples as statistically, there was no difference between the concentration of the vanadium species with that of the total vanadium concentration from an independent method.

4.5. Analytical results of V(V) in plant samples

The analytical results of V(V) in plants taken in the vicinity of the vanadium mine are summarized in Table 2. The results shows that the concentration of V(V) in plants around this area ranges between 17.8 and 24.3 $\mu\text{g g}^{-1}$ and that of total vanadium concentration are in the range 190–350 $\mu\text{g g}^{-1}$. The total vanadium concentrations exceed 2 ppm upper limit recommended by [22] for safe vanadium levels in 75% of the samples analyzed. These could have somehow affected both plants and animals

grazing in the area. The continuous consumption of plants with these levels of V(V) by animals may somehow result in the accumulation of this toxic species in their cells. This may results in symptoms of vanadium poisoning in the long-term.

Cattle that died after grazing in this area could be attributed to drinking water released after the collapse of the vanadium dam or the consumption of grass covered with dust as the upper limit of total vanadium concentration found in plants was not exceeded by a big factor.

5. Conclusion

It can be concluded that vanadium species, viz. V(IV) and V(V), can be determined successfully with ETAAS after their separation by treating the sample with 1 M (NH₄)₂HPO₄. The method of leaching V(V) with (NH₄)₂HPO₄ was validated by statistical evaluation which indicated that the sum of concentrations of vanadium species is the same as the total concentration of vanadium at 95% level of confidence. The plants samples collected around the vanadium mine were found to contain vanadium concentrations that exceed acceptable levels.

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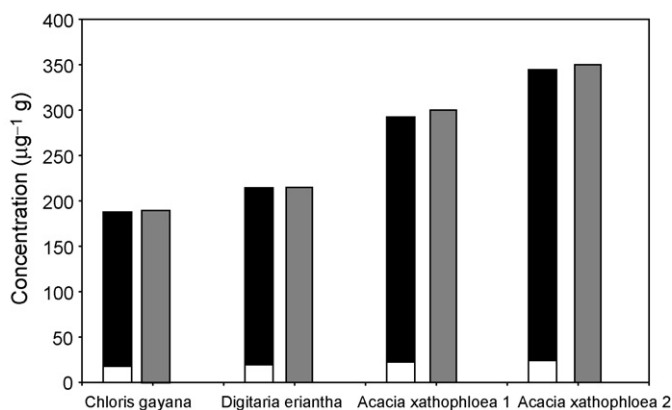


Fig. 4. Correlation of the sum of [IV] and [V] with the total concentration of vanadium determined by an independent method: (■) [IV], (□) [V(V)] and (▒) total vanadium concentration.

Determination of copper and nickel in vegetable oils by direct sampling graphite furnace atomic absorption spectrometry

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Abstract

The quality of food products has been receiving great attention due to its influence on human nutrition and health. In this sense, the determination of trace metals in foods has turned an important field on food analysis. Concerning vegetable oils, its metal trace composition is an important criterion for the assessment of their quality once it is known that trace metals affect their rate of oxidation, influencing freshness, keeping properties as well as storage. In the present work an analytical method which enables the direct determination of Cu and Ni in vegetable oils by graphite furnace atomic absorption spectrometry (GFAAS), using a “solid” sample strategy is presented: in nature, samples are directly weighed on the graphite platform boat and inserted in the graphite tube. An adequate temperature program permitted the calibration by external aqueous analytical curves. Good concordance between the proposed procedure and EPA procedures was found in the analysis of real samples. Limits of detection of 0.001 and 0.002 $\mu\text{g g}^{-1}$ were found for Cu and Ni, respectively, in the original samples, and they were comfortably below the concentrations found.

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Keywords: GFAAS direct determination; Vegetable oils; Copper; Nickel

1. Introduction

Trace metals present in oils and fats may be of natural origin or present due to processing procedures such as bleaching (Fe), hardening (Ni, Cu) as well as corrosion of the processing equipments (Fe, Ni) [1]. The concentration of trace metals in vegetable oils is an important criterion for the assessment of their quality in regard to freshness, keeping properties and storage [2]. Many reports have described the deleterious effects that trace metal have on the flavor and oxidative stability of oils. In particular copper and nickel have a catalytic effect on the mechanism of autoxidation. Furthermore, some of these metals are subject to food legislation [3–5]. However, the accurate determination of trace metals in vegetable oils is still an analytical challenge, owing to their low concentration level and the difficulties that arise due to the characteristics of the matrix. Several procedures have been employed for this determination, encompassing techniques such as molecular absorciometry

[6,7], neutron activation analysis [8] potentiometry [9,10], as well as voltammetry [11,12]. Concerning atomic spectrometric methods determinations have been performed by ICP–OES [5,13–15] and ICP–MS [2,16,17]. However, AAS is still the most cited technique [1,18–38]. Nevertheless, due to the high organic content of vegetable oils, sample pre-treatment is frequently necessary. It may involve acid extraction [9,19–22], solid phase extraction [23] as well as dry [24–26] or wet ashing [7,13,14], eventually assisted by microwave heating [27,28]. Alkaline solubilization was also proposed [29]. However, these procedures are time consuming and present the potential risks of sample contamination and analyte loss. Dilution with a suitable solvent [4,30–34] as well as emulsification [15–17,35] are also alternatives; however, they impoverish the overall limit of detection and the maintenance of emulsions stability for an acceptable period of time is frequently problematic. Work devoted to the comparison of some of these techniques may also be found [36–38].

In general, for most atomic spectrometric techniques the samples must be presented as low viscosity and density liquids. In contrast, GFAAS is well fitted even for solids. However, viscous samples pose special difficulties, since their direct dispensing is troublesome, impairing sample introduction with good volume

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repeatability, if sample dilution is to be avoided. Recent proficiency tests [39] have shown that Cu determination in edible oils is still somewhat difficult, with individual recoveries ranging from 10 to 250%, while for Ni these results range from 50 to 144%. In order to overcome such difficulties, the present work presents an alternative procedure for the direct GFAAS determination of Cu and Ni in vegetable oils making use of a “solid” sampling strategy.

2. Experimental

2.1. Instrumentation

The measurements were carried out with an Analytik Jena (Jena, Germany) Model Zeenit 60 atomic absorption spectrometer with Zeeman effect background correction and transversely heated graphite atomizer. A SSA6Z solid sampler device was used for the sample introduction. Hollow cathode lamps (Analytik Jena, cat. no. 450.014, Germany, for Cu and VWR Scientific, cat. no. 58137-452, USA, for Ni) were used as line sources. Integrated contact tubes (Analytik Jena, part no. 407-152.013) and IC-Sample Platforms (Analytik Jena, part no. 407-152.023) were also used. The operating parameters and the instrumental settings were adjusted according to the manufacturers' recommendations, unless otherwise specified. All measurements were based on integrated absorbance and the results are the average of at least three measurements. An AD-4 microbalance (Perkin-Elmer, part no. C655-001, 0.1 µg sensitive) was used for the sample weighing, while a DGT 100 Plus microwave oven (Gehaka, S. Paulo, Brazil) was used for the microwave-assisted acid digestion in the EPA comparative procedures.

2.2. Reagents, solutions and samples

All chemicals were of analytical reagent grade and Milli-Q water (Millipore, Bedford, MA, USA) was used throughout. Nitric acid was purified by sub-boiling distillation using a Teflon sub-boiling still (Kuerner Analysentechnik, Rosenheim, Germany). Argon (99.96%, Aga, Rio de Janeiro, Brazil) was used as purge and protective gas. Aqueous Ni and Cu analytical solutions were prepared by adequate dilution of 1000 µg ml⁻¹ solu-

tions prepared from Titrisol concentrates (Merck, Darmstadt, Germany) diluted with 0.2% (v/v) nitric acid. Hydrochloric, sulfuric and nitric acids, all of them concentrated (Vetec, Rio de Janeiro, Brazil) and potassium permanganate (Vetec, Rio de Janeiro, Brazil, cat. no. 275.07) were used for the microwave acid digestions. Corn oil and soybean oil samples of three different Brazilian trademarks were obtained from the local market. During the manipulations, the samples were stored in screw-capped 50 ml polypropylene tubes with conical bottom (Sarstedt, part no. 62.547.004, Nümbrecht, Germany).

2.3. Proposed procedure

Approximately 0.5 mg of sample was accurately weighed in the micro analytical balance directly onto the IC sample platform (boat). In sequence, the IC sample platform was held by the holder of the solid sampler device. The holder was then adjusted at the solid sampler device, pushed forward the graphite atomizer and cautiously deposited into the graphite tube. It is important to take into account the right positioning of the boat for repetitive results. The temperature program was then run. Aqueous (0.2%, v/v, HNO₃) analytical curves were used for the quantification of Ni and Cu. They ranged from 20 to 100 and 25 to 100 µg/l, for Ni and Cu, respectively, showing linear ($r^2 > 0.99$) behavior in these intervals. Since the autosampler has to be removed during the use of the solid sampling device, the calibration solutions were manually dispensed onto the IC platform, which were then introduced into the furnace as previously described.

2.4. Comparative procedures

Comparative procedures, namely EPA 3031 [40] and EPA 3051 [41] were used. In short, one of them (EPA 3031) consisted in an open-vessel digestion with an appropriate acids and oxidant sequence (sulfuric acid, nitric acid, hydrochloric acid and potassium permanganate), followed by a “close to dryness” evaporation. The mineralized samples were then made up to 30 ml with 0.2% (v/v) HNO₃. This method was originally developed for determinations at the µg ml⁻¹ concentration level, using FAAS or ICP–AES as the final quantification technique. However, due to the final concentrations attained these tech-

Table 1
Temperature programs for the determination of Ni and Cu in vegetable oils

Step	Ni			Cu		
	Temperature (°C)	Ramp (°C/s)	Hold (s)	Temperature (°C)	Ramp (°C/s)	Hold (s)
1	90	10	5	90	10	5
2	120	10	5	150	10	5
3	350	4	10	600	40	10
4	800	40	15	1000 ^a	40	20
5	1200 ^a	40	20	1000 ^a	0	6
6	1200 ^a	0	6	2000 ^b	1800	5
7	2400/2300 ^{b,c}	2200	6	2650	1000	5
8	2600	2000	7			

^a Pyrolysis temperature.

^b Atomization temperature.

^c For corn and soybean oils, respectively.

Table 2

Proposed and comparative (EPA 3031 and EPA 3051) procedures results ($\mu\text{g g}^{-1}$) in the determination of Ni and Cu in soybean oil (S) and corn oil (C) samples ($\pm\text{S.D.}$; $n=6$)

Samples	Ni			Cu		
	Proposed	EPA 3031	EPA 3051	Proposed	EPA 3031	EPA 3051
S ₁	3.17 $\pm$ 0.02	3.53 $\pm$ 0.02	3.37 $\pm$ 0.04	2.82 $\pm$ 0.01	3.10 $\pm$ 0.02	3.08 $\pm$ 0.05
S ₂	3.26 $\pm$ 0.02	3.60 $\pm$ 0.03	3.35 $\pm$ 0.02	3.63 $\pm$ 0.02	3.87 $\pm$ 0.02	3.75 $\pm$ 0.03
S ₃	4.42 $\pm$ 0.02	4.37 $\pm$ 0.02	4.48 $\pm$ 0.04	3.02 $\pm$ 0.02	3.06 $\pm$ 0.04	3.11 $\pm$ 0.06
C ₁	3.92 $\pm$ 0.02	4.32 $\pm$ 0.04	4.30 $\pm$ 0.03	2.96 $\pm$ 0.02	3.06 $\pm$ 0.03	2.95 $\pm$ 0.03
C ₂	4.53 $\pm$ 0.01	4.64 $\pm$ 0.02	4.18 $\pm$ 0.03	3.63 $\pm$ 0.03	3.73 $\pm$ 0.03	3.65 $\pm$ 0.04
C ₃	4.12 $\pm$ 0.01	4.34 $\pm$ 0.02	4.26 $\pm$ 0.06	2.63 $\pm$ 0.02	2.73 $\pm$ 0.02	2.66 $\pm$ 0.03

niques were not sensitive enough. Thus, in the present work, the original sample pre-treatment was kept but the final determination was performed by GFAAS, using aqueous (0.2%, v/v, HNO₃) calibration. The EPA 3051 method consists in a nitric acid digestion assisted by microwave heating. The sample and acid are placed into a fluorocarbon (PFA or TFM) vessel, which is capped according to the manufacturer recommendation and heated in the microwave oven unit. After cooling, the vessel content is filtered, centrifuged and then diluted to volume for the quantification. Alike the EPA 3031 method, the original sample pre-treatment was kept but the final determination was performed by GFAAS using aqueous (0.2%, v/v, HNO₃) calibration. In both cases, analyte addition tests have confirmed the adequacy of aqueous calibration.

3. Results and discussion

3.1. Pyrolysis and atomization temperatures optimization

The temperature programs for the present work were based on those of a previous work [1]. However, the pyrolysis and atomization temperatures were further optimized by pyrolysis and atomization temperature curves. The curves were performed with the samples introduced as described in Section 2.3. Since along these experiments it was not possible to weigh exactly the same sample mass for each measurement, each result of the pyrolysis and atomization temperatures curves was normalized for its respective mass. Table 1 summarizes the optimized temperature programs. The same temperature programs were used for calibration.

3.2. Sample analysis, validation and limits of detection

The samples were analyzed as already described in Section 2.3. Since in the present study no adequate certified reference material for the investigated samples is available, accuracy was assessed by the analysis of the same samples by two comparative independent procedures, namely EPA 3031 and EPA 3051. Table 2 shows the results obtained with the three procedures, and good concordance was obtained between the proposed and reference methods. ANOVA analysis has shown no statistically significant difference between the proposed and the EPA 3051 procedure. However, although showing very close results, the same cannot be stated for the EPA 3031 method. Considering

that this method involves an open-vessel mineralization procedure, contamination may respond for this small difference. Moreover, since the original EPA 3031 procedure indicates flame AAS for detection, it was certainly supposed for concentrations larger than those found here.

Since for the direct sampling, there is no reagent or field blanks, the limits of detection were calculated according to Burdge [42] that take into consideration the dispersion of the calibration points in relation to the analytical curve. In this way, if a sample mass of 0.5 mg is considered the limits of detection are 0.001 and 0.002 $\mu\text{g g}^{-1}$, for Cu and Ni, respectively, in the original samples.

4. Conclusion

The direct determination of Ni and Cu in vegetable oils by GFAAS using the solid sampling strategy has proved to be possible and reliable. No sample dilution was necessary and the limits of detection obtained compare favorably with those of other procedures in the literature. Calibration with aqueous analytical solution was possible, making the analysis even simpler. Since no sample digestion or extraction was necessary, risks of contamination and losses were minimized and time saved. Similar to the liquid samples GFAAS analysis, the sample throughput (30 h⁻¹) was defined by the length of the temperature program if the microbalance is posed in an ergonomic position in relation to the equipment and operator. Also, the background attenuation for both elements was comfortably in the range of the background correction system. Although six replicate determinations were used in the validation experiment, triplicates would be enough for routine analysis, since coefficient of variation better than 5% were observed even in this case. It also means that the number of weighing operations is not larger than any traditional procedure, usually a disadvantage of direct solid sampling analysis. The Cu and Ni concentrations found in the investigated samples were at the low $\mu\text{g g}^{-1}$ range, somewhat high in comparison to other results reported in the literature for similar samples, deserving a specific investigation.

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Free aluminium extraction from various reference materials and acid soils with relation to plant availability

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Abstract

The single extractions with 15 extractants (agents) (H_2O , KCl , NH_4Cl , NH_4F , CaCl_2 , BaCl_2 , CuCl_2 , LaCl_3 , $\text{Na}_2\text{S}_2\text{O}_4$, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, $\text{Na}_4\text{P}_2\text{O}_7$, NTA, EDTA, DTPA, HCl), the optimised BCR (Community Bureau of Reference) three-step sequential extraction procedure (SEP) and the solid phase extraction (SPE) by the chelating ion-exchanger Iontosorb Salicyl (cellulose resin containing covalently bound salicylic acid functional groups) were used for the partitioning of Al in very acid soil samples taken from an area influenced by acid mine solutions. The precision, accuracy and repeatability for all steps of the optimised BCR SEP were checked on the various reference materials (CRM 483 sewage sludge amended soil, CRM BCR 701 freshwater sediment, SRM 2710 and SRM 2711 Montana soils). Also the new indicative values of the optimised BCR SEP fractional Al concentrations were obtained for these reference materials. The aluminium amounts obtained by the used extraction procedures were valued and discussed from the aspect of the Al concentration in the plants (grass) growing on the same studied soils. The aluminium toxicity indexes (ATI) calculated for the studied soils, the BaCl_2 and acetic acid soil extracts and the grass stems and roots were used for the assessment of the Al toxicity to the plants. The ATI value was defined as the ratio of the nutrient cations (Ca, Mg, K, Na) concentration sum to the Al concentration. The flame atomic absorption spectrometry ($\text{LOQ} = 0.2 \text{ mg l}^{-1}$) and the inductively coupled plasma optical emission spectrometry ($\text{LOQ} = 0.03 \text{ mg l}^{-1}$) were used for the aluminium quantification.

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1. Introduction

Although aluminium is the most abundant metal in the Earth's crust, it is nonessential for plants. Because of its low solubility in a solution under neutral conditions, Al was regarded as a nontoxic element and its environmental and biological effects were not investigated until recently [1–6]. During the last two decades it has been found that the acid deposition leads to the considerable increases of the dissolved Al concentrations in the acidified soils and surface waters [7–18]. The continuing acidification of soils with the low buffering capacity leads to an increase of the aluminium mobilisation in the environment and may be potentially hazardous to the all terrestrial and aquatic systems. Aluminium is a strongly hydrolyzing metal and it is relatively insoluble in the neutral pH range (from 6.0 to 8.0). Under the acidic ($\text{pH} < 6.0$) or alkaline ($\text{pH} > 8.0$) conditions,

and/or in the presence of the complexing ligands, the solubility of Al is enhanced, making it more available for the biogeochemical transformations.

The toxic effect of certain Al species to plants is a phenomenon that has been reported by a number of the studies [1,2,4,6,7,9–18]. The bioavailability and toxicity of aluminium is dependent on its distribution among the various forms or species coexisting in the environment. So the form of soil Al is a key factor of its potential risk impact to living organisms. It can be stated that the certain soil Al species represent a limiting factor for the plant growth under the acidic conditions [19].

Aluminium occurs in the various chemical species, of which the free Al^{3+} , AlOH^{2+} , and $\text{Al}(\text{OH})_2^+$ species were found to be the most crucial to evaluate its toxicity and to predict the impact of the proton inputs in the soils and surface waters. The toxicity of AlSO_4^+ is not always accepted and even though polymer Al_{13} (not in a form of phosphates or silicates) seems to be the most toxic Al species, its actual risk in the soil environment is probably smaller [19]. The concentration of such Al species may be difficult to determine directly [20]. For that reason these

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species are determined in one or more fractions. Generally, these fractions are defined operationally or functionally, respectively, and they are termed according to the used separation method, e.g., the reactive species fraction, or the function of the isolated analyte, e.g., bioavailable species fraction [21–27].

In the present paper the single, sequential and solid phase extraction procedures were used for the partitioning of Al in the acid soils. The aim of our work was to investigate the aluminium interactions in the soil–plant system subjected to the strong acidification. Also the calculation of the aluminium toxicity indexes (ATI) was used for the assessment of Al toxicity to the plants (grass).

2. Experimental

2.1. Description of the sampling area

The serious negative environmental effects connected with the open quartzite mine were observed in the Šobov area, the central Slovakia [28–39]. The pyrite oxidation in the mine dump and the subsequent acid mine drainage (AMD) attack on other mineral phases are the main negative environmental factors [30,33,34]. The created sulphuric acid attacks other minerals and causes the high migration rate of the different toxic elements including aluminium originally fixed in the mineral lattices. The dissolved clay minerals (the most abundant are aluminosilicates, illite and pyrophyllite) release the high amount of aluminium by the neutralisation reaction with the H^+ ions resulted from acid sulphate weathering [28,29,36]. The pollution of the surface and ground water as well as the degradation of the soil and vegetation are distributed in the region mainly by the extremely acid water [35,38,39]. The secondary minerals identified by the X-ray diffraction analyse, e.g., jarosite $KFe_3(OH)_6(SO_4)_2$ and copiapite $Fe^{2+}Fe_4^{3+}(OH)_2(SO_4)_6 \cdot 20 H_2O$ [30–32,37], supported the presence of the oxidation reactions in the studied area (they are formed due to the neutralisation of sulphates and the presence of iron).

2.2. Samples collection and treatment

Ten soil samples (No. 1–10; pH 1.8–5.0; EC 40–1720 $\mu S cm^{-1}$; TOC 0.1–7.9%) were collected with a

different distance from the quartzite mine area at a depth of 0–20 cm; see the map of the sampling area [34]. The samples were air-dried and sieved through a 2 mm sieve and milled in an agate pot to the fine powder which was used for the chemical analysis. The pH measurement was done by the standard potentiometric method. The pH values of the samples were determined in a suspension after 2 h mixing of the sample with redistilled water (v/w ratio = 2.5/1). The electrical conductivity (EC) measurement was done by the standard conductometric method. The EC values of the samples were determined in a suspension after 2 h mixing of the sample with redistilled water (v/w ratio = 5/1). Total organic carbon (TOC) in the samples was determined by wet combustion of organic matter with a mixture of $K_2Cr_2O_7$ and H_2SO_4 . The nonconsumed dichromate was back titrated with $FeSO_4$.

Five plant materials (grass *Festuca rubra*) growing on the studied soils (No. A–E; pH 2.8–5.0) from the same locality were sampled at the same time. The grass stems and roots were rinsed with redistilled water and cut on the small pieces after an air drying.

2.3. Reference materials

The description of six used soil, sediment and plant reference materials (RM) [40–45] is listed in Table 1. The data of the total Al concentration are certified for four soil and plant reference materials (SRM 2710, SRM 2711, CRM GBW 07603, CRM GBW 07604). The indicative (not certified) values of the fractional and total Al concentrations obtained after the optimised BCR three-step SEP application are available for the soil reference materials SRM 2710, SRM 2711, CRM 483 and CRM BCR 701 in the literature [46,47] only.

2.4. Instrumentation and reagents

The flame AAS Perkin-Elmer Model 5000 (USA) spectrometer (conditions for Al: a wavelength 309.3 nm, a nitrous oxide-acetylene flame, LOQ = 0.2 $mg l^{-1}$) and the ICP-OES Kontron Plasmakon Model S 35 (Germany) sequential spectrometer (conditions for Al: a wavelength 396.152 nm, LOQ = 0.03 $mg l^{-1}$ for Al) were used for the Al, Na, K, Ca, Mg and P quantification. All employed reagents were of the ana-

Table 1
Standard (SRM) and certified (CRM) reference materials used in this study

Reference material	Description	Reference
SRM 2710 (NIST)	Highly contaminated soil collected from the top 10 cm of pasture land along Silver Bow Creek in the Butte, Montana area	[40]
SRM 2711 (NIST)	Moderately contaminated agricultural soil collected in the till layer of a wheat field, Montana area	[41]
CRM 483 (BCR)	Sewage sludge amended soil collected from Great Billings Sewage farm, Northampton	[42]
CRM BCR-701 (BCR)	Freshwater sediment collected from lake Orta, Piemonte	[43]
CRM GBW 07603/GSV-2/NCS DC 73349 (CNACIS)	Bush branches and leaves collected from an arid area, Qinghai province	[44]
CRM GBW 07604/GSV-3/NCS DC 73350 (CNACIS)	Leaves of poplar collected from Beijing	[45]

NIST, National Institute of Standards and Technology. BCR, formerly Community Bureau of Reference, now Institute for Reference Materials and Measurements or Standards, Measurements and Testing Programme. CNACIS, China National Analysis Center for Iron and Steel.

Table 2

The experimental conditions for the single extraction procedures

Extracting agent	H ₂ O	KCl	NH ₄ Cl	NH ₄ F	CaCl ₂	BaCl ₂	CuCl ₂	LaCl ₃	Na ₂ S ₂ O ₄	(NH ₄) ₂ C ₂ O ₄	Na ₄ P ₂ O ₇	NTA	EDTA	DTPA	HCl
Concentration (mol l ⁻¹)		1	1	0.5	0.01	0.1	0.5	0.3	0.2	0.2	0.1	0.005	0.005	0.005	0.5
Ratio volume/weight	5/1	10/1	10/1	10/1	10/1	10/1	10/1	10/1	10/1	20/1	10/1	10/1	10/1	10/1	20/1
Extraction time (h)	24	1	1	1	2	1	1	1	1	1	1	1	1	1	1

lytical grade (HNO₃ and HCl were purified by the sub-boiling distillation in a quartz apparatus). The Al, Na, K, Ca, Mg and P standard stock solutions (Merck) were diluted with a redistilled water to a required concentration with the HNO₃ addition (5 ml conc. HNO₃ per 100 ml solution).

2.5. Determination of the total concentrations

The total concentrations of all elements (Al, Na, K, Ca, Mg) in the reference materials and the real samples were determined after the decomposition with the HF, HNO₃, HClO₄ and H₂O₂ hot mixture in an open system (for soils and soil and sediment RMs) and the HF and HNO₃ hot mixture in an autoclave at 160 °C (for plants and plant RMs) [48]. The reference materials listed in Table 1 were used for the validation of the determination of the total concentrations of all elements.

2.6. Single extraction procedures

Fifteen extracting agents were applied to 10 soil samples according to the conditions listed in Table 2. The redistilled H₂O extracts were filtered by the polysulphone filtration apparatus with the cellulose nitrate membrane filter of the nominal pore size 0.4 µm.

2.7. Sequential extraction procedure (SEP)

The optimised BCR (Community Bureau of Reference) three-step SEP (Table 3) was applied to four soil and sediment reference materials and 10 soil samples.

2.8. Solid phase extraction (SPE)

The operationally defined reactive Al fraction (Al_r) represents the reactive aqua, hydroxy and probably sulphato Al

Table 3

The comparison between the optimised BCR three-step sequential extraction and some single extraction procedures

Step	Fraction	Chemical reagents and analytical conditions
1	Acid extractable	0.11 mol l ⁻¹ CH ₃ COOH
2	Reducible	0.5 mol l ⁻¹ NH ₂ OH·HCl (in 0.05 mol l ⁻¹ HNO ₃)
3	Oxidisable	H ₂ O ₂ followed by 1.0 mol l ⁻¹ CH ₃ COONH ₄ adjusted to pH 2.0 by HNO ₃
4	Residual	HF, HNO ₃ , HClO ₄ , H ₂ O ₂ hot mixture in open system

cationic monomeric forms which are the most responsible for the toxicity effects of aluminium.

The fraction of Al_r in the filtered H₂O soil extracts was determined after the solid phase extraction by the chelating ion-exchanger Iontosorb Salicyl (the cellulose resin containing covalently bound salicylic acid functional groups; fy Iontosorb, Czech Republic [49]) in the polyethylene bottles. The theoretical capacity of this resin is 0.5 mmol of active groups per 1 g of dry resin, the water content is 84% and the maximal sorption capacity for Al is 1.255 ± 0.049 mg g⁻¹ [50].

The portion of the filtrate (25 ml) was stirred with a magnetic stirrer at a room temperature in the presence of the damp resin (10 g). At appropriate interval of 1 min the stirring was stopped momentarily and after the flash centrifugation an aliquot of the supernatant was removed and analysed for Al. The optimal extraction/sorption time 1 min was found after the experimental equilibrium study of the Al complexation by resin in the synthetic solutions containing sulphate, fluoride, citric acid, oxalic acid, tartaric acid, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA) and fulvic acid at the various pH values (2.5–6.0) [33,39]. The effect of the other usual major and minor components (K, Fe, Ca, Mg, Mn, Zn, Cu) on the Al extraction/sorption by Iontosorb Salicyl (IS) was investigated in our previous works [33,39]. The concentration of Al_r was calculated as the difference between the initial (before the sorption) and final (after the sorption) concentration of Al in the solution. The latter portion of Al is considered for the nonreactive (Al_{nr}) species.

3. Results and discussion

3.1. Testing of the used sequential extraction procedure (SEP)

The specific fraction precision of the optimised BCR three-step sequential extraction procedure (SEP) was tested on four reference materials, see Table 4. The standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated from four replicates. The precision with the most R.S.D. values less than 5% except a few cases (R.S.D. from 8 to 10%) was very satisfactory.

The specific fraction repeatability of the used SEP was tested on SRM 2710. The optimised BCR three-step SEP was performed five times within 2 years (from august 2003 to august 2005). The repeatability of the results is acceptable (R.S.D. of the grand mean was less than 4% for the steps 1 and 4, overall and total concentration). The greater R.S.D. values (<10%) were found for the steps 2 and 3 which can be connected with the

Table 4

Optimised BCR three-step SEP fraction specific Al concentrations for testing of method precision, accuracy and repeatability on various reference materials (RM)

RM (MM/YY) parameter	Step 1 (mg kg ⁻¹)	Step 2 (mg kg ⁻¹)	Step 3 (mg kg ⁻¹)	Step 4/residual (mg kg ⁻¹)	Overall (mg kg ⁻¹)	Total (mg kg ⁻¹)	Recovery ^a (%)
SRM 2710 (August 2003) ^b	154 ± 1 (0.6)	1806 ± 15 (0.8)	1413 ± 39 (0.3)	59833 ± 615 (1.0)	63206 ± 616 (1.0)	61130 ± 290 (0.5)	103/98/351
SRM 2710 (April 2004) ^b	168 ± 17 (10)	1571 ± 42 (2.7)	1826 ± 114 (6.2)	59350 ± 1250 (2.1)	62915 ± 1256 (2.0)	61130 ± 290 (0.5)	103/98/350
SRM 2710 (May 2004) ^b	158 ± 2 (1.3)	1468 ± 28 (1.9)	1650 ± 75 (4.5)	59000 ± 1000 (1.7)	62276 ± 1003 (1.6)	61130 ± 290 (0.5)	102/97/346
SRM 2710 (June 2004) ^b	159 ± 2 (1.3)	1868 ± 32 (1.7)	1715 ± 35 (2.0)	59080 ± 800 (1.4)	62822 ± 801 (1.3)	61130 ± 290 (0.5)	103/98/349
SRM 2710 (August 2005) ^b	156 ± 2 (1.3)	1788 ± 8 (0.4)	1630 ± 62 (3.8)	59112 ± 860 (1.5)	62686 ± 862 (1.4)	61130 ± 290 (0.5)	103/97/348
Grand mean	159 ± 5 (3.1)	1700 ± 172 (10)	1647 ± 151 (9.2)	59275 ± 338 (0.6)	62781 ± 341 (0.5)	61130 ± 290 (0.5)	103/98/349
Indicative value ^c	171 ± 9 (5.3)	1660 ± 57 (3.4)	1040 ± 61 (5.9)	22400 ± 1970 (8.8)	25271 ± 1972 (7.8)	64400 ± 800/18000 ^d	NA/39/140
Difference (%)	−7	+2	+58	+165	+148	−5/+240	NA/+151/+149
SRM 2711 (August 2004) ^b	94.9 ± 2.2 (2.3)	2011 ± 11 (0.5)	1234 ± 73 (5.9)	61500 ± 426 (0.7)	64840 ± 432 (0.7)	65270 ± 250 (0.4)	99 / 99 / 360
Indicative value ^c	124 ± 4 (3.2)	1540 ± 25 (1.6)	845 ± 23 (2.7)	19900 ± 300 (1.5)	22409 ± 302 (1.4)	65300 ± 900/18000 ^d	NA/34/124
Difference (%)	−23	+31	+46	+209	+189	0/+263	NA/+191/+190
CRM BCR 483 (August 2004) ^b	50.1 ± 2.6 (5.2)	2247 ± 50 (2.2)	1179 ± 93 (7.9)	30542 ± 219 (0.7)	34018 ± 243 (0.7)	30970 ± 770 (2.5)	110/NA/NA
Indicative value ^c	50 ± 2 (4.0)	2420 ± 52 (2.1)	952 ± 74 (7.7)	12000 ± 220 (1.8)	15422 ± 238 (1.5)	NA	NA/NA/NA
Difference (%)	0	−7	+24	+155	+121	NA	NA/NA/NA
CRM BCR 701 (August 2004) ^b	182 ± 2 (1.1)	3526 ± 46 (1.3)	1319 ± 57 (4.3)	68958 ± 1054 (1.5)	73985 ± 1057 (1.4)	71470 ± 150 (0.2)	104/NA/NA
Indicative value ^e	NA	NA	NA	NA	NA	75700 ± 500	NA/NA/NA
Difference (%)	NA	NA	NA	NA	NA	NA	NA/NA/NA

NA, not available.

^a Recoveries data of overall from total determined values/total certified values/pseudototal certified values concentration of Al.^b Data measured by us as average values ± S.D. (R.S.D.).^c Indicative value from Guevara-Riba et al. [47].^d Certified value for total concentration of Al/pseudototal leachable concentration of Al by EPA Method 3050B (median value).^e Indicative value from Sutherland and Tack [46].

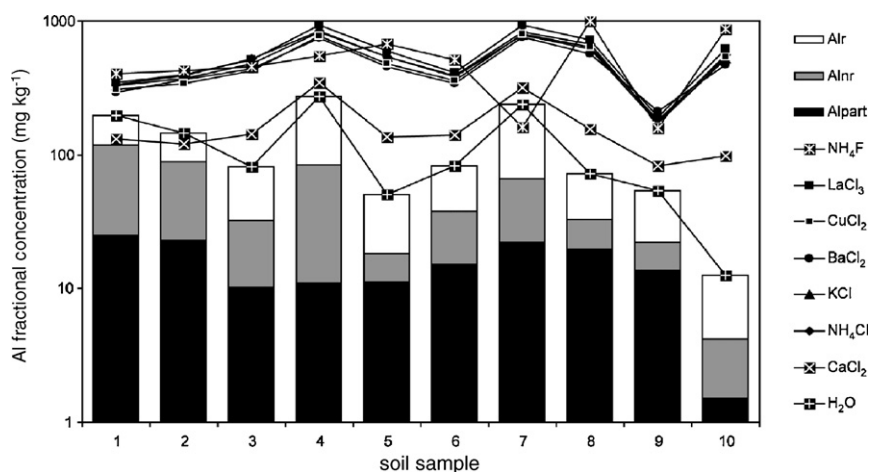


Fig. 1. The distribution of Al in the H₂O soil extracts and comparison with some other single extractions (Alr, reactive Al; Alnr, nonreactive Al; Alpart, particulate Al).

increasing number of the sample manipulations and the possible analyte readsorption and redistribution.

The specific fraction accuracy of the optimised BCR three-step SEP was checked on SRM 2710, SRM 2711 and CRM 483, see Table 4. The accuracy of the results is very good for the first two steps and two reference materials (SRM 2710 and CRM 483). The greater differences between our results and the indicative literature values [46] (from 23% to 58%) for the first three-steps can be explained by the varied extent of the analyte readsorption and redistribution, respectively. The positive deviations from 155% to 209% for the step 4 and 121% to 189% for the overall concentrations are certainly caused by the different used procedures of Al determination in the solid residue. For the total Al determination we treated the solid sample with hydrofluoric acid which attacks the alumino-silicate lattice. On the other hand Sutherland and Tack [46] determined so called the pseudototal Al (not total) concentration by the aqua regia (3HCl + HNO₃ reflux) extraction protocol according to the ISO Norm 11466.

The accuracy of the used SEP was also evaluated by the comparison of the total Al concentrations with the sum of four Al individual fractions (the overall Al concentrations). The recovery values of the overall Al concentrations calculated from the our total and from certified Al concentration are in the all cases close to 100%, see Table 4. From the calculated recoveries for SRMs 2710 and 2711 (346–360%) using the certified values of Al concentrations obtained after the pseudototal decomposition by EPA Method 3050 (the treatment with HNO₃ and H₂O₂ reflux) it can be concluded that nonadequate decomposition method was used. As aluminium is strongly bound in the alumino-silicate lattice, neither the aqua regia extraction protocol according to the ISO Norm 11466 nor the EPA Method 3050 are not suitable for the determination of the residual or total Al concentrations in the samples because of the used reagents (HCl, HNO₃ and H₂O₂) do not destroy the alumino-silicate lattices.

3.2. Partitioning of aluminium in soil samples

The procedures described in the section Experimental were used in order to evaluate the aluminium soil distribution

and mobility in the monitoring mine area with the sulphidic deposits.

The determination of the reactive aluminium fraction (Alr) in the filtered H₂O soil extracts after the solid phase extraction (SPE) by the chelating ion-exchanger Iontosorb Salicyl was performed in order to assess the most labile Al fraction. The aqueous extracts [51–55] simulate Al release to the soil solution under normal conditions; it can thus exhibit the actual Al toxicity. The prevailing Al forms (more than 70%) in the aqueous soil extracts were Al(L)⁺, e.g., Al(OH)₂⁺, AlSO₄⁺, AlF₂⁺, AlOrgL⁺ [19]. The distribution of soluble Al forms in ten nonfiltered H₂O soil extracts (0.02–0.36% of total soil Al) is obvious from Fig. 1. The particulate Al (Alpart) ranges from 4 to 27% of total soil soluble Al and from 0.002% to 0.04% of total soil Al, respectively. It is obvious that the particulate Al, retained on the membrane filter of the nominal pore size 0.4 μm, relates probably to the high molecular inorganic polymerised or organic Al compounds in the samples. The fraction of nonreactive Al (Alnr) was measured in the interval 14–48% of total soil soluble Al and 0.004–0.086% of total soil Al, respectively. The concentration of the reactive Al in the samples ranges from 39% to 72% of total soil soluble Al and from 0.01% to 0.26% of total soil Al, respectively. Thus, the Al concentrations in the soluble soil fractions decrease in the order Alr > Alnr > Alpart.

The simple salt extractants (KCl, NH₄Cl, NH₄F, CaCl₂, BaCl₂, CuCl₂, LaCl₃) release the soluble and exchangeable forms of Al [19,53,56–64] in the studied soils. The efficiencies of the KCl, NH₄Cl, BaCl₂, CuCl₂ and LaCl₃ extractants with the concentrations from 0.1 to 1 mol l⁻¹ are very similar (0.22–1.4% of total soil Al) for the all samples. The Al amounts extracted by the more dilute 0.01 mol l⁻¹ solution of CaCl₂ (0.1–0.5% of total soil Al) well correlate with Alr ($R = 0.8691$). These results confirm that these single extraction procedures with the low agent concentrations, can be used as an effective and rapid tool for the assessment of risk of Al toxicity. This conclusion corresponds well with our previous results [35,37,38] and with the fact that KCl, CaCl₂, NH₄Cl, BaCl₂ are often utilised in the functionally defined fractionation for the bioavailable species determination [51]. Fluorides are used as the extracting agents in the aluminium partitioning because of their complexing power.

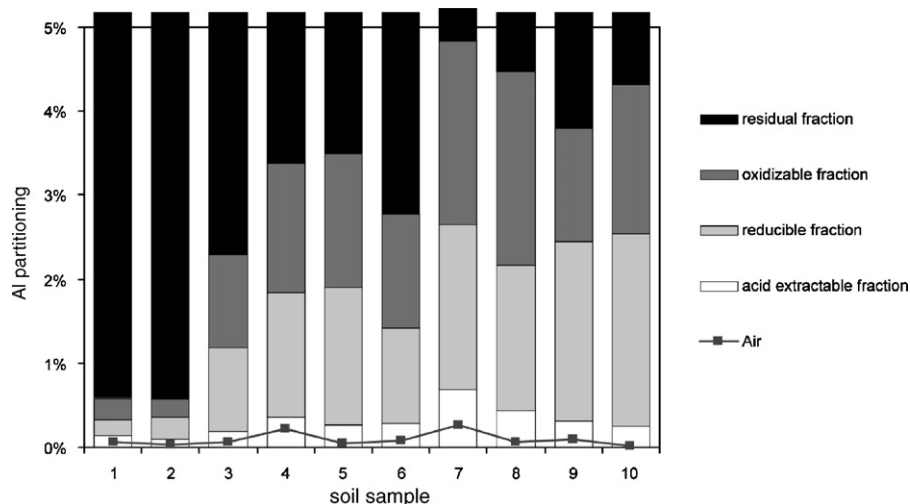


Fig. 2. The partitioning of Al in soil samples after the application of the optimised BCR three-step sequential extraction procedure and the solid phase extraction by the chelating ion-exchanger Iontosorb Salicyl (Alr, reactive Al).

They are suitable to release polymerised Al compounds [51]. The NH_4F does not dissolve sulphides and should not mobilise the trace metals fixed in the organic matter [51]. The amounts of Al leached by NH_4F range from 0.27% to 1.5% of total soil Al.

The results of the Al partitioning by the optimised BCR three-step sequential extraction procedure (SEP) applied to 10 soil samples are presented in Fig. 2. The Al concentrations in the obtained soil fractions decrease in the order residual > oxidisable = reducible > acid extractable. It is evident that the vast majority of Al (95.2–99.4% of total soil Al in the residual fraction) is bound in the aluminosilicate lattice in all samples. This reflects the fact that Al is present in the samples mainly in the form of the primary and secondary silicate minerals. The second most abundant fraction is Al bound to the oxidisable phase (0.2–2.2% of total soil Al) and Al bound to the reducible phase (0.2–2.3% of total soil Al). In the first case hydrogen peroxide destroys the oxidisable matter and acidic

ammonium acetate with the complexing ability leaches released Al. It is evident that the obtained results reflect the mineral and chemical composition of the samples, e.g., the presence of the organic matter or sulphides. The reducible fraction of Al was extracted by hydroxylammonium chloride in nitric acid. This fraction represents Al in the oxide form, in particular as hydroxides or oxyhydroxides. The least amount of Al (0.1–0.7% of total soil Al) occurs in the dilute acetic acid extractable fraction which includes the water and acid soluble, exchangeable or adsorbed Al species. The dilute acetic acid also dissolves the sample carbonate phase. The acetic acid is often used also in the single extraction procedures because of its ability to release the available fraction of the trace metals [51].

It is obvious that the dilute acetic acid extractable fraction of Al fairly correlate ($R=0.5810$) with the Alr concentrations (0.01–0.26% of total soil Al) determined in the filtered H_2O soil extracts after the solid phase extraction (SPE) by the chelating ion-exchanger Iontosorb Salicyl, see Fig. 2. Thus, the first (acid

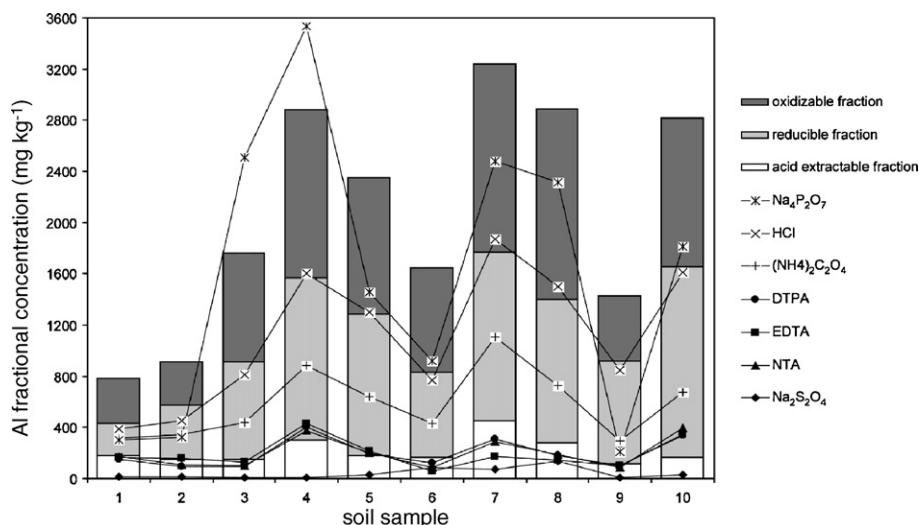


Fig. 3. The comparison between the optimised BCR three-step sequential extraction and some single extraction procedures.

Table 5

The correlation coefficients for relative Al concentrations obtained by optimised BCR three-step sequential extraction and some single extraction procedures

Fraction	Na ₂ S ₂ O ₄	NTA	EDTA	DTPA	(NH ₄) ₂ C ₂ O ₄	HCl	Na ₄ P ₂ O ₇
Acid extractable	0.2884	0.2900	0.0755	0.4647	0.8707	0.6460	0.4047
Acid extractable + reducible	0.1120	0.5833	0.4085	0.5717	0.7736	0.9834	0.3020
Acid extractable + reducible + oxidisable	0.2294	0.5668	0.3526	0.6879	0.8592	0.9806	0.4398

extractable) step of the optimised BCR three-step SEP is related to the reactive Al concentration in the acid samples.

The mobilisable (the sum of first two or three-steps of the optimised BCR three-step SEP) Al fractions are highly related to Al extracted by dilute HCl (0.29–2.8% of total soil Al) and ammonium oxalate (0.22–1.67% of total soil Al), see Fig. 3 and Table 5. Thus, dilute HCl can supersede the laborious optimised BCR three-step SEP and it can be an effective and rapid approach for the assessment of Al contamination or for some other applications in the Al fractionation. Oxalates are often used to estimate the reducible fraction in many sequential extraction protocols, but in our case the Al amounts released by acid hydroxylammonium chloride in the second reducible step of the used SEP were about 2 times greater than in the single (NH₄)₂C₂O₄ extracts. It seems that the hydroxylammonium chloride agent has the more reducible effect than ammonium oxalate.

The sodium dithionite (Na₂S₂O₄) agent is often used as an extractant because of its ability to reduce the crystalline iron oxides and the most organic-iron complexes and to release the occluded trace metals [51]. Dithionite may also dissolve aluminium coprecipitated in the iron oxides [51]. The dithionite agent appears as less suitable extracting agent for our purpose (the Al amounts released from the all samples are similarly low, ranging from 0.008 to 0.2% of total soil Al). The low Al recoveries are probably due to the formation of the sulphides as the result of the dithionite disproportionation [37]. It is presumably about the short-term precipitation of aluminium or the Al adsorption on other metals created sulphides.

The complexing agents NTA, EDTA and DTPA are used usually as extractants in the functionally defined fractionation [51,65]. Concentrations of many metals in the fractions extracted by these agents, for example, well correlate with the relevant concentrations of these metals in plants. From Fig. 3 it is obvious that the Al amounts extracted by NTA, EDTA and DTPA are very similar (0.06–0.6% of total soil Al). The high concentrations of Al in the Na₄P₂O₇ soil extracts (0.2–4.2% of total soil Al) are probably connected with the complexation ability of the agent to impair clay minerals or weathered secondary minerals [35].

3.3. Selectivity study of the used methods

The selectivity of the solid phase extraction (SPE) by the chelating ion-exchanger Iontosorb Salicyl, fifteen single extractants and the optimised BCR three-step sequential extraction procedure (SEP) was investigated with the relation to the Al plant availability. The relationship between the Al concentrations in the individual soil fractions and the Al concentrations in the grass stems is presented in Fig. 4. The slopes of these dependences decrease with the increased extraction efficiency of the used methods (from the Na₂S₂O₄ and Alr to Na₄P₂O₇ and dilute HCl extraction procedures). Likewise, the selectivity of the used methods decrease in the same order.

It is obvious that the used methods in accordance with their extractive efficiency can be divided into three groups. The first group is consisting with the weakly efficient extraction proce-

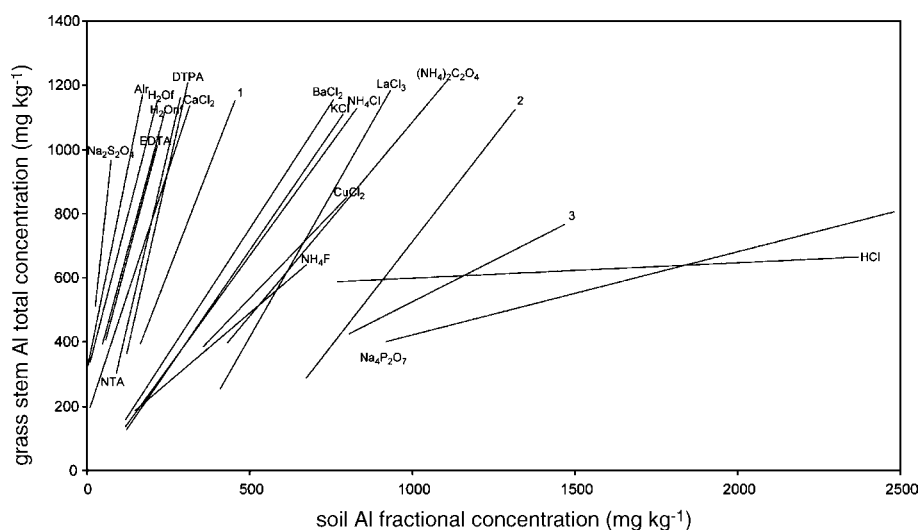


Fig. 4. The relationship between the Al concentrations in the individual soil fractions and the Al concentrations in the grass stems (H₂O extract; filtered H₂O extract; H₂O extract; nonfiltered H₂O extract; 1, acid extractable fraction; 2, reducible fraction; 3, oxidisable fraction of the optimised BCR three-step sequential extraction procedure).

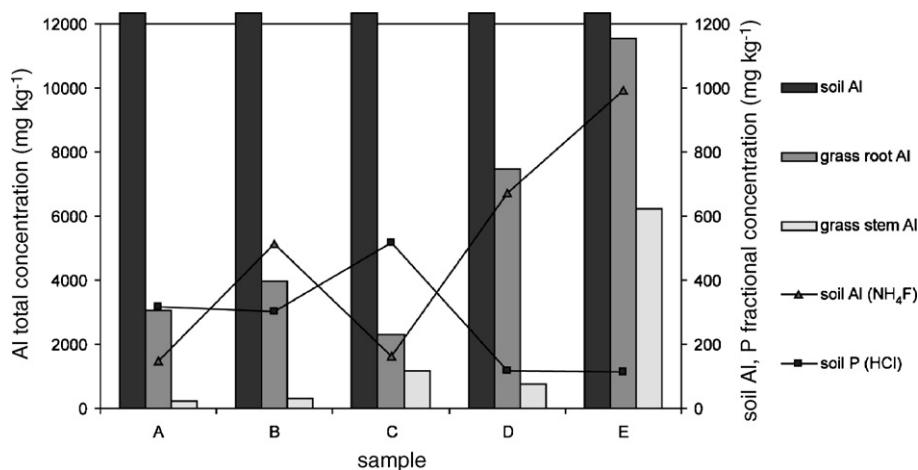


Fig. 5. The comparison between the concentrations of total Al in the soils and grass and the extracted Al and P amounts.

dures (H_2O , CaCl_2 , $\text{Na}_2\text{S}_2\text{O}_4$, NTA, EDTA, DTPA and dilute acetic acid from the first acid extractable step of the optimised BCR three-step SEP) and the chelating ion-exchange SPE which release a small amounts of the available Al fractions. The more efficient extraction agents as KCl , NH_4Cl , BaCl_2 , CuCl_2 , LaCl_3 , NH_4F and $(\text{NH}_4)_2\text{C}_2\text{O}_4$ leach from the soil samples approximately the same amounts of Al as the total Al concentrations measured in samples of the grass stem. These extractions better describe a potential threat in a case of the strong disturbance of natural soil conditions [19]. There is a strong probability that highly concentrated CaCl_2 solution ($0.1\text{--}1\text{ mol l}^{-1}$) could be also placed in the second group of extractants. The third group of extractants comprises of most aggressive agents as $\text{Na}_4\text{P}_2\text{O}_7$, dilute HCl , hydroxylammonium hydrochloride/ HNO_3 and H_2O_2 /ammonium acetate used in the second and third step of the optimised BCR three-step SEP. These extractants release the highest amounts of soil Al, approximately double as the total Al concentrations measured in the grass stem samples. The most suitable agents for the extraction of bioavailable Al forms are those from the first and partly from

second group. They have the ability to ion-exchange or complex the bioavailable forms of Al.

The NH_4F extractant is used in the aluminium partitioning because of its complexing power [51]. From Fig. 5 it is evident that NH_4F releases from the soils about ten times less Al than the plant roots contain ($R = 0.9134$) and that one seems to be more suitable than NTA, EDTA and DTPA agents for the estimation of plant available Al in the acid soils. Aluminium extracted by NH_4F sufficiently correlate with phosphorus extracted from the soils by dilute HCl ($R = 0.7194$). The concentrations of Al in the NH_4F soil extracts decrease with increased concentrations of P measured in the dilute HCl and vice versa.

3.4. Calculation of aluminium toxicity indexes (ATI)

The calculation of the aluminium toxicity index (ATI) is a useful and promising approach for the estimation of Al toxicity in plants. The ATI values, defined as the ratio between the sum of nutrient cations (Ca , Mg , Na , K) concentration to the Al concentration in mmol kg^{-1} , was used for this purpose. From

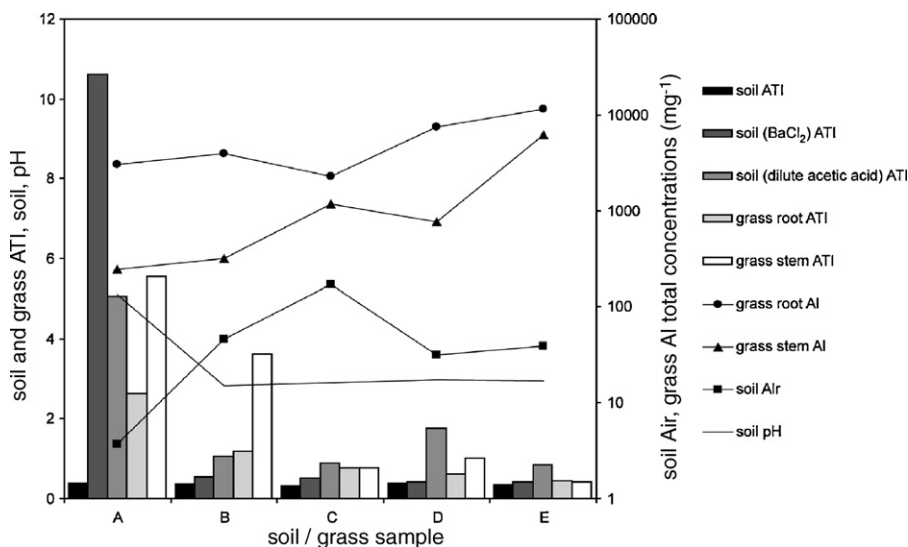


Fig. 6. The values of aluminium toxicity indexes (ATI) in the soils, soil extracts and grass.

the low ATI data results the high toxic impact to plants and vice versa. From Fig. 6, it is evident that the values of aluminium toxicity index (ATI) calculated for the original soil samples are nonselective considering the soil pH and Alr values but the calculation of the ATI values for two soil extracts and grass samples is more useful (ATI values <1 mean the toxicity risk of Al to living organisms). The selective ATI values calculated for the BaCl₂ and dilute acetic acid soil extracts and the grass root and stem samples very well correspond to soil pH, soil Alr and the total grass root and stem Al values. The highest ATI values relate to the highest soil pH and the lowest Alr values (the sample A) and the lowest ATI values relate to the highest concentration of Al in the grass root and stem (the sample E).

4. Conclusions

The different Al partitioning methods were applied to the acid soils from the location influenced by very acid drainage water. The aluminium interactions in the soil–plant system subjected to the strong acidification were studied and the calculation of the aluminium toxicity indexes (ATI) was used to assess Al toxicity to the grass.

The reliability of the optimised BCR three-step sequential extraction procedure (SEP) was valued on the CRMs only with the certified values for total Al concentrations. Therefore, obtained data can be utilised as indicative values for another studies of the Al fractionation by SEP in the interlaboratory comparisons. The dilute HCl extraction can be the alternative for routine monitoring of the Al mobility instead optimised BCR three-step SEP when the phase associations estimation is not necessary. The acid extractable Al fraction is the most Alr selective from the optimised BCR three-step SEP.

From the selectivity study of fifteen single extractants related to plant available Al can be concluded that the group of weakly efficient extractants (H₂O, CaCl₂, NTA, EDTA, DTPA, dilute acetic acid) have the ability to solubilise, ion-exchange or to complex the available forms of Al, so they are more suitable than more aggressive extractants. They can be a simple and rapid tool for some applications in the Al fractionation. The most sensitive indicator of the increasing aluminium toxicity in plants is the determination of reactive Al in the H₂O soil extracts by the chelating ion-exchange solid phase extraction.

The calculation of the aluminium toxicity index is not only a useful tool for the estimation of the Al toxic influence to plants but this value can indicate the important losses of the exchangeable nutrients Ca, Mg, Na and K due to acidification and the increase of the available aluminium species.

Acknowledgements

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Influence of different bentonites on the rare earth element concentrations of clarified Romanian wines

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Abstract

The rare earth element (REE) concentrations of 19 Romanian young wine samples originating from the Dealurile Moldovei viticulture area were determined by double focusing inductively coupled plasma mass spectrometry (DF-ICP-MS) after microwave-assisted digestion with nitric acid. The determination of Eu was hampered by the BaO molecular interference. Generally, the red wine samples were more concentrated for REEs than the white wine samples studied. The REE concentrations of the four bentonites (Gelbenton, Evergel, BW200, Tükrös) determined after their fusion were higher by three orders of magnitude than those of the wine samples. After a simulated wine purification process performed with these bentonite samples and a red and white pool samples, the REE concentrations of the clarified wine samples increased by 1.2–1.5 times for red, and 1.3–3 times for white wines in case of the fibrous bentonite sample (Gelbenton), by about 2–5 times in case of the bentonite containing ovalbumin, caseine and gelatine (Evergel), meanwhile this factor was about 20–25 for Na bentonite powder samples (BW200, Tükrös). On basis of the chemometric evaluation using the REE concentrations as input data, the majority of the Feteasca wines belonged to the same cluster as well as the two Cabernet Sauvignon to another subcluster. The adequate choice of the bentonite may allow the use of REEs as fingerprints for determining the wine provenance.

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Keywords: Rare earth elements; Wines; Bentonites; ICP-MS; Cluster analysis

1. Introduction

Inductively coupled plasma mass spectrometry (ICP-MS) is a multi-element technique suitable for the analysis of young–finished, red–white, table–fortified wine and even sweet wines with over-ripe grape samples by (semi)quantitative mode providing high selectivity, sensitivity and lower detection limits than other multi-element techniques [1–3].

Wine is of a great economic importance, however, falsification, wrong declaration and blending of wines cause a serious financial shortage. Therefore, there is a strong need to develop fingerprint techniques assisted by mathematical statistical meth-

ods in order to determine the provenance of wines. The biggest restriction to the fingerprinting is the representative selection of the group of elements or compounds found in wine samples, which ideally should depend only on the soil composition, but not on the wine technology, transport or storage. Biology, soil mineralogy and solution geochemistry, can all contribute to controlling element uptake by plants [4].

Rare earth elements (REEs) would be ideal candidates for fingerprinting as they have similar chemical properties. The greatest risk for introducing REEs and other elements in wines represents the purification of wines by bentonites [3]. Therefore, up to now, volatile organic compounds like 1-hexanol and cyclohexane [5], amino acids [6,7] or anthocyanins [8] have been chosen for wine fingerprinting. Medina and Van Zeller [9] differentiated among red wines from different parts of France using volatile compounds, phenols, metals, anthocyanins and organic acids. Maarse et al. [10] and García-Jares et al. [11] used volatile

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compounds, organic acids, amino acids, and major and minor metals to distinguish between German wines from the Rhein-Pfalz and Mosel regions and between some white wines from Rias Baixas (Galicia), respectively. However, metals are the best to perform a differentiation according to the geographical origin due to the direct relationship with soil composition. This differentiation can be carried out by using major, trace and ultra-trace elements [12]. Cluster analysis is proved to be suitable for mathematical statistical evaluation of major and trace element concentrations of wine samples [13–20]. The initial assumption of this analysis is that the nearness of the objects, defined by their variables, reflects the similarity of their properties [21]. In case of Czech wine samples, differentiation was made by making a comparison with the vineyard soil composition for 27 major and trace elements, but not for REEs [13]. The authors concluded that both wine and soil samples were very well clustered according to their locality. However, the clustering of wines did not follow clustering of soils and it was possible that major and trace element fingerprints of wines reflect soil chemistry, pollution and wine manufacturing practices by individual producers.

An excellent way of comparing the REE concentration ratios for different matrices, consists of dividing the concentration of each element by the corresponding value for chondrite and then plotting their logarithm values versus the atomic mass of REEs [22]. Chondritic meteorites are regarded as a reference, because they contain REEs in their primitive relative abundance. The plotted curves obtained should have a smooth shape with the possible exceptions for Ce and Eu. The anomaly of Ce is due to the fact that Ce occurs in tetravalent form under oxidising condition, and that of Eu because Eu(III) can be reduced to Eu(II) causing fractionation relative to other REEs. A smooth profile can therefore be used as an additional assessment of analytical accuracy [23]. Hence, this curve can, for instance, reveal possible anthropogenic contamination; demonstrate that the environmental habitats were well protected from pollution [24]; or show the bioavailability of the low solubility REEs for living organisms [25,26].

The aim of the present work was to detect and quantitatively determine REEs in selected Romanian young white and red wine samples; to investigate the suitability of the REEs as a fingerprint for wine provenance by chemometric means; to evaluate the effect of wine purification by different types of (fibrous, powder, natural or natural gelling agents containing) bentonites on the fingerprinting attempt.

2. Experimental

2.1. Instrumentation

For the determination of rare earth elements (REEs), an Element2 DF-ICP-MS instrument (Thermo Finnigan, Germany) was used. The instrumental conditions of the DF-ICP-MS analytical procedure are summarised in Table 1. For the fusion of bentonites, a KMM6/1200 Kalória GMK (Hungary) temperature controlled furnace (Model LT-S/1200) and Pt crucibles were used. Microwave-assisted digestion of the wine samples

Table 1

Instrumental conditions of the DF-ICP-MS measurements for REE determination

Power (W)	1200
Ar gas carrier flow (dm ³ /min)	16.0
Ar gas auxiliary flow (dm ³ /min)	0.8
Ar nebuliser flow (dm ³ /min)	1.1
Measurement mode	Low resolution ($R = 300$)
Acquisition mode	E-scan
No. scans	20 (5 runs, 4 passes)
Search window (%)	60
Integration window (%)	60
Calibration	External
Internal standard	50 pg cm ⁻³ In
Points per peak	30
Isotopes	¹³⁹ La, ¹⁴⁰ Ce, ¹⁴¹ Pr, ¹⁴⁶ Nd, ¹⁴⁷ Sm, ¹⁵¹ Eu, ¹⁵⁷ Gd, ¹⁵⁹ Tb, ¹⁶³ Dy, ¹⁶⁵ Ho, ¹⁶⁶ Er, ¹⁶⁹ Tm, ¹⁷³ Y, ¹⁷⁵ Lu
Nebuliser	Meinhard
Spray chamber	Scott
Sampler cone	Ni, Ø 1.0 mm orifice
Skimmer cone	Ni, Ø 0.4 mm orifice

was performed by using a Multiwave Paar Physica device (Paar, Austria). For this procedure, six HQ50 quartz bombs (Paar, Austria) were used simultaneously.

2.2. Reagents and materials

Throughout the experiments ion-exchanged water (Purite, UK) with a resistivity of 17 MΩ cm was used. Suprapure® 65% HNO₃ (Merck, Germany) was used for the microwave-assisted digestion of the samples. For tuning and mass calibration of the DF-ICP-MS instrument, an acidic (5% HNO₃) Merck Multi-element standard solution in concentration of 1 ng cm⁻³ for each element was employed. For the determination of REEs, a Spex Certi Prep Claritas ppt multi-element standard solution containing Ce, Dy, Er, Eu, Gd, Hf, La, Lu, Nd, Pr, Sc, Sm, Tb Tm, Yb each in a concentration of 10 µg cm⁻³ was applied. For internal standardisation, an acidic (0.5 mol dm⁻³ HNO₃) In Merck stock solution in a concentration of 1000 µg cm⁻³ was used. All solutions were prepared daily from their stock solutions by appropriate dilution with deionised water. The final HNO₃ concentration of the solutions was 5%. Four, commercially available bentonite samples, Gelbenton, Evergel, BW200 and Tükrös were used for wine clarifying without any further purification. Gelbenton and Evergel bentonites were produced in Italy. BW200 and Tükrös bentonites are originating from Germany and Hungary, respectively. Lithium metaborate (LiBO₂) and ammonium nitrate (NH₄NO₃), used for the fusion of the bentonite samples, were purchased by Spex.

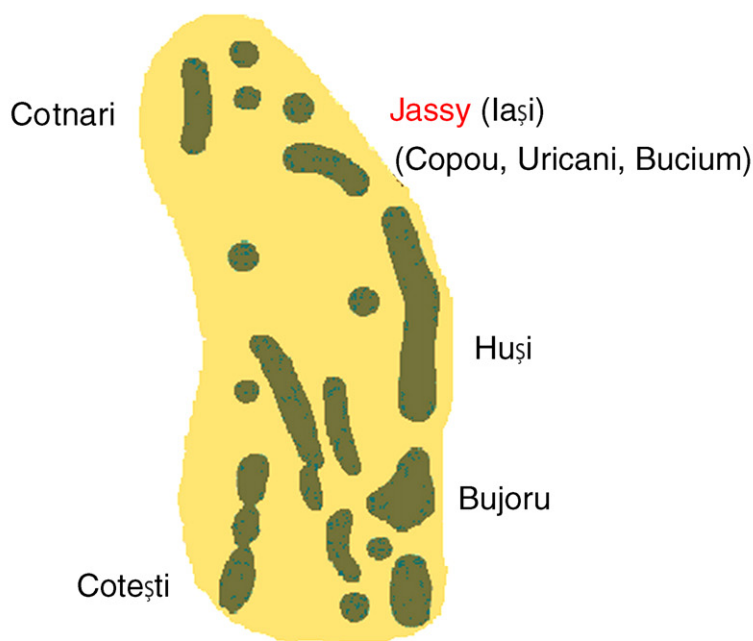
2.3. Procedures

2.3.1. Wine selection and clarifying by bentonites

Nineteen young wine samples – 12 white and 7 red – of the 2003 vintage prepared according to the valid EU regulations were selected from the Dealurile Moldovei viticultural region, namely from the hills of Cotnari, Jassy (Copou, Uri-



(a)



(b)

Fig. 1. Map of Romania indicating the Dealurile Moldovei viticulture area (black ellipsis) (a) and its sampling sites (b).

cani, Bucium Hills), Huși, Bujoru and Cotești (Fig. 1). The label assignment as well as the names of the sampling sites of the samples collected are presented in Table 2. Samples were transported in polypropylene bottles to the laboratory and kept at 4 °C. Soil composition of the hills from where samples are orig-

inating is similar: cambic–chernozemic, grey, anthropic soils, regosol, except for Cotești characterised by chernozemic, grey, brown, alluvial, anthropic soils, alluvial clays, regosol. For the wine clarifying experiments, identical aliquots from the original white and red samples were pooled separately and homogenised.

Table 2

Sample labels of the investigated wine samples (provenance)

Label	White wine (provenance)	Label	Red wine (provenance)
1	Fetească Albă (Cotnari)	8	Fetească Neagră (Bujoru)
2	Fetească Albă (Cotești)	9	Fetească Neagră (Bujoru)
3	Fetească Albă (Bucium)	10	Fetească Neagră (Stațiunea Didactică, Iasi)
4	Fetească Regală (Bucium)	11	Fetească Neagră (Uricani)
5	Fetească Regală (Cotești)	17	Cabernet Sauvignon (Bujoru)
6	Fetească Regală (Copou)	18	Cabernet Sauvignon (Uricani)
7	Fetească Regală (Huși)	19	Băbească Neagră (Bujoru)
12	Tămâioasă Românească (Cotnari)		
13	Muscat Ottonel (Bucium)		
14	Grasă (Cotnari)		
15	Aligoté (Bucium)		
16	Sauvignon (Bucium)		

The volume of the resulted two pool samples (1 dm³, each) was divided into five equal parts. One part from each pool sample of 200 cm³ was kept as reference sample. The remaining parts underwent to wine clarifying. Two hundred cubic centimeter of a pool white and a red wine sample, each, were stirred with 60 mg of Gelbenton, 240 mg of Evergel, 400 mg BW200 and 160 mg of Tükrös and left to decantate for 5 days at 20 °C. The wine: bentonite mass ratio was chosen according to the tag prescription on the bentonite packaging.

2.3.2. Fusion of bentonites

Fusion of bentonite samples were made according to a previous method [27]. Briefly, 0.5 g bentonite sample were fused in Pt crucibles with 1.16 g LiBO₂ and a tiny amount of NH₄NO₃ in a temperature controlled electric furnace at 1000 °C for 30 min. The fused samples were transferred into glass beakers, covered with deionised water and then dissolved, using also a magnetic stirrer, in 10 cm³ 1:1 HNO₃: deionised water solution mixture. The resulted solutions were transferred each into PP flasks and made up to 250 cm³ with deionised water. Only the

fibrous bentonite sample (Gelbenton) had to be powdered in a ball mill conferring an average grain size of 60 μm to the samples. The REE concentrations of the fused samples were determined by DF-ICP-MS after appropriate dilution with deionised water.

2.3.3. Microwave-assisted digestion of the wine samples for REE analysis

Aliquots of 2 cm³ of the wine as well as clarified wine samples, filtered through Millex PDVF filters of 0.2 μm pore size, were placed into quartz bombs. Aliquots of 2 cm³ HNO₃ were carefully added. Then, the samples were subjected to microwave-assisted digestion for 40 min at 1000 W (nominal power value per six bombs) achieved with a ramp of 50 W/min. Six bombs were used simultaneously for one digestion procedure. Between two digestions, the bombs were cleaned with 5 cm³ HNO₃ Suprapur® at 1000 W for 20 min. The cleanness of the resulting solutions was checked by the DF-ICP-MS method.

2.3.4. Hierarchical cluster analysis

Hierarchical cluster analysis was carried out by using XLMiner software working with Excel. The settings of the procedure were: Ward's method as linkage type, Euclidean distance method and normalisation was also been applied.

3. Results and discussion

REE concentrations of the samples were measured by using a previously reported method [3]. In order to investigate the effect of interferences on the REE analysis, the first step in the analysis consisted of making pool samples from the 7 red and from the 12 white wine samples by taking aliquots of 2 cm³ from each and mixing them. After this step, the pool samples were prepared according to the protocol described in Section 2 and analysed by DF-ICP-MS in low ($R=300$), medium ($R=4000$) and high ($R=10000$) resolution. Formation of BaO

Table 3

Concentration ranges, recovery (%) and detection limits (DL) of REEs (except for Eu) for the investigated Romanian wine samples

REE	Concentration range		Pool white wine				Pool red wine				DL (pg cm ⁻³)
	White wines ^a	Red wines ^a	Original ^a	Spiked ^{a,b}	Expected	%	Original ^a	Spiked ^{a,c}	Expected	%	
La	389–6490	106–7247	528 ± 20	1460 ± 52	1528	96	792 ± 30	2643 ± 118	2792	95	0.27
Ce	71.3–5000	74.4–7721	895 ± 27	1893 ± 30	1895	100	1633 ± 28	3933 ± 135	3634	108	0.25
Pr	4.53–675	21.0–945	120 ± 1	992 ± 14	1120	89	214 ± 10	2035 ± 50	2214	92	0.36
Nd	14.0–2647	74.3–3540	507 ± 12	1481 ± 28	1507	98	952 ± 18	2830 ± 64	2952	96	0.19
Sm	2.76–561	13.3–778	117 ± 2	1227 ± 12	1117	110	239 ± 5	2456 ± 18	2239	110	0.02
Gd	5.02–591	16.3–867	169 ± 3	1430 ± 14	1169	122	306 ± 8	2839 ± 45	2306	123	0.07
Tb	1.08–110	1.90–184	57.1 ± 1.34	1045 ± 9	1057	99	119 ± 1	2074 ± 68	2119	98	0.04
Dy	3.94–427	46.8–664	88.2 ± 2.21	1105 ± 8	1088	102	214 ± 4	2277 ± 38	2214	103	0.05
Ho	0.87–78.5	14.1–128	16.7 ± 0.77	996 ± 21	1016	98	44.1 ± 1.7	2064 ± 35	2044	101	0.01
Er	3.64–275	4.26–446	93.4 ± 2.07	1136 ± 12	1093	104	223 ± 9	2312 ± 27	2223	104	0.03
Tm	0.69–25.1	7.62–48.4	6.00 ± 0.51	1056 ± 9	1006	105	20.3 ± 0.9	2140 ± 13	2020	106	0.004
Yb	5.37–196	3.33–350	59.7 ± 2.78	1188 ± 12	1059	112	175 ± 1	2470 ± 28	2175	114	0.03
Lu	1.15–24.0	0.68–52.5	5.29 ± 0.26	1058 ± 5	1005	105	23.2 ± 4.2	2153 ± 15	2023	104	0.32

^a c/pg cm⁻³.^b Spiked with 20 μl 100 ng cm⁻³ multi-elemental REE standard.^c Spiked with 40 μl 100 ng cm⁻³ multi-elemental REE standard. % = recovery expressed as [(spiked REE concentration)/(expected REE concentration)] × 100.

in the plasma produced a significant interference on the two Eu isotopes in the low-resolution mode, especially in the case of the samples treated with bentonites, the Ba:Eu ratio for the pool samples being about 180:1 for the white pool and 170:1 for the red pool samples. The BaO-produced interference on the Eu isotopes can be a serious problem in case when the Ba:Eu ratio is >200:1, which may impede Eu determination [23] by ICP-MS. As the Ba:Eu ratios established for the pool samples were close to this ratio, mathematical correction was not made for Eu. However, all the other REEs could be monitored in the wine samples in low-resolution mode. Although, the employment of high resolution would have been the best solution to determine all REEs, the precision of the measurements ($RSD > 20\%$) impeded the application of this operational mode. A doubling of the acquisition spectra could not either reduce the RSD of the measurements. The linear fit obtained by fitting the counts of the REEs, whose concentrations in the standard solutions were between 1 and 100 pg cm^{-3} , gave r^2 values >0.9990. The intra- and inter-day RSD were lower than 5%. Generally, the REE concentrations of the red wine samples were higher than that of white wine samples. The concentration ranges of the white and red samples as well as the detection limits for the REEs determined are listed in Table 3. The concentration of La and Ce, and in some cases for Nd, were in the ng cm^{-3} concentration range. Due to the lack of a wine sample certified for REEs and the too low concentration values to be measured by alternative analytical techniques, the accuracy of the measurements could not be checked. However, a recovery study was performed by spiking the white and red pool samples with 20 and 40 μl multi-elemental standard solution in concentration of 100 ng cm^{-3} for each REE, respectively. Then, the spiked samples underwent to the whole analytical procedure used for the REE determination. The REE concentrations resulted after spiking the samples were in good agreement with the expected values (Table 3). The REE concentrations of the four bentonites (Gelbenton, Evergel, BW200, Tükrös) were higher by approximately three orders of magnitude than those of the wine samples (Table 4).

Table 4
REE concentrations of the bentonites samples investigated

REE	Bentonite			
	Gelbenton ($\text{c}/\mu\text{g g}^{-1}$)	Evergel ($\text{c}/\mu\text{g g}^{-1}$)	BW200 ($\text{c}/\mu\text{g g}^{-1}$)	Tükrös ($\text{c}/\mu\text{g g}^{-1}$)
La	28.4 ± 0.54	16.83 ± 0.73	53.82 ± 0.28	31.5 ± 2.35
Ce	52.5 ± 1.17	32.05 ± 0.86	109 ± 1.48	62.7 ± 4.81
Pr	5.31 ± 0.18	3.37 ± 0.06	13.1 ± 0.06	7.24 ± 0.25
Nd	17.8 ± 0.06	11.07 ± 0.20	50.3 ± 0.25	26.5 ± 0.77
Sm	3.30 ± 0.01	2.03 ± 0.04	10.4 ± 0.05	5.8 ± 0.15
Gd	4.10 ± 0.16	2.88 ± 0.19	1.60 ± 0.11	6.5 ± 0.31
Tb	0.69 ± 0.01	0.45 ± 0.01	2.27 ± 0.01	1.32 ± 0.03
Dy	3.06 ± 0.11	1.95 ± 0.04	9.32 ± 0.03	6.42 ± 0.13
Ho	0.65 ± 0.02	0.42 ± 0.01	1.83 ± 0.01	1.35 ± 0.02
Er	2.29 ± 0.02	1.53 ± 0.03	6.19 ± 0.04	4.43 ± 0.03
Tm	0.32 ± 0.01	0.22 ± 0.01	0.75 ± 0.01	0.60 ± 0.01
Yb	2.28 ± 0.05	1.57 ± 0.06	5.26 ± 0.06	3.96 ± 0.05
Lu	0.37 ± 0.01	0.25 ± 0.01	0.73 ± 0.01	0.58 ± 0.01

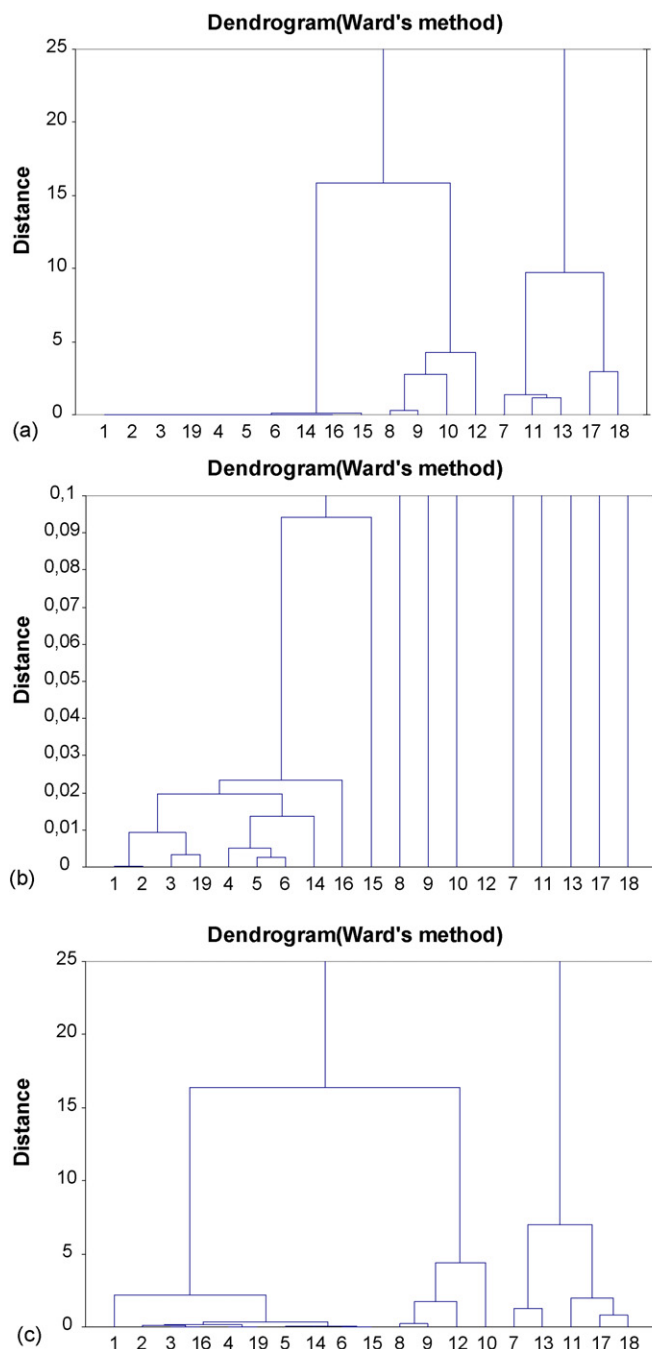


Fig. 2. Dendrogram of the cluster analysis of the Romanian wine samples (a) with a magnified part (b) as well as after clarifying with Gelbenton bentonite (c) employing Euclidean distance. Legend: see Table 2.

The hierarchical cluster analysis of the wine samples, according to their REE concentrations, grouped the samples in two clusters each having two subclusters (Fig. 2). A part of the dendrogram (Fig. 2b) had to be magnified, because the distance between its objects (generally, Feteasca wines) was very small, proving their strong similarity. The 82% of the Feteasca wine samples belong to the same cluster. Also, the two Cabernet Sauvignon samples belong to the same subcluster. As the classification of the samples did not reveal any dependence of the sample sites and taking into consideration that the wines are not cuvée ones,

the differences obtained for the cluster analysis suggest that the grouping of wine samples is traceable, first of all, to the sort of grapes. In some cases, the wine classification seems to be disturbed by the differences in REE concentrations of the soils of

the sampling sites. However, the REE determination of the soils of the sampling sites were not an object of this investigation.

By determining the REE concentrations of young and finished wine samples, Jakubowski et al. [3] found that wine purification with bentonites increased the REE concentrations of the samples shaken for 48 h. Their results were normalised with the REE concentrations of chondrite. Similar results were obtained by performing the bentonite extraction with 1% tartaric acid (major acidic compound of wines). Therefore, in our study, the bentonite extraction by tartaric acid was not applied, wine purification was performed only with red and white pool samples. Moreover, the samples were left to be clarified for 5 days, as it is usual practice in oenology. Special attention was paid to the types of bentonites used: purified fibrous (Gelbenton) versus powder (Evergel, BW200, Tükrös); Na bentonites (BW200, Tükrös) versus bentonites containing other natural clarifying additives like caseine, gelatine and ovalbumin (Evergel). Chondrite normalisation according to Nakamura [28] has been applied for the REE concentration results obtained for the bentonites, control and purified pool wine samples (Fig. 3). The logarithmic plots revealed similarity for the chemical structure of the two Na bentonite samples (BW200 and Tükrös) and, also, similar behaviour in case of using them for wine purification. The patterns obtained for the fibrous (Gelbenton) and the natural clarifying additives containing (Evergel) bentonites were also similar in case of bentonites. Moreover, the pattern of the logarithmic plots for the Gelbenton and Evergel were similar to those of the control wines, proving that these two bentonites did not affect significantly the REE concentrations of the purified wine samples. The REE concentrations of the clarified wine samples increased by 1.2–1.5 times for red, and 1.3–3 times for white wines in case of the fibrous bentonite sample (Gelbenton) and by about 2–5 times in case of the bentonite containing ovalbumin, caseine and gelatine (Evergel). Wine purification by Na bentonite powder samples (BW200 and Tükrös) increased significantly the REE concentrations of the samples (by about 20–25 times). However, this increase was appalling compared with the REE concentrations of the bentonite samples. This is in concordance with the results of Mihaljevič et al. [29] who established that the fractionation of REE during a simulated wine purification process with bentonites was not significant. The relative REE concentrations values of the purified white pool samples, obtained by relating them to the control ones, were higher than in case of the red wine purification, because the REE concentrations of the pool white wine sample were lower than those of the pool red wine sample. As Gelbenton increased the least the REE concentration of the samples, 100 cm³ each sample was clarified with it and analysed following the previously described procedure. The application of Ward's method employing Euclidean distance for the samples treated with Gelbenton did not affect their classification into clusters (Fig. 2c).

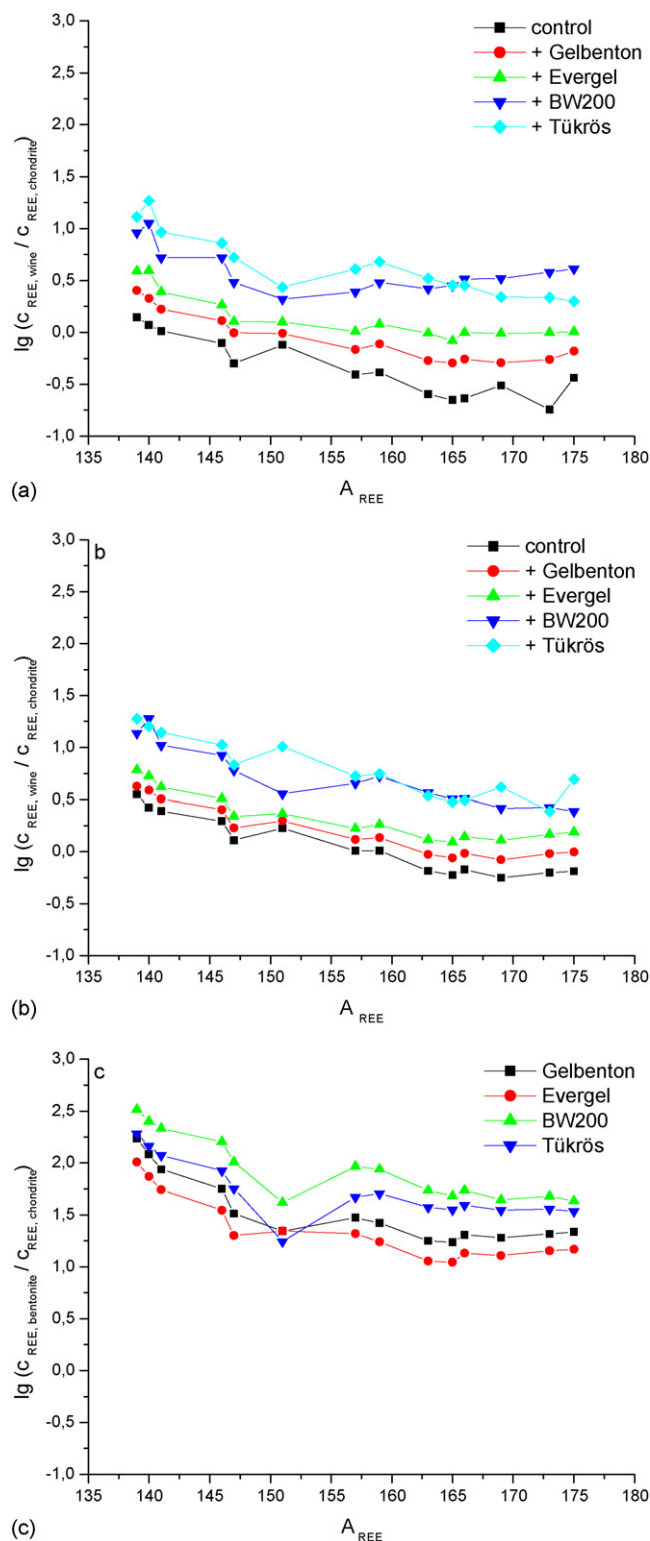


Fig. 3. REE concentrations with chondrite normalisation for control and a pool white (a) and red wine sample (b) purified by the bentonite samples investigated as well as for the previously fused bentonites (c).

4. Conclusions

- (i) From the first time, REEs could be detected and determined (except Eu) in 19 Romanian wine samples from the

Dealurile Moldovei viticulture area by DF-ICP-MS instrument in ng cm^{-3} and pg cm^{-3} concentration ranges.

- (ii) Generally, the REE concentrations of red wine samples are higher than that of white wine samples.
- (iii) Taking into consideration the scarce technology applied for the elaboration of these young wines, hierarchical cluster analysis suggests that wines can be classified according to the sort of grapes used for their production. Thus, the majority of the Feteasca wine samples belong to the same cluster and the two Cabernet Sauvignon samples also belong to a separate subcluster. The better understanding of the results of the cluster analysis needs the exact knowledge of the REE concentrations of the soils of the sampling sites.
- (iv) Depending on the bentonites used, wine clarifying influences the REE concentrations of the wines. An Italian fibrous bentonite sample alters the least REE concentrations of the pool wine samples (1.2–1.5 times for red, and 1.3–3 times for white wines). Therefore, the use of REEs as fingerprint for determining the wine provenance by chemometric methods after a proper selection of bentonites (i.e., Gelbenton) seems to be applicable.

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Polarographic chemometric determination of zinc and nickel in aqueous samples

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Abstract

Polarographic chemometric methods were applied to the determination of zinc and nickel in aqueous solutions previously acidified with 0.1 M acetate buffer (pH 4.2). The studied methods are multivariate methods including classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS); derivative ratio methods (first, ^1D and second, ^2D derivative ratio). A comparative study was considered. The studied chemometric methods do not need the presence of any reduction potential shift reagent in spite of the great overlap between the two metals polarograms. A training set consisting of 10 binary mixture solutions in the possible combinations containing 0.13–9.30 $\mu\text{g/ml}$ Zn(II) and 0.20–12.25 $\mu\text{g/ml}$ Ni(II) was used to develop the chemometric calibrations (CLS, PCR and PLS). A validation set containing the synthetic mixtures in the range of 0.29–9.00 $\mu\text{g/ml}$ for Zn(II) and 0.30–11.60 $\mu\text{g/ml}$ for Ni(II) was used to validate the multivariate calibrations. Same mixtures were used to develop the derivative ratio methods. The polarograms were recorded and their current values were measured within the potential range -920 to -1052 mV at 2 mV intervals. The mean percentage recoveries obtained using CLS, PCR and PLS were found to be $99.5 \pm 1.5\%$, $100.0 \pm 1.1\%$ and $100.0 \pm 1.0\%$ for Zn(II) and $99.4 \pm 1.3\%$, $99.7 \pm 1.2\%$ and $99.9 \pm 1.0\%$ for Ni(II), respectively. The mean percentage recoveries obtained using ^1D at -950 mV, ^1D at -1010 mV, ^1D at -950 mV– ^1D at -1010 mV and ^2D at -986 mV for Zn(II) were found to be $99.7 \pm 1.2\%$, $99.2 \pm 1.6\%$, $99.4 \pm 1.4\%$ and $99.4 \pm 1.4\%$; and using ^1D at -1030 mV and ^2D at -1010 mV for Ni(II) were found to be $100.5 \pm 1.3\%$ and $100.4 \pm 1.3\%$, respectively. Interferences due to the presence of Cd, Co, Pb, Fe, Mn, Ca, Mg, Cu and Al were studied. The applicability of the proposed methods was assessed through the determination of both metals in tap drinking-water. Samples were subjected if required up to a 20-fold preconcentration step by microwaving in pyrex vessels. The results were compared with those obtained using the zincon and the heptoxime colorimetric reference methods for the determination of zinc and nickel, respectively.

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Keywords: Polarography; Chemometrics; Zinc; Nickel; Water

1. Introduction

Differential pulse polarography (DPP) is a highly sensitive technique, though its applicability to the determination of mixtures of several components is rather limited when they display strongly or partially overlapped polarograms. In this case, one may try to optimize conditions for maximum separation [1]. This usually involves time-consuming manipulation of the sample in order to obtain a specific and/or selective signal. These problems can alternatively be overcome by using certain techniques as derivative voltammetry [2], square-wave adsorptive stripping voltammetry [3] or kalman filter technique [4]. The use

of chemometric procedures, viz. partial least squares and principal component regression provides a more convenient means for the same purpose.

Diverse chemometric methods have been applied to improve results in the multi-component analysis by different analytical techniques which have been mainly spectroscopic [5] and, more recently, electroanalytical [6,7]. Partial least squares have been used for the simultaneous polarographic determination of some binary and ternary mixtures [6–9]. Principal component regression has been applied to the determination of captopril by DPP with no prior removal of dissolved oxygen [10]. Multiple linear regression with baseline subtraction and fast smoothing has been applied to resolve overlapped polarographic peaks [11]. Derivative ratio method has been applied to the polarographic determination of copper, cadmium and nickel [12,13] and to the determination of amaranth and sunset yellow in mixtures [13].

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Chemometric methods have been used for the simultaneous analysis of metals by electroanalytical techniques [12–17].

In the present work, polarographic chemometric methods were applied to the determination of zinc and nickel. The studied methods are multivariate methods including classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS); derivative ratio methods (first, ^1D and second, ^2D derivative ratio). A comparative study was considered. The applicability of the proposed methods was assessed through the determination of both metals in tap drinking-water. The results were compared with those obtained using the zinc-con and the heptoxime colorimetric reference methods for the determination of zinc and nickel, respectively [18].

Zinc is an essential element in all living organisms. Nearly 200 zinc-containing enzymes have been identified [19]. Zinc is used in galvanizing steel and iron products. It is also used in the production of corrosion-resistant alloys, paint pigments, pharmaceuticals, dyes, cosmetics, several kinds of insecticides and pesticides [20].

Major sources of zinc in natural waters come from zinc containing fertilizers and industrial effluents [20]. In natural surface waters, the concentration of zinc is usually below 10 $\mu\text{g/L}$. In ground waters, it ranges from 10 to 40 $\mu\text{g/L}$ [21]. In tap water, the zinc concentration can be much higher as a result of the leaching of zinc from piping and fittings [22]. Zinc in tap water from galvanized pipes may range from 3 to 2100 $\mu\text{g/L}$ [20].

The WHO guidelines for drinking-water quality states that drinking-water containing zinc at levels above 3 mg/L tends to be opalescent, develops a greasy film when boiled and has an undesirable astringent taste [23]. The water supply regulations for the United Kingdom [24], the U.S. Public Health Service Drinking Water Standards [25] and the Egyptian Ministry of Health [26] put 5 ppm as a guideline value for zinc in drinking-water.

Nickel and its compounds are toxic in the conventional sense [20]. Nickel is used in a large number of alloys including stainless steel. It acts as a catalyst in chemical reactions. It is also used in the electrolytic coating of items such as chromium-plated taps and fittings used for tap water [23]. Being highly soluble in water, nickel salts may be discharged to surface or ground waters [20]. The WHO health-based guideline value of nickel in drinking-water is 0.02 mg/L [23].

Zinc and nickel have been determined by atomic emission spectrometry [27], flame atomic absorption spectrometry [28], inductively coupled plasma atomic emission spectrometry [29] and ion chromatography [30].

The polarographic determination of zinc and nickel was made difficult due to their strongly overlapping waves. The difference in reduction potentials between zinc and nickel is very small in conventional supporting electrolytes such as KCl, KNO_3 or CH_3COONa [31]. The determination of these two ions, however often became possible in the presence of a reagent which forms a complex with the target ion, causing a larger difference in reduction potentials [32]. Crown ethers [31] and polyethylene glycol [33] worked as useful reduction potential shift reagents in the polarographic determination of zinc and nickel. Simultaneous determination of zinc, nickel, cobalt, iron, cadmium and

manganese by kalman filter polarography has been studied [34]. The differential pulse polarographic determination of nickel, zinc, cobalt and manganese in a 2-amino-2-methylpropionic acid-thiocyanate mixed system has been reported [35]. Determination of Cu(II), Cd(II), Zn(II), Ni(II) and Mn(II) in solid production wastes by ac square-wave polarography with use of graphite and dropping-mercury electrodes has been described [36]. The supporting electrolyte used was the ammonium buffer with addition of sulphosalicylic acid which acted as a reduction potential shift reagent [36]. Separation of polarographic waves of Zn(II), Ni(II) and Co(II) in potassium chloride by differential complexation with potassium salicylate and iminodiacetic acid has been performed [37].

2. Theoretical background

2.1. Multivariate methods

Classical least squares (CLS), sometimes known as K-matrix calibration, can be applied to simple systems where the concentration values for all of the components present in the training samples are provided [38,39].

Principal component regression (PCR) is sometimes described as performing a least squares regression of the projections of the data onto the basis vectors of a factor space using inverse least squares [5,38–40].

The partial least squares (PLS) calibration technique using the orthogonalized PLS algorithm was developed by Wold [41,42] and extensively discussed by Martens and Naes [43]. PLS involves simultaneously the independent and the dependent variables in the data compression and decomposition operations [38,39,44].

2.2. Derivative ratio method

In order to understand the principle of this method, consider a mixture M of two components X and Y. The differential pulse polarographic waves of which are not influenced by the presence of the other, resulting in the additive signals obtained for the mixtures of the two compounds. If a linear relationship exists between response and concentration for all the potential values selected, the polarographic signal of the mixture, I_{Mi} (the current ' I in nA' measured over the potential range ' E in mV'), is defined by the equation

$$I_{Mi} = \alpha_{Xi} C_X + \beta_{Yi} C_Y \quad (1)$$

where I_{Mi} is the current I in nA of the mixture M at potential i ; α_{Xi} and β_{Yi} are the coefficients, analogous to extinction coefficient in spectrophotometric analysis of the pure compounds, X and Y at potential i , respectively; and C_X and C_Y are the concentration of X and Y, respectively.

In order to obtain an analytical signal for the mixture depends on a single analyte (e.g. Y), the following mathematical treatment is suggested: Dividing Eq. (1) by the corresponding equation for the signal of a standard X, normalized to unit concen-

tration, the following equations can be written:

$$\frac{I_{Mi}}{\alpha_{Xi}} = C_X + \left(\frac{\beta_{Yi}}{\alpha_{Xi}} \right) C_Y \quad (2)$$

$$I_{ri} = a + b C_Y \quad (3)$$

Plotting the current ratio value, I_{ri} , at a given potential, i , as a function of C_Y provides a straight line whose intercept, a , is dependent on C_X . The slope, b , of the straight line is equal to the ratio of (β_{Yi}/α_{Xi}) . The current ratio spectrum, I_{ri} can be considered the sum of two curves: (i) a true curve (bC_Y) and (ii) the constant, a . The constant term, a , that appears in Eq. (3) can be eliminated by differentiation. The current ratio spectra thus obtained are then differentiated with respect to potential and the derivative values for a given potential are plotted against C_Y to give a calibration graph. Application of the method to the sample containing both X and Y, and use of the calibration graph, will then give the value of C_Y in the mixture. X can be determined by an analogous procedure.

3. Experimental

3.1. Apparatus and software

The polarograms were obtained with a Metrohm 693 VA processor. A Metrohm 694 VA stand was used in the dropping mercury electrode (DME) mode. The three electrodes system was completed by means of a Ag/AgCl (3 M KCl) reference electrode and a Pt auxiliary electrode. The pH measurements were carried out by means of a Schott Gerate pH-Meter CG 820 at 25 °C. A visual BASIC program [45] was used for the differentiation of ratio data. The Chemometrics Toolbox 3.02 Software [46] was used for the CLS, PCR and PLS calculations.

3.2. Reagents and standard solutions

Analytical grade of acetic acid and sodium acetate were used for the preparation of 0.1 M acetate buffer (pH 4.2). Analytical grade of ZnSO₄, NiSO₄ and deionized H₂O were used all over the work.

Stock standard solution (1.0 mg ml⁻¹) of each metal was prepared in deionized H₂O. Suitable dilutions were made using the same solvent.

A training set consisting of 10 binary mixture solutions in the possible combinations containing 0.13–9.30 µg/ml Zn(II) and 0.20–12.25 µg/ml Ni(II) was used for the chemometric calibrations. A validation set containing the synthetic mixtures in the range of 0.29–9.00 µg/ml for Zn(II) and 0.30–11.60 µg/ml for Ni(II) was prepared by using the above stock solutions.

3.3. Sample preparation

Six tap drinking-water samples were collected. They were subjected up to a 20-fold preconcentration step by microwaving in pyrex vessels (if required for the determination of one or both

metals). Then, they were analysed after acidification with 0.1 M acetate buffer (pH 4.2).

3.4. Polarographic measurements

A 10 ml aliquot of standard (or sample) solution, previously acidified with 0.1 M acetate buffer (pH 4.2), was transferred into the polarographic cell. The solution was de-aerated by bubbling nitrogen for 5 min. The differential pulse polarograms were obtained between –700 and –1200 mV with a –100 mV pulse amplitude, 6.667 mV/S scan rate and a drop size of ca. 0.60 mm² drop area.

Quantitative results were obtained by the standard additions method. Appropriate aliquots of the standard solutions of the two metals were added and the concentration was evaluated.

4. Results and discussion

Acetate buffer has been selected, in between other tested buffers, and it was found to show relatively better separation of the polarographic waves. The effect of pH on the intensity of the polarographic waves has been studied and it was found to be optimum at pH 4.2.

The differential pulse polarograms of 1 µg/ml Zn(II) and 1 µg/ml Ni(II) in aqueous solutions previously acidified with 0.1 M acetate buffer (pH 4.2) in the range of –700 to –1200 mV potential region are shown in Fig. 1. The strong overlap between the two metals polarograms can be seen. The reduction potentials of both metals are very similar (–988 and –996 mV, respectively). This fact shows the impossibility

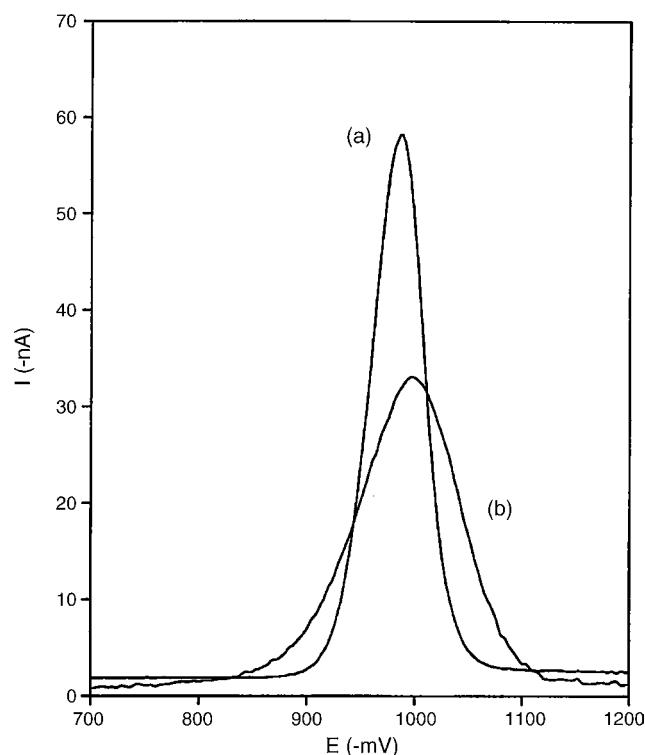


Fig. 1. Differential pulse polarograms of 1 µg/ml Zn(II) (a) and 1 µg/ml Ni(II) (b) in aqueous solutions previously acidified with 0.1 M acetate buffer (pH 4.2).

Table 1
Composition of the training and the validation sets

Mixture no.	Training set (μg/ml)		Validation set (μg/ml)	
	Zn(II)	Ni(II)	Zn(II)	Ni(II)
1	0.13	6.50	0.29	11.60
2	0.30	12.25	0.50	11.00
3	0.50	10.00	0.60	4.80
4	1.00	2.00	1.50	3.00
5	1.50	1.50	2.00	2.00
6	2.40	1.20	3.00	1.50
7	5.00	1.00	5.00	0.70
8	6.00	0.60	6.50	0.50
9	9.30	0.31	8.00	0.35
10	8.00	0.20	9.00	0.30

of their simultaneous determination by DPP directly in a mixture.

4.1. Multivariate methods

Ten mixtures of Zn(II) and Ni(II) standard solutions containing different proportions of both metals were selected as the training set (Table 1). Their composition is orthogonally designed in order to obtain maximum information on each metal from the calibration procedure. The lower and higher concentrations used were 0.13 and 9.30 μg/ml, respectively, for Zn(II); 0.20 and 12.25 μg/ml, respectively, for Ni(II). Other concentrations out of the training set were tested but invalid results were obtained. So, samples dilution or concentration might be

required prior to analysis. Below the lower concentration limits of both elements in the training set, the noise contribution increased significantly, whereas above the higher limits, both metals did not show linear response. The number of calibration mixtures in the training set was selected according to the rule of five. This rule states that using five times the number of samples as there are components provides enough samples to reasonably represent all possible combinations of concentration values [38]. The polarograms of this calibration set were recorded and their current values were measured within the potential range −920 to −1052 mV at 2 mV intervals.

The chemometric calibrations were computed with the CLS, PCR and PLS algorithms, using the correlation for the training set concentration and its current data. The developed calibrations were validated by determining the content of Zn(II) and Ni(II) in the validation set mixtures. The composition of this validation set is shown in Table 1. The mean percentage recoveries obtained using CLS, PCR and PLS were found to be $99.5 \pm 1.5\%$, $100.0 \pm 1.1\%$ and $100.0 \pm 1.0\%$ for Zn(II) and $99.4 \pm 1.3\%$, $99.7 \pm 1.2\%$ and $99.9 \pm 1.0\%$ for Ni(II), respectively (Table 2). These results contributed to the high accuracy and precision of the proposed multivariate methods. The obtained results were compared with each other and with those obtained by derivative ratio methods using *t*- and *F*-tests between the smallest and highest mean percentage recoveries and variances. The calculated values were not exceeded the theoretical ones, therefore, there is no significant difference between the proposed methods of analysis with regards to accuracy (*t*-test) and precision (*F*-test).

Table 2
Assay results of Zn(II) and Ni(II) combinations in synthetic mixtures (validation mixtures) by the proposed chemometric methods

Mixture no. ^a	Recovery (%)											
	Multivariate methods						Derivative ratio methods ^b					
	Zn(II)			Ni(II)			Zn(II)				Ni(II)	
	CLS	PCR	PLS	CLS	PCR	PLS	¹ D _{−950}	¹ D _{−1010}	¹ D _{−950} − ¹ D _{−1010}	² D _{−986}	¹ D _{−1030}	² D _{−1010}
1	99.3	100.1	99.9	100.0	100.4	100.3	99.8	98.8	99.2	99.4	101.2	101.0
2	101.7	101.5	101.4	98.1	98.1	98.8	101.6	101.3	101.0	101.5	99.1	99.2
3	98.8	99.7	99.7	101.3	101.0	101.5	99.1	98.5	98.6	98.8	102.6	102.5
4	99.4	100.0	100.0	99.5	99.8	99.9	99.7	99.6	99.7	99.5	99.9	99.9
5	98.5	99.4	99.1	97.4	97.8	98.7	98.9	97.9	98.3	98.3	98.9	99.0
6	97.5	98.4	98.4	100.2	101.3	100.6	98.0	97.0	97.8	97.4	101.7	101.5
7	99.9	100.0	100.2	99.2	99.3	99.7	100.3	100.2	100.3	99.9	100.2	100.1
8	101.8	102.0	101.8	97.8	98.5	98.5	101.2	101.6	101.6	101.6	98.8	98.8
9	97.7	99.1	99.0	100.9	100.7	100.8	97.9	97.2	97.5	97.7	101.9	101.8
10	99.9	100.2	100.3	99.8	100.0	99.9	100.2	99.7	100.0	99.8	100.5	100.5
Mean	99.5	100.0	100.0	99.4	99.7	99.9	99.7	99.2	99.4	99.4	100.5	100.4
Variance	2.3	1.2	1.0	1.7	1.4	1.0	1.4	2.6	2.0	2.0	1.7	1.7
±S.D.	1.5	1.1	1.0	1.3	1.2	1.0	1.2	1.6	1.4	1.4	1.3	1.3
R.S.D.	1.5	1.1	1.0	1.3	1.2	1.0	1.2	1.6	1.4	1.4	1.3	1.3
<i>t</i> ^c		1.33	(Zn(II))					1.89	(Ni(II))			
<i>F</i> ^c		2.60	(Zn(II))					1.70	(Ni(II))			

R.S.D., relative standard deviation.

^a Same mixtures described in Table 1.

^b Subscripts stand for the potential in mV.

^c Theoretical *t*(0.05)=2.101 and *F*(0.05)=3.18.

4.1.1. Statistical analysis

The predictive ability of a model can be defined using several validation diagnostics. These include the standard error of prediction (SEP), the mean squared error of prediction (MSEP), the root mean standard error of prediction (RMSEP) and the variance of prediction (s^2) [38,39]. The MSEP and the RMSEP characterize both the accuracy and the precision of prediction [39].

$$\text{SEP} = \left[\frac{\sum_{i=1}^n (C_i^{\text{True}} - C_i^{\text{Predicted}})^2}{n-1} \right]^{1/2}$$

$$\text{MSEP} = \frac{\sum_{i=1}^n (C_i^{\text{True}} - C_i^{\text{Predicted}})^2}{n}$$

$$\text{RMSEP} = \left[\frac{\sum_{i=1}^n (C_i^{\text{True}} - C_i^{\text{Predicted}})^2}{n} \right]^{1/2}$$

$$s^2 = \frac{\sum_{i=1}^n (C_i^{\text{Predicted}} - C_i^{\text{True}} - \text{bias})^2}{n-1}$$

where C_i^{True} is the true concentration, $C_i^{\text{Predicted}}$ is the predicted concentration and n is the total number of samples. The numerical values of SEP, MSEP, RMSEP and s^2 are indicated in Table 3.

Another way to validate the models and to examine the results is the predicted versus known concentration plot. Points in this plot are expected to fall on a straight line with a slope of one and a zero intercept [39]. The regression analysis for these linear relationships has been carried out and the results are shown in Table 3. The absence of bias has been proved by determining the confidence limits for the intercept, a , and the slope, b , at the 95% significance level [47]. The upper and lower confidence limits are shown in Table 3. For Zn(II) and Ni(II) using the three

proposed chemometric models, the 95% confidence interval of the intercept includes the ideal value of zero and that of the slope includes the ideal value of one. This indicates no problems with the models (no bias nor lack of fit). Absence of bias confirms the trueness of the studied methods. Furthermore, there do not appear to be any sample(s) that are unusually far from the line than the rest of the data.

Determining how many factors to use in the calibration is a key step in factor based techniques. Only those factors that contain analytical information must be kept. The discarded factors should contain only noise [38,39,48–50]. The chemometrics Toolbox 3.02 Software offers several indicators or functions which could be used for determining the optimum number of factors (rank). The cross-validation procedure leaving out one sample at a time, is used for this purpose [38] and the predicted residual error sum-of-squares, PRESS is calculated.

$$\text{PRESS} = \sum_{i=1}^n (C_i^{\text{True}} - C_i^{\text{Predicted}})^2$$

where C_i^{True} represents the true concentration, $C_i^{\text{Predicted}}$ denotes the predicted concentration and n is the total number of samples.

A better criterion for selecting the optimum number of factors involves the generation of a calibration for every possible rank. Each calibration is used to predict the concentrations for a set of independently measured, independent validation samples. Then the PRESS is calculated [38]. Another way to determine the number of factors is the two-way F -test on reduced eigenvalues (REV's) according to the method of Malinowski [38]. A rank of two factors was found to be the optimum system rank for both the PCR and the PLS models according to the three studied criteria. Fig. 2 shows the obtained (PRESS) values with an independent validation set, those with cross-validation technique (CROSS) and the reduced eigenvalues (REV) for the PCR and PLS methods using the different number of factors.

Table 3
Statistical parameters of the validation synthetic mixtures

Method	SEP ($\times 10^2$)	MSEP ($\times 10^3$)	RMSEP ($\times 10^2$)	s^2 ($\times 10^3$)	a ($\times 10^3$)	Lower ^a 95%	Upper ^a 95%	b	Lower ^b 95%	Upper ^b 95%	r
Zn(II)											
CLS	7.77	5.44	7.38	5.62	−4.21	−0.092	0.084	0.996	0.977	1.014	0.99974
PCR	5.26	2.49	4.99	2.76	−4.86	−0.067	0.057	1.002	0.989	1.015	0.99987
PLS	5.12	2.36	4.86	2.62	−5.88	−0.067	0.055	1.002	0.989	1.015	0.99988
¹ D _{−950}	6.59	3.91	6.25	4.12	−2.17	−0.078	0.073	0.997	0.981	1.013	0.99981
¹ D _{−1010}	8.94	7.19	8.48	7.11	−3.30	−0.100	0.094	0.993	0.973	1.014	0.99969
¹ D _{−950} – ¹ D _{−1010}	7.94	5.67	7.53	5.89	−2.82	−0.093	0.087	0.996	0.977	1.014	0.99973
² D _{−986}	7.63	5.24	7.24	5.27	−4.06	−0.088	0.080	0.995	0.977	1.013	0.99976
Ni(II)											
CLS	7.50	5.07	7.12	5.07	6.42	−0.057	0.070	0.992	0.980	1.004	0.99989
PCR	7.50	5.06	7.11	5.35	7.17	−0.063	0.077	0.994	0.981	1.007	0.99987
PLS	5.23	2.47	4.97	2.71	6.92	−0.044	0.058	0.997	0.987	1.006	0.99993
¹ D _{−1030}	7.15	4.60	6.78	4.79	6.06	−0.064	0.076	1.003	0.990	1.016	0.99988
² D _{−1010}	6.38	3.66	6.05	3.82	6.39	−0.056	0.069	1.002	0.991	1.014	0.99990

SEP, standard error of prediction; MSEP, mean squared error of prediction; RMSEP, root mean standard error of prediction; s^2 , variance of prediction; a , intercept; b , slope; r , correlation coefficient.

^a Lower and upper confidence limits for the intercept at the 95% confidence level.

^b Lower and upper confidence limits for the slope at the 95% confidence level.

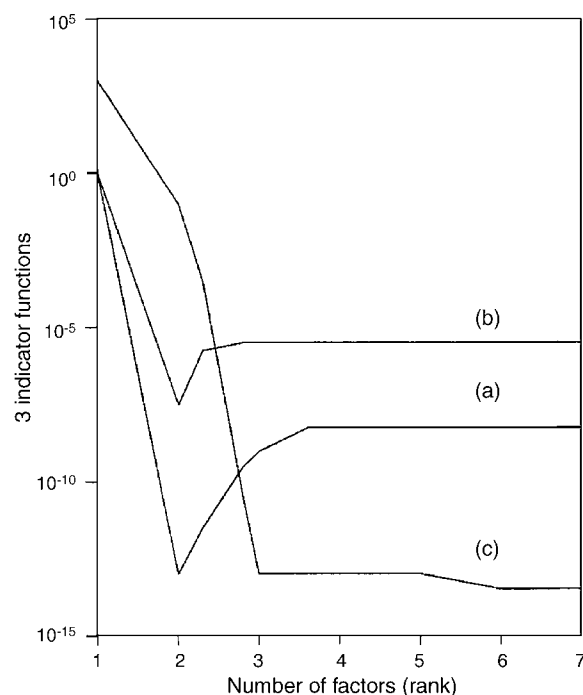


Fig. 2. PRESS (a), CROSS (b) and REV (c) vs. number of factors for the PCR and PLS models of Zn(II) and Ni(II).

4.2. Derivative ratio methods

Fig. 3(a) shows the current ratio curve of 1 $\mu\text{g/ml}$ Zn(II) divided by Ni(II) normalized to unit concentration. Its first, ^1D and second, ^2D derivative curves are shown in Fig. 3(b and c). The optimum working potentials were found to occur at $E = -950$ and -1010 mV in the first derivative curve and at $E = -986$ mV in the second derivative curve.

Similarly Fig. 4(a) shows the current ratio curve of 1 $\mu\text{g/ml}$ Ni(II) divided by Zn(II) normalized to unit concentration. Its first and second derivative curves are shown in Fig. 4(b and c). The optimum working potentials were found to occur at $E = -1030$ and -1010 mV in the first and second derivative curves, respectively.

All the selected working potentials coincide with maxima in the derivative curves.

The ^1D and ^2D values calculated for Zn(II) and Ni(II) polarograms were correlated to concentration and the regression data are listed in Table 4.

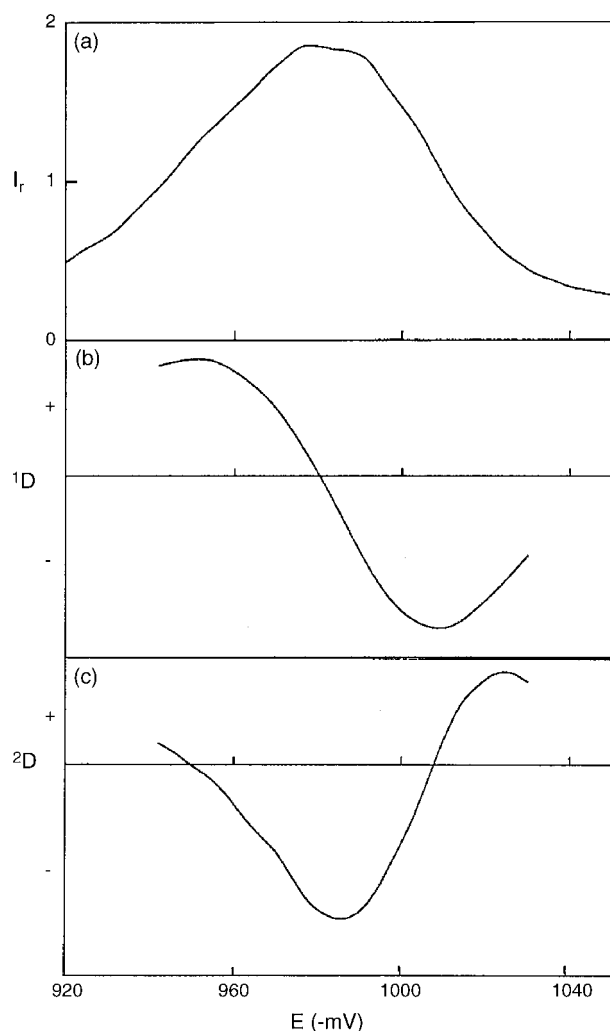


Fig. 3. Current ratio curve of 1 $\mu\text{g/ml}$ Zn(II) (Zn(II)/Ni(II)) (a); its first derivative curve (b) and its second derivative curve (c).

In order to demonstrate the validity of the proposed derivative ratio methods for the determination of Zn(II) and Ni(II) in combination, 10 synthetic mixtures of both metals were prepared in different ratios (same mixtures of the validation set (Table 1)). The polarographic measurements were taken for all solutions within the potential range -920 to -1052 mV at 2 mV intervals. The current ratio curves were developed. The ^1D and ^2D curves were computed [45] ($\Delta E = 22$ and 14 mV) for the current

Table 4
Regression data of the derivative ratio methods used for the determination of Zn(II) and Ni(II)

Metal	Measurement	Linearity range ($\mu\text{g/ml}$)	Regression data					
			<i>a</i>	<i>b</i>	$S_{Y/X}$	S_a	S_b	<i>r</i>
Zn(II)	$^1\text{D}_{-950}$	0.28–9.30	1.26×10^{-2}	1.284	4.45×10^{-2}	3.13×10^{-2}	5.70×10^{-3}	0.99996
	$^1\text{D}_{-1010}$	0.28–9.30	1.49×10^{-2}	1.666	6.30×10^{-2}	4.43×10^{-2}	8.07×10^{-3}	0.99995
	$^1\text{D}_{-950}$ – $^1\text{D}_{-1010}$	0.28–9.30	1.91×10^{-2}	2.953	9.44×10^{-2}	6.63×10^{-2}	1.21×10^{-2}	0.99997
	$^2\text{D}_{-986}$	0.28–9.30	4.79×10^{-3}	0.583	2.39×10^{-2}	1.68×10^{-2}	3.06×10^{-3}	0.99995
Ni(II)	$^1\text{D}_{-1030}$	0.25–12.25	2.51×10^{-2}	1.865	0.1222	8.57×10^{-2}	1.16×10^{-2}	0.99990
	$^2\text{D}_{-1010}$	0.25–12.25	-2.25×10^{-3}	0.254	1.58×10^{-2}	1.11×10^{-2}	1.49×10^{-3}	0.99991

a, Intercept; *b*, slope; $S_{Y/X}$, residual standard deviation; S_a , standard deviation of the intercept; S_b , standard deviation of the slope.

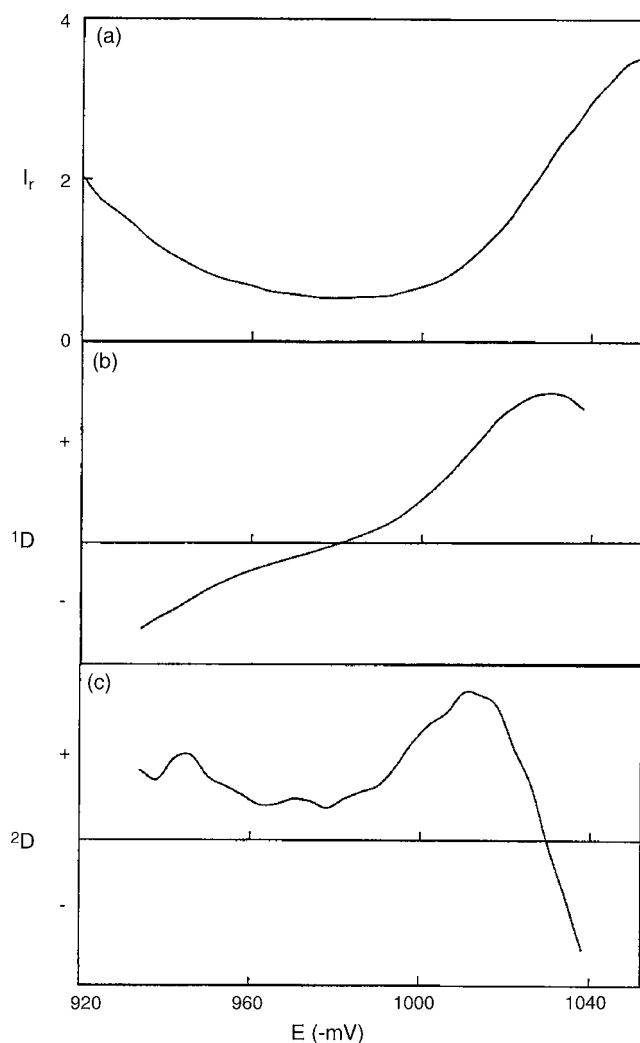


Fig. 4. Current ratio curve of 1 µg/ml Ni(II) (Ni(II)/Zn(II)) (a); its first derivative curve (b) and its second derivative curve (c).

ratio curves data of both metals, respectively. The 10 mixtures have been analysed for their Zn(II) and Ni(II) content using the first and second derivative ratio methods at the selected optimum working potentials. Furthermore, peak-to-peak difference of the ^1D curve of the current ratio curve of Zn(II) has been calculated at -950 and -1010 mV.

The mean percentage recoveries obtained using $^1D_{-950}$, $^1D_{-1010}$, $^1D_{-950}-^1D_{-1010}$ and $^2D_{-986}$ for Zn(II) were found to be $99.7 \pm 1.2\%$, $99.2 \pm 1.6\%$, $99.4 \pm 1.4\%$ and $99.4 \pm 1.4\%$;

using $^1D_{-1030}$ and $^2D_{-1010}$ for Ni(II) were found to be $100.5 \pm 1.3\%$ and $100.4 \pm 1.3\%$, respectively (Table 2). These results show good accuracy and precision of the proposed derivative ratio methods.

4.2.1. Statistical analysis

The predictive ability of the proposed derivative ratio methods can be defined using the same validation diagnostics previously discussed with the multivariate methods. The numerical values of SEP, MSE, RMSEP, s^2 and the regression data for the predicted versus known concentration plot were calculated and indicated in Table 3.

4.3. Interferences of other elements

To study interferences, polarograms were recorded in the range from -400 to -1500 mV. Metal ions tested included Cd, Co, Pb, Fe, Mn, Ca, Mg, Cu and Al. The recoveries of 0.28 µg/ml Zn(II) after the addition of different elements were ~ 99 – 102% within the potential range of -400 to -1500 mV. A similar study was carried out for 0.25 µg/ml Ni(II). Good recoveries ~ 98 – 101% were obtained.

Only Co exhibits a neighbouring peak at -1218 mV but without interference with the Zn(II) and the Ni(II) signals up to a 100-fold excess of Co(II).

4.4. Determination of Zn(II) and Ni(II) in tap drinking-water samples

The concentrations of Zn(II) and Ni(II) were determined in six tap drinking-water samples using the proposed multivariate and derivative ratio methods (Table 5). The results were compared with those obtained using the zincon and the heptoxime colorimetric reference methods for the determination of zinc and nickel, respectively [18] (Table 5). The differences between the studied methods and the reference methods were found to be within -1.20 to 1.50% and -2.08 to 0.69% for the levels of Zn(II) and Ni(II) found, respectively.

4.4.1. Recovery of added metals

To determine the recovery of Zn(II) and Ni(II), appropriate volumes of the standard solutions were added to the investigated samples. The results listed in Table 6 show mean percentage recoveries ranging from 99.5 to 100.9% for both metals.

Table 5
Determination of Zn(II) and Ni(II) in tap drinking-water samples

	Concentration found (µg/ml $\times 10^3$)													
	Multivariate methods						Derivative ratio methods						Reference methods	
	Zn(II)			Ni(II)			Zn(II)			Ni(II)			Zn(II)	Ni(II)
	CLS	PCR	PLS	CLS	PCR	PLS	$^1D_{-950}$	$^1D_{-1010}$	$^1D_{-950}-^1D_{-1010}$	$^2D_{-986}$	$^1D_{-1030}$	$^2D_{-1010}$		
Mean ^a	331	330	333	14.3	14.4	14.5	339	335	337	336	14.1	14.3	334	14.4
R.S.D.	1.7	1.4	1.6	1.8	1.9	1.7	1.9	1.9	1.8	1.6	1.8	2.0	1.6	1.8

Samples were subjected to a 20-fold preconcentration step before analysis; R.S.D., relative standard deviation.

^a Mean of six samples.

Table 6

Recovery experiments of Zn(II) and Ni(II) added in drinking-water samples

Sample no.	Zn(II) added ($\mu\text{g/ml}$)	Ni(II) added ($\mu\text{g/ml}$)	Recovery (%)												
			Multivariate methods						Derivative ratio methods						
			Zn(II)			Ni(II)			Zn(II)				Ni(II)		
			CLS	PCR	PLS	CLS	PCR	PLS	$^1\text{D}_{-950}$	$^1\text{D}_{-1010}$	$^1\text{D}_{-950}-^1\text{D}_{-1010}$	$^2\text{D}_{-986}$	$^1\text{D}_{-1030}$	$^2\text{D}_{-1010}$	
1	0.1	0.010	101.2	100.6	100.7	100.0	99.9	99.8	100.4	100.6	100.5		100.6	100.4	100.0
2	0.2	0.015	99.8	99.2	99.6	101.4	101.1	100.7	98.7	98.7	98.7		98.9	101.9	101.6
3	0.3	0.020	101.4	101.0	100.8	98.4	98.5	98.7	100.9	101.3	101.0		101.0	98.6	98.2
4	0.5	0.100	98.4	97.8	98.4	101.7	101.7	101.6	97.5	97.6	97.6		97.7	102.3	102.1
5	0.8	0.150	102.1	101.5	101.5	99.2	99.5	99.4	101.6	102.0	101.5		101.6	99.2	98.8
6	1.0	0.200	98.9	98.4	98.5	102.6	102.1	101.6	97.9	98.1	98.1		98.3	102.9	102.7
Mean			100.3	99.8	99.9	100.6	100.5	100.3	99.5	99.7	99.6		99.7	100.9	100.6
$\pm\text{S.D.}$			1.5	1.5	1.3	1.6	1.4	1.2	1.7	1.8	1.6		1.6	1.8	1.8

5. Conclusion

The studied polarographic chemometric methods multivariate (CLS, PCR and PLS) and derivative ratio (first and second) proved to be accurate and precise in the determination of Zn(II) and Ni(II) in aqueous solutions previously acidified with 0.1 M acetate buffer (pH 4.2). The applicability of the methods was assessed through the determination of both metals in tap drinking-water. The results were compared with those obtained using the zincon and the heptoxime colorimetric reference methods for the determination of zinc and nickel, respectively [18].

The proposed methods are useful for the determination of analytes in mixtures by DPP without the need for a prior separation or special conditions to resolve the analytes waves. The ensuring methods are simple, inexpensive, precise and affordable; also they require no complex pretreatment. So, they are suggested to be used in routine analysis of mixtures of analytes giving overlapped polarographic waves.

The superiority of the multivariate methods over the derivative ratio methods is manifested in being fast, easy, simple and do not require prerequisite for the successful application. The derivative ratio methods require that the polarogram of each metal is divided by that of the other to obtain two ratio curves. A suitable derivative order curve (first or second) should be derived from each of the two ratio curves in order to correct for the constant term resulted from the above mentioned divisions to obtain the concentration of the studied components.

The factor based methods (PLS and PCR) are suggested to be used if there are interferences, taking in consideration that the interfering component(s) must be included in the training set. Interferences should be absent whenever the derivative ratio method is used.

Chemometric methods open up new prospects for the resolution of mixtures with highly overlapped polarographic waves.

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Fast steel-cleanliness characterization by means of laser-assisted plasma spectrometric methods[☆]

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Abstract

Laser-assisted plasma spectrometry is a palette of analytical techniques (L-OES, LA-ICP-MS) capable of fast spatially-resolved elemental analysis in the micrometer range. For fast estimation of the occurrence in steel samples of non-metallic inclusions, which degrade the material's technical properties, simultaneous OES detection and sequential ICP-MS detection were compared. Histograms were obtained for the intensity distribution of the acquired signals (laser pulse statistics). The skewness coefficient of the histograms for Al (indicator of non-metallic inclusions) was found to be clearly dependent on the fraction of non-metallic inclusions in the case of scanning L-OES. For LA-ICP-MS less clear dependence was observed, which was influenced by the acquisition characteristics. In fact, less measurement throughput limited for LA-ICP-MS the counting statistics to an extent that overrides the benefit of higher detection power as compared to L-OES.

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1. Introduction

Steel is a complex high-tech material with elaborated characteristics for several industrial applications. Its properties are a sensitive function of cleanliness, and in fact non-metallic inclusions are local defects in the steel matrix and could degrade the mechanical properties of the material drastically. The negative impact of non-metallic inclusions becomes dramatic especially during reforming, in the manufacture of products with reduced end size, e.g. steel sheets for the light and secure body of motor vehicles or for deep drawn beverage cans, or steel cords for automotive tires.

Therefore, there is a significant interest of steel industry in the potential of several direct solid micro-analytical techniques, which has impacts on quality assurance close to production process. Fast estimation of the number, the size distribution, and the chemical composition of non-metallic inclusions in a metal-

lic steel matrix is important and implemented in process and product control. In addition to the representativity of the analytical results, mostly obtained on statistical basis of the scanned ensemble, high sample throughput and low time consumption are critical requirements put forward by the steel industry.

Several techniques have the potential to be the method of choice, i.e. allowing a reliable and fast in situ access to the population of non-metallic inclusion in a sample. Spark-induced optical emission spectrometry (S-OES) is operated since decades in terms of bulk analysis in steel plants. S-OES with signal recording of each single spark discharge has been reported for few years [1,2]. Unfortunately the spark discharge is influenced by the grain structure, so that the evaluation of individual spark intensities could be used only for a limited number of steel grades, up to now. Electron-probe microanalysis (EPMA) [3] is a time consuming technique due to both, the extensive sample preparation (mirror polishing) as well as the relatively long single analysis time in element mapping mode. Furthermore, the requirement for vacuum conditions does not allow direct online analysis. Laser-assisted plasma spectrometry [4], i.e. laser-induced optical emission spectrometry (L-OES, also known as 'laser-induced breakdown spectrometry', LIBS) and inductively coupled plasma mass spectrometry with

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micro-sampling by laser ablation (LA-ICP-MS), are rapid and powerful techniques for these kinds of industrial applications. LA-ICP-MS in combination with quadrupole mass filters is a sequential detection mode characterized by high detection power. Detection limits are reported to be in the low $\mu\text{g g}^{-1}$ or even in the ng g^{-1} -range depending on the target element and detection unit. OES is typically less sensitive as a technique for the analysis of most metallic elements [4–6], but with photo multiplier detection it can be coupled to laser ablation which enables to perform simultaneous element analysis and improves counting statistics. Additionally, L-OES is capable to detect also the light elements like B, C, P and S in the low $\mu\text{g g}^{-1}$ range.

Scanning element mapping by L-OES, focussing distinct laser pulses onto the sample surface, has already been described by a number of workers like a group around Geertsen et al. [7], Kim et al. [8], Cravetchi et al. [9] as well as by Menut et al. [10]. Element mapping by means of scanning LA-ICP-MS has been performed by Van der Putten et al. [11] to determine the spatial distribution of trace elements in the calcite shell of mussels as well as by Latkoczy et al. [12] for the determination of the surface element distribution of Mg, Al, and Zn in multi-phase magnesium-base alloys. Characterisation of inclusions in clean steels *via* LA-ICP-MS has been reported by Dubuisson et al. [13]. Inclusions generating coincident signal spikes for Mn–S, Mg–Al, and Mg–Al–Ca–Ti–Si were studied. Laserna and co-workers [14] proposed a two dimensional inclusion mapping by use of a cylindrical lens for focussing a pulsed laser beam onto the sample surface. This leads to ‘micro-line plasma’ which can be analysed in spatially-resolved mode using an optical emission spectrometer with charge-coupled device (CCD) detection. The detection power is highly variable along the micro-line plasma, because of different plasma characteristics in the inner and outer

parts of the plasma line. Low pulse repetition rate of 10 Hz does not enable to perform really fast 2D mapping. Two dimensional L-OES mapping by means of a 1 kHz repetition rate laser for scanning in a 2D raster and estimation of inclusion contents in ferrous materials is reported more detailed elsewhere [1,15].

2. Experimental

2.1. Instrumentation

2.1.1. L-OES

A 1 kHz Nd:YAG laser system at the Fraunhofer ILT (Aachen, Germany) was implemented at fundamental harmonic of 1064 nm and at 6 ns pulse duration (FWHM). The beam is characterized by a gaussian intensity profile (TEM_{00}), $M^2 < 1.3$. Self designed measuring chamber with pre-chamber for Ar 5.0 measuring atmosphere equipped with automatic x and y stages was used as sample holder. The OES detection unit was a Paschen–Runge spectrometer (Thermo ARL LaserSpark) with removed laser, sample stand, and recording electronics. The system comprised 41 element channels (photo multiplier tubes, PMTs) for simultaneous recording in the 130–777 nm spectral range. After a delay of 500 ns after the laser pulse, an interval of 9.5 μs for integral recording of emission radiation was used. The entire L-OES measurement is performed by scanning a raster of 500×500 steps with 15 μm step width, leading to 250,000 individual measurements on each sample within less than 15 min.

2.1.2. LA-ICP-MS

An in-house modified Nd:YAG laser system (DCR-11, Spectra Physics) owned by the Swiss Federal Laboratories for Materials Testing and Research (EMPA) was implemented in combi-

Table 1
Certified element contents for low-alloy steel CRMs, mean, median, standard deviation, and skewness coefficient of LA-ICP-MS and L-OES data (both: blank subtracted, except background values; S.D.: standard deviation)

CRM	Background	BCS 271 (SS 11)	BCS 272 (SS 12)	BCS 274 (SS 14)
Certificate				
Al _{tot} (wt.%)		0.008	0.068	0.032
Al _{sol} (wt.%)		0.008	0.064	0.029
Calculated from certificate				
Al _{insol} (wt.%)		0	0.004	0.003
Al _{insol} /Al _{tot}		0	0.059	0.094
LA-ICP-MS ²⁷ Al (a.u.)				
Mean	5507	180	1988	10300
Median	5506	27	2162	6197
S.D.	299	2209	1982	50408
Skewness coefficient	0.01	0.21	−0.26	0.24
L-OES at 396.15 nm (a.u.)				
Mean	61.8	23.0	260.5	134.9
Median	62.0	21.0	255.0	115.0
S.D.	6.5	25.4	78.4	130.5
Skewness coefficient	−0.10	0.23	0.21	0.46
L-OES at 308.22 nm (a.u.)				
Mean	21.7	19.7	149.7	81.1
Median	22.0	19.0	146.0	71.0
S.D.	2.3	14.6	49.5	77.2
Skewness coefficient	−0.43	0.15	0.22	0.39

nation with a quadrupole ICP-MS. The laser was operated at the fourth harmonic of 266 nm, with a Q-switch delay of 300 ns, 6 ns pulse duration (FWHM), 10 Hz, 4 mJ pulse energy distributed in a ring profile (Bessel shape). The focussing lens was $f = 40$ mm, used in combination with a pinhole of adjustable diameter to obtain flexible spot size at constant fluence. A 30 cm³ ablation cell with inlet nozzle for the carrier gas (0.9 l/min, Ar 4.8) was used. The ICP-MS was PE/Sciex ELAN6000, run under standard operating conditions, namely RF power of 1300 W, plasma gas flow rate of 15 l/min. Ar 4.8 plasma gas. The isotope measurement method was edited as follows: dwell time, 100 ms for ²⁷Al; 30 ms for ⁵⁷Fe, in peak hopping mode. The measurement on each sample was performed by delivering 300 pulses on each of five sample positions (i.e. five replicates of each 300 pulses).

2.2. Samples and standard materials

Sample preparation was performed either by filing the sample slabs for LA-ICP-MS or by milling them for L-OES, each immediately before analysis to avoid surface contamination and oxidation. A set of low-alloyed steel standards (certified reference materials, CRMs), namely BCS 271 (SS11), BCS 272 (SS12), and BCS 274 (SS14), with certified contents (reported in Table 1) of total aluminium and of acid soluble aluminium (representing the metallic aluminium content in opposite to alumina content) were analysed by LA-ICP-MS as well as by L-OES.

3. Results and discussion

3.1. Data evaluation

The first step was to distinguish qualitatively among non-metallic inclusion and metallic matrix related signal. This was done using the emission signals of specific elements that are characteristic of each of the two phases and the intensity level of them. In fact, two classes of signal intensities were distin-

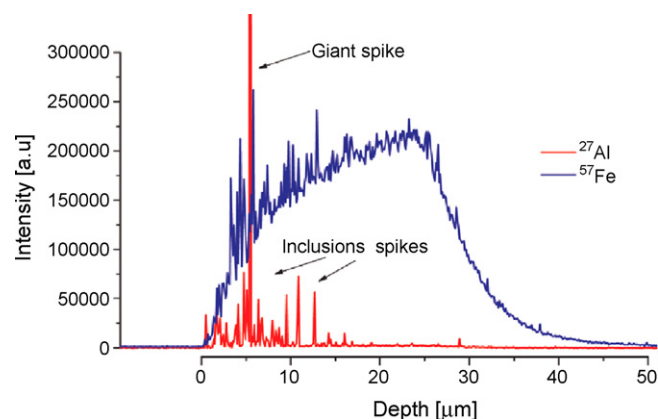


Fig. 2. LA-ICP-MS aluminium and iron signal (analyte: ²⁷Al, matrix ⁵⁷Fe) plotted vs. ablation depth.

guished in terms of the inclusion-related elements. A lower and continuous signal component was related to the ablation of elements in the metallic matrix, occurring in the 1–10 μg g⁻¹ concentration range. Superposed on such signal profiles, high spikes could be detected in concomitance with the ablation of individual or groups of inclusions. High intensity spikes were recognised and counted for L-OES (Fig. 1) as well as for LA-ICP-MS (Fig. 2). The ‘high’ and especially the ‘giant spikes’ are produced by the relative enrichment of the element within non-metallic inclusions, namely 53 wt.% Al in alumina (Al₂O₃) inclusions embedded in the steel matrix.

Various algorithms for inclusion analysis by means of spark and laser-induced plasma OES are discussed in the literature [1]. Histograms plotting the number distribution as a function of signal intensity were prepared to separate the inclusion-related and the matrix-related signal components, which is shown by the asymmetric shape (Fig. 3 for L-OES and Fig. 4 for LA-ICP-MS). The distribution is an indicative measure for the number and/or size of inclusions ablated. Actually, a giant pulse could be obtained either as an effect of number, i.e. many

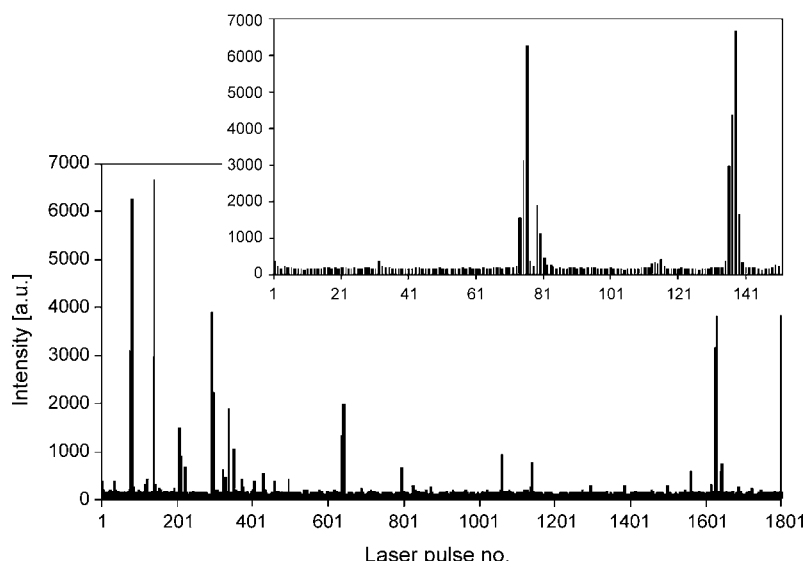


Fig. 1. Scanning L-OES aluminium signal (Al I) 308.22 nm) plotted vs. laser pulse number.

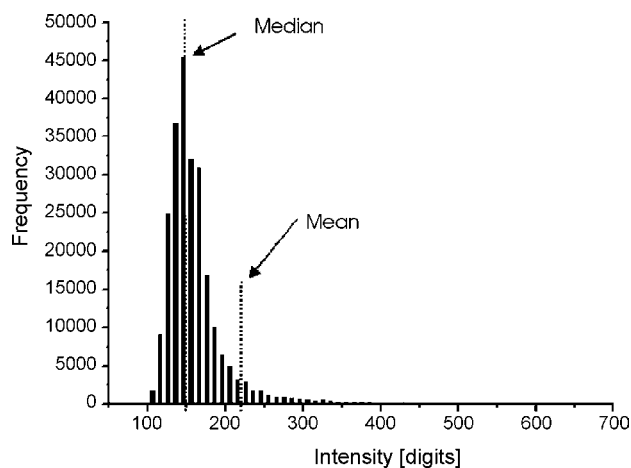


Fig. 3. Pulse intensity distribution (histogram) of Al (I) 308.22 nm signals of scanning L-OES on steel CRM with Al_2O_3 inclusions.

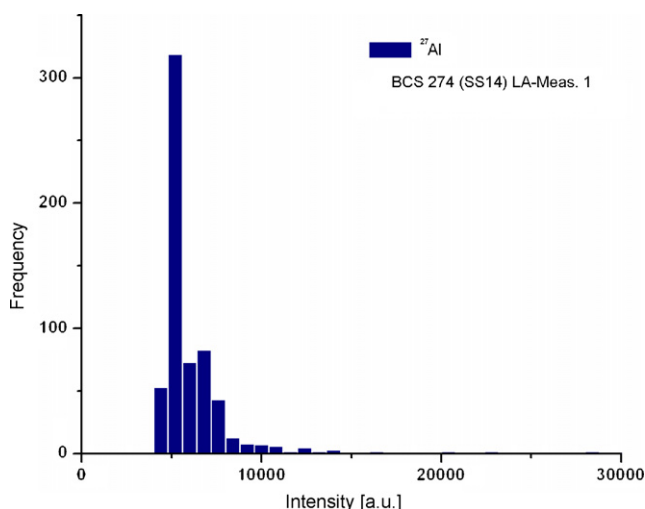


Fig. 4. Pulse intensity distribution (histogram) of ^{27}Al signals of LA-ICP-MS on steel CRM with Al_2O_3 inclusions.

small inclusions ablated altogether, or as an effect of size, i.e. few large inclusions. The density of the spikes might provide indication on the spatial distribution of the inclusion, and so indirectly can help to unravel the real source of signal build-up.

As an example, the spatial distribution of the Al (I) and the Ca (II) emission intensities at 308.22 nm, respectively, at 396.85 nm for a scan on a steel (CRM BCS 274) bearing Al and Ca rich inclusions are given in Fig. 5a and b. The positive tailing in the histogram, i.e. the extension of the signal hinge on the high intensity side from the distribution maximum, suggested that the inclusions under investigations were more prone to yield a clearly high signal above the continuum matrix-related intensity within a certain range. Thus, the size of the inclusion would be the most significant factor in spike build-up.

The skewness coefficient P (calculated following Pearson [16] formula) depends on the difference of the mean M and the median A as well as on the standard deviation s of the entire data set, as follows:

$$P = 3 \left(\frac{M - A}{s} \right) \quad (1)$$

3.2. Analytical results

For a preliminary study, a set of certified standard materials with low-alloyed steel matrix was analysed with both, LA-ICP-MS and L-OES. The total aluminium content as well as the so-called ‘acid soluble aluminium’ content was certified. Generally, one has to take into account that within the ‘acid soluble aluminium’ content [17,18] a fraction of some Al-precipitates as aluminium nitride (AlN) is contained, which usually can be enclosed in the case of real steels. Especially the small particles of these precipitates are known to tend to be (partly) dissolved under the recommended conditions for this test [19]. Thus, the fraction of aluminium-bearing inclusions can vary due to the different compounds and particle size distributions, and laser-based analysis could result in excess of inclusions detected in opposite to the Al-insoluble value.

The skewness coefficients for the simultaneously recorded and blank subtracted atomic emission intensities at Al (I) 308.22 nm and at Al (I) 396.15 nm as well as the ^{27}Al signal are depicted versus the ratio of the acid insoluble aluminium content and the total aluminium content in Fig. 6. The skewness increases with increasing inclusion fraction, most clearly for the

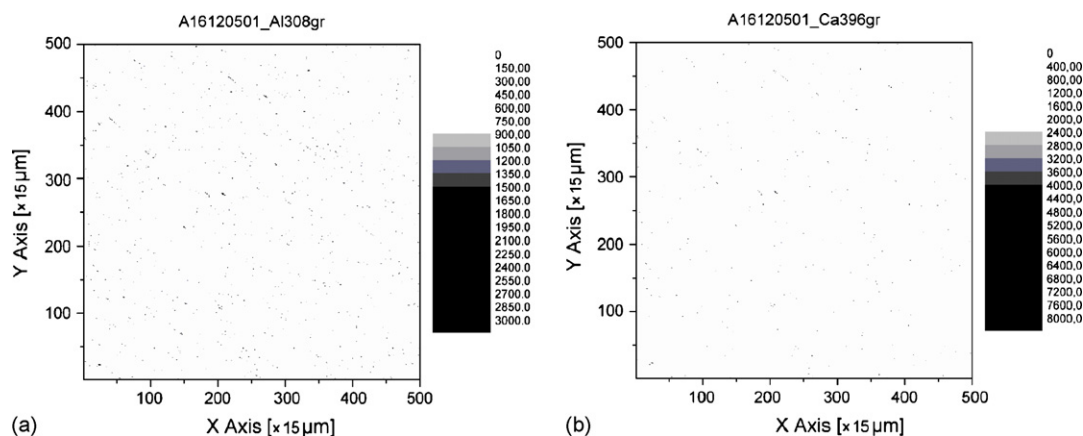


Fig. 5. Two-dimensional map of (a) Al (I) 308.22 nm and (b) Ca (II) 396.85 nm signals of scanning L-OES on steel CRM BCS 274 (SS 14) with $\text{CaO-Al}_2\text{O}_3$ inclusions (7.5 mm $\times$ 7.5 mm mapped area, 15 μm step with).

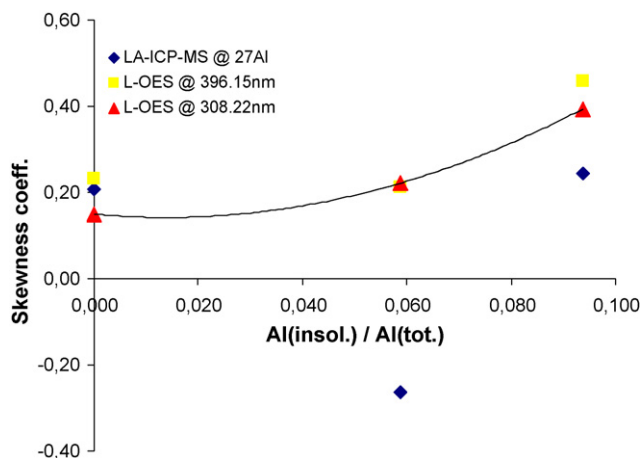


Fig. 6. Skewness coefficient of Al histograms for LA-ICP-MS and for L-OES vs. $Al_{\text{insol.}}:Al_{\text{tot}}$ ratio.

308.22 nm line. This dependence of the skewness on the fraction of insoluble Al, i.e. the $Al_{\text{insol.}}:Al_{\text{tot}}$ ratio, was independent of the total Al content, as far as the Al signal is characterized by high signal-to-background ratio. Otherwise the skewness coefficient would deviate, due to the fact that the difference between median and mean is more influenced by rounding-off and due to the increasing standard deviation next to the background level.

For the LA-ICP-MS measurements, the skewness coefficient and the $Al_{\text{insol.}}:Al_{\text{tot}}$ ratio do not show any clear dependence (Fig. 6). This could be the consequence of the lower number of single measurements which could be obtained at 10 Hz laser repetition rate within appropriate time for fast steel-cleanness characterisation. The lower number of single measurements is supposed to lead to poorer statistics in terms of hitting inclusions in the metallic matrix by random event occurring. Additionally, high Al blank level has an adverse influence on detection power. Another effect which has to be taken into account is the “pulse mixing”, as reported recently [20]. This mixing of sample aerosols within the ablation cell and the tubing on its path to the ICP torch produced from fast delivery of multiple laser pulses as compared to the flush frequency, reduces depth-related resolution.

From the current investigation, LA-ICP-MS and L-OES have equivalent detection performance. In fact, in the former case the detection limit is mostly limited by noise introduced during the transport stage and peak hopping detection mode, whereas in the latter case the optical absolute detection power is the limiting factor. The high repetition rate of 1000 Hz implemented in the L-OES method enabled to enhance measuring efficiency and speed by a factor of 100 compared to the 10 Hz sampling rate for LA-ICP-MS, with consequent higher counting statistics. Better pulse-to-pulse signal resolution and truly simultaneous element analysis are further advantages of the L-OES method.

A correlation between the $Al_{\text{insol.}}:Al_{\text{tot}}$ ratio and the skewness of the pulse height distribution was observed in terms of scanning L-OES for a set of iron-base certified reference materials.

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A preconcentration system using polyamine Metalfix-Chelamine resin for the on-line determination of palladium(II) and platinum(IV) by inductively coupled plasma optical emission spectrometry

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Abstract

A new matrix separation/preconcentration method is developed for the on-line determination of palladium(II) and platinum(IV) in complex matrices using a sequential ICP-OES instrument. These metals are preconcentrated in a microcolumn packed with Metalfix-Chelamine, a polymeric functionalised resin containing the tetraethylenepentamine group. The hydrodynamic and chemical conditions of the flow system affecting the loading and elution steps are optimised off-line using a mixture of 1.0 mol L⁻¹ thiourea and 2.0 mol L⁻¹ NaClO₄ in 4.0 mol L⁻¹ HCl which proved to be the most effective solution for the simultaneous elution of Pd(II) and Pt(IV). High enrichment factors of nearly 35 are achieved for both metals and the detection limits (LOD) are 22 ng L⁻¹ for platinum and 2.5 ng L⁻¹ for palladium. The accuracy of the method was tested by analysing a used pellet catalyst (certified reference material NIST 2556) and trace metal solutions resulting from the leaching of this material. Despite the fact that this CRM contains zirconium and large amounts of aluminium and lead, a high level of agreement was achieved demonstrating the efficiency of the resin in eliminating interfering elements.

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1. Introduction

Platinum group elements (PGE) have become more widely used in recent years due to new applications both in the pharmaceutical industry, particularly in cancer drugs such as cisplatin, carboplatin, and oxaliplatin [1–4] and in the car manufacturing industry in the production of catalysts [5]. Although the benefits of car catalysts are indisputable, highly toxic elements including Pt, Rh and Pd are still released into the atmosphere [6–8]. The release of these PGE's has been found to increase the levels of contamination in soils [9], plants [10] and road sediments [11] next to highways as well as in airborne particles [12]. The development of analytical methods for the determination of trace amounts of PGE's in water solutions [13], soils and sediments

[14] and biological matrices [15–17] is important not only as a control of the state of the environment [18] but also as a first step in the development of techniques for the recovery of PGE's from waste materials [19].

PGE concentrations in environmental matrices are usually at trace levels and detection is made more difficult by the presence of interfering metals making it necessary to conduct a preconcentration/matrix elimination step. Solid phase extraction has been demonstrated to be a highly effective preconcentration method when applied prior to spectrometric determination. Among the different resins tested for this preconcentration phase are anion exchangers containing quarternary phosphonium chloride [20]; amberlite XAD-16 [21]; SSPS, a polystyrene-based sorbents sorbent [22]; modified silicagel [23]; Dowex 1-X10 [24]; activated alumina [25] and various chelating resins such as polyacrylisothiourea [26] dithizone anchored poly(vinylpyridine) [27], imidazoline [28], aminoisopropylmercaptan [29] and Metalfix-Chelamine [30,31]. The fast kinetics, selectivity, and great adsorption capacity of this last resin have led us to choose it for use in the present study.

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Various techniques such as atomic absorption spectrometry (AAS, both by flame atomisation and electrothermal atomisation), inductively coupled plasma mass spectrometry (ICP-MS), neutron activation analysis (NAA) and stripping voltammetry are used for the determination of PGE's [32]. Atomic absorption [33] and atomic emission spectrometry [34] are widely used despite problems caused by the presence of matrix spectral interferences [24,35]. The coupling of online flow injection systems to atomic emission or atomic absorption instruments has been shown [36,37] to have a series of advantages in comparison with off-line systems such as the near contamination-free sample handling, higher analytical throughput, low sample and reagent consumption and the automation of the preconcentration/clean up step by the incorporation of small sorbent-containing columns. An example of this experimental set-up coupled to ICP-OES has proved to be a reproducible, rapid and low cost system for the analysis of small volumes [38,39].

In this study, Metalfix-Chelamine, a polyamine chelating resin, is used both for the off-line and on-line preconcentration of Pt and Pd. Metalfix-Chelamine resin has been found to be suitable for the selective preconcentration of PGE's [31,40]. Its sorption capacity is pH dependent due to the protonation of the nitrogen atoms of the functional group. Cd, Cu, Mn, Zn, Ni and Pb are not adsorbed at $\text{pH} < 2$ [41]. At pH 1 (0.1 mol L^{-1} HCl) the resin is highly selective for Pt(IV), Pd(II) and Au(III) with capacities of 2.8 mmol g^{-1} , 2.0 mmol g^{-1} and 3.1 mmol g^{-1} , respectively [42]. The preconcentration conditions in our study were optimised using an off-line extraction system before finally, prior to ICP-OES detection, developing an on-line preconcentration method.

The study aims to characterise the loading and elution processes of Pd(II) and Pt(IV) in a flow system comprised of a minicolumn filled with Metalfix-Chelamine and then to use the results in the development of an on-line preconcentration method for the determination of Pt and Pd by ICP-OES. We then apply this method in the determination of Pd(II) and Pt(IV) in a certified reference pre-powered catalyst (NIST SRM-2556) and trace metal solutions resulting from the leaching of this material.

2. Experimental

2.1. Reagents

One thousand milligrams per litre stock solutions of Pd(II) and Pt(IV) were prepared from PdCl_2 (Merck, Germany) and PtCl_4 (Johnson Matthey, Germany) and the required amount of standardised HCl solution. Working mixtures were prepared by dilution of stock solutions.

Thiourea and sodium perchlorate solutions in HCl were prepared from solid reagents (Panreac, Spain) and the required amount of standardised HCl solution. Sodium peroxide (Fluka Chemie AG, Buchs, Switzerland) was used in the digestion of the sample.

All solutions were prepared with analytical quality chemicals and Milli Q water. The functional group of the Metalfix-Chelamine resin (Fluka Chemie AG, Buchs, Switzerland) is 1,4,7,10,13-pentaazatridecane, or the tetren group.

Table 1

Composition of used pellet catalytic converter (certified reference material NIST 2556)

Element	Concentration (mg Kg^{-1})
Certified values ^a	
Pt	697.4
Pd	326.0
Rh	51.2
Pb	6228.0
Non-certified values ^b	
Ba	100
Zn	600
Zr	300
Al	400000
Ca	1000
Ce	10000
Fe	800
La	700
Si	200

^a Values determined by two analytical methods.

^b Either only one analytical method used or comparison failed.

Used automobile catalyst pellets (standard reference material 2556) were purchased from NIST. The certified values for platinum, palladium, rhodium, lead and the concentration of other elements present in the reference material are presented in Table 1 [43].

2.2. Instrumentation

The flow system (Fig. 1) consisted of a six-way peristaltic pump (Gilson, Minipuls 3, Model M312, France), a flow manifold with two rotary valves (Rheodyne, USA), polyethylene tubing (Watson Marlow Limited, England) and a resin column. The flow system was connected to an ICP nebulizer. A sequential ICP-OES instrument (Varian-Liberty Series I, Australia) with a radial torch equipped with the V groove nebulizer and a gravity spray chamber was used. The sorption columns were prepared as has been earlier described [40].

A muffle furnace (Hobersal HD-230, Spain) was used in the pre-treatment of samples for their digestion.

2.3. Procedure

Metal preconcentration was carried out under dynamic conditions using a peristaltic pump, a collector, and a minicolumn

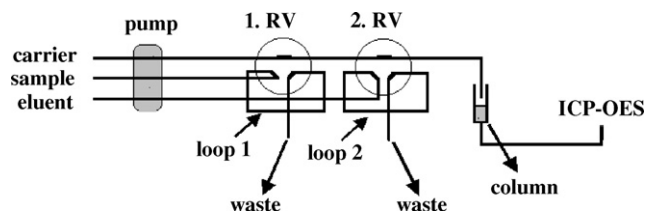


Fig. 1. Experimental set-up for on-line system: loop 1 (17.3–50 mL), loop 2 (2.4 mL), flow rate (adsorption— 5 mL min^{-1} , elution— 2.7 mL min^{-1}), RV (rotary valve).

filled with different sorbent masses (0.02 and 0.1 g). The column was washed with 3 mL of 0.1 mol L^{-1} HCl in order to protonate the nitrogen atoms of the sorbent's amine group. The working metal solutions were then pumped through the resin and the degree of adsorption was determined through ICP analysis of the outflowing solution. The efficiency of Metalfix-Chelamine in adsorbing Pd and Pt was calculated by applying a mass balance equation (the difference between the metal concentration in the initial and outflowing solutions).

In a previous work [42], quantitative elution of Pd was obtained with 0.5 mol L^{-1} thiourea in 0.1 mol L^{-1} HCl and 70% elution of Pt with 0.5 mol L^{-1} NaClO_4 in 1 mol L^{-1} HCl. In order to obtain quantitative elution for both metals in this study we have evaluated different flow rates and the eluent composition.

2.4. Manifold design

The manifold used for the on-line preconcentration is shown in Fig. 1. In the first step, the injection and elution valves were in the load position and carrier electrolyte (0.1 mol L^{-1} HCl) were pumped through the microcolumn at a flow rate of 5 mL min^{-1} . The carrier electrolyte eliminated the remaining eluent in the microcolumn. In the second step the elution valve was in the injection position and the sample was passed through the resin. In the third step both valves were again set in the load position, the column was washed by the carrier electrolyte and the matrix elements were removed. In the final step the elution valve was in the injection position and the eluent was introduced into the column. In order to introduce the eluent directly into the ICP-OES, the flow rate was decreased to 2.7 mL min^{-1} . The same manifold was used in all the experiments.

2.5. Sample pretreatment

The certified sample was heated for 2 h at 500°C . A 0.3 g of sample was then weighed in a dry zirconium crucible and a 3 g portion of sodium peroxide was added. The sample was thoroughly mixed and fused in a muffle furnace for 30 min at

600°C . Once the mixture was cool, it was dissolved in aqua regia (15 mL HNO_3 and 45 mL HCl) and diluted to 100 mL in a flask [19].

3. Results and discussion

3.1. Adsorption

As has been mentioned above, the effect of various parameters on Pt and Pd adsorption were studied with 0.02 g and 0.1 g of resin. The results (Table 2) show that Pt and Pd can be quantitatively adsorbed by 0.1 g of resin independently of the loading parameters of the Pt content in the loading solution and 100% of the Pd were adsorbed. When 0.02 g of resin were used with a flow rate of $>2 \text{ mL min}^{-1}$, Pt adsorption decreased. Taking all these results into account, we selected 0.1 g of resin and a flow rate of 5 mL min^{-1} for on-line preconcentration.

3.2. Elution

In earlier studies using Metalfix-Chelamine resin for PGE preconcentration, the quantitative elution of PGE was not achieved [30,42]. In the present study, the elution of palladium and platinum from the resin was first attempted with thiourea and HCl at different concentrations. The complete elution of palladium was achieved with a solution of 0.5 mol L^{-1} thiourea and 12 mol L^{-1} HCl, but it was only possible to obtain 55% Pt recovery in a 4 mL eluent fraction. In order to improve Pt recovery, and taking into account the fact that the adsorption of this metal occurs via an ion-exchange mechanism, we used elution solution consisted in sodium perchlorate and thiourea in HCl medium. Different fractions of eluent (1, 2, 3 mL) were then collected to monitor Pd and Pt elution. In the 1 mL fraction approximately 90% of Pd and 80% of Pt were eluted. With 2 mL of eluent, quantitative elution of Pd and approximately 85% recovery of Pt were achieved. A further increase in eluent volume did not significantly improve recovery and hence 2 mL was chosen for the elution. We then investigated the influence of flow rate and loop volume on elution and found that these factors have almost no effect on Pd recovery. In the case of Pt, a 2 mL

Table 2
Percentage of Pd and Pt sorption as a function of the Metalfix-Chelamine resin quantities, flow rate, loading concentrations and volumes

Element	0.02 g of resin					0.1 g of resin				
	Loading concentration (mg L^{-1})	Loading volume (mL)	Flow rate (mL min^{-1})			Loading concentration (mg L^{-1})	Loading volume (mL)	Flow rate (mL min^{-1})		
			1	2	4			1	2	4
Pt	2	25	96	94	90	2	25	96	96	96
	2	50	95	92	89	1	50	95	95	95
						0.5	100	96	96	95
Pd	2	25	98	98	97	2	25	97	99	99
	2	50	98	97	97	1	50	98	99	99
						0.5	100	98	97	100

Standard deviations were in the 1–2% range ($n = 3$). Samples in 0.1 mol L^{-1} HCl media.

Table 3
Study of the composition of the elution solution

Eluent	Flow rate (mL min ⁻¹)	First fraction elution (%)		Second fraction elution (%)	
		Pt	Pd	Pt	Pd
0.5 M T + 0.5 M P in 4 M HCl	4	82	98	15	3
	6	80	98	10	4
0.5 M T + 0.5 M P in 6 M HCl	2	83	97	9	3
	4	82	98	7	5
1 M T + 1.5 M P in 4 M HCl	4	87	100	5	<2
	6	83	99	9	<2
1 M T + 2 M P in 4 M HCl	2	88	102	4	<2
	4	85	102	7	<2
	6	84	101	8	<2

T represents thiourea and P represents sodium perchlorate. Each fraction is 2 mL. Standard deviations were in the 1–3% range ($n = 3$).

eluent loop volume and a flow rate of 4 mL min⁻¹ were found to be the most efficient (Table 3). We selected an elution loop volume of 2.4 mL and a flow rate of 2.7 mL min⁻¹ for on-line processing.

3.3. Elution curves

Since a sequential ICP-OES instrument was used, it was necessary to study the elution curve profiles of Pd and Pt (Fig. 2) in order to find the best determination conditions for the two metals. Each metal's elution curve was determined separately and in both cases peaks were observed 20 s after opening the elution valve. The similarity between the profiles of the elution curves of the two metals indicates similar elution kinetics. As can be seen in Fig. 2, Pd was eluted from the resin slightly faster and the signal intensity was higher. The maximum signal was observed on the elution curve 32 s after the start of elution in the case of Pd and 36 s later for Pt after which the signal tailed off. The on-line quantitative determination was designed exploiting this difference.

3.4. On-line method

Pt and Pd have typically been analysed using instrumental parameters such as power and grating order to find the optimal

conditions for high signal intensity. For the online determination of these metals it is necessary to optimise the time delay involved in the change of instrumental conditions. A time delay of 10 s was achieved by using the instrumental conditions of Pt for the measurement of the two metals although this change led to a slight reduction in the intensity of the Pd signal. We first measured the intensity of Pt at the top of its elution curve with an integration time of 3 s, and then Pd with the same time (Fig. 2). These conditions proved to be reproducible at the different concentrations tested. In order to measure platinum intensity exactly at the top of its peak, sample uptake delay was optimised to 20 s. The elution curve profiles obtained for the different metal concentrations between 0.4 and 2 mg L⁻¹ gave similar peaks.

3.4.1. Calibration curves

Calibration curves were determined using the previously described on-line analysis manifold and standard solutions without matrix components. Two sets of calibrations, with and without a preconcentration step, were obtained. Good linearity was achieved for the calibration graphs in the 0–20 mg L⁻¹ range. Two curves in the 0–2 mg L⁻¹ and 0–0.1 mg L⁻¹ ranges were used for real sample analyses (17.3 and 50 mL of loading sample, respectively) in order to analyse low loading sample concentrations and improve the precision of the calibration (Table 4).

The sensitivity of calibrations curves was characterised by their slope. Preconcentration factors were calculated from the ratio of the slopes obtained using the preconcentration method and those obtained by direct analysis. Enrichment factors of nearly 35 were obtained for both metals and the detection limits (LOD) of the calibration step with the on-line procedure were 22 ng L⁻¹ for Pt and 2.5 ng L⁻¹ for Pd (Table 4).

3.4.2. Interferences and matrix removal

Spectral interferences in the determination of Pd and Pt by ICP-OES are mainly caused by matrix elements. The presence of Al and Fe affects the Pt signal whereas Fe and V modify the signal of Pd. The determination of Pd and Pt in the catalyst sample may be affected by the Al, Pb, Zr present in the catalyst matrix [35]; moreover the overlap of Pd line $\lambda = 340.458$ nm by Zr line $\lambda = 340.464$ nm makes it difficult to determine the Pd by ICP-OES [24]. The use of Metalfix-Chelamine permitted the

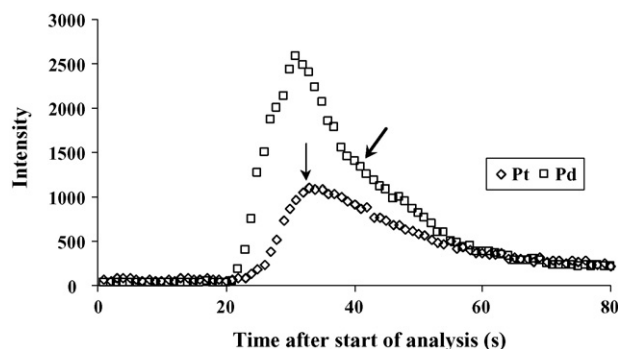


Fig. 2. On-line elution curve profiles of Pt and Pd. Metal loading concentration: 0.4 mg L⁻¹. Elution solution: 1 mol L⁻¹ thiourea + 2 mol L⁻¹ NaClO₄ in 4 mol L⁻¹ HCl. Integration time: 1 s. Replicates: 80; ↓ marks the spot where signal intensity was measured.

Table 4

Figures of merit (signal based on peak height)

Without preconcentration step					With preconcentration step			
Curve	Analyte	Slope	Intercept	Correlation factor	Slope	Intercept	Correlation factor	Enrichment factor
a	Pd	423	47	0.9963	4382	193	0.9976	10.4
	Pt	253	387	0.9992	2193	378	0.9955	8.7
b	Pd	252	77	0.9918	8569	22	0.9905	34.0
	Pt	272	375	0.9971	9734	221	0.9906	35.8

Calibration curves: (a) loading volume of sample 17.3 mL, linear range of response 0–2 mg L⁻¹ (10 standard solutions and $n=3$); (b) loading volume of sample 50 mL, linear range of response 0–0.1 mg L⁻¹ (5 standard solutions and $n=3$).

Table 5

Determination of Pd and Pt in certified material NIST 2556

Platinum (mg kg ⁻¹) 697.4 ± 2.3			Palladium (mg kg ⁻¹) 326.0 ± 1.6		
Determined in solution (μg L ⁻¹)	Calculated in solution ^a (μg L ⁻¹)	Determined in solid (mg kg ⁻¹)	Determined in solution (μg L ⁻¹)	Calculated in solution ^a (μg L ⁻¹)	Determined in solid (mg kg ⁻¹)
(1) 0.870	0.837	725 ± 45	(1) 0.359	0.391	299 ± 27
(2) 0.389	0.418	648 ± 55	(2) 0.189	0.196	315 ± 36
(3) 0.191	0.210	637 ± 36	(3) 0.103	0.098	344 ± 28
(4) 0.032	0.035	645 ± 35	(4) 0.018	0.016	358 ± 36

(1–4) indicate the results of the analysis of the certified sample solution obtained after diluting 2.5, 5, 10, 60 times, respectively. The number of measurements made for each solution was 7.

^a Values calculated from the initial concentrations.

elimination of interfering elements. This was particularly important in the case of Zr as it was not until this had been successfully removed that the peak of Pd was observed. The loading and out-flowing concentrations of interfering metals were determined and more than 98% of major matrix elements were removed by the preconcentration step.

3.5. Analysis of real samples

In order to verify the accuracy of the method, the palladium and platinum content of a certified reference material, NIST 2556 (used catalyst pellets), was determined. The solid was dissolved as described in the experimental section resulting in a sample solution containing 2.33 mg L⁻¹ Pt and 1.10 mg L⁻¹ Pd in 5.0 mol L⁻¹ HCl and 2.0 mol L⁻¹ HNO₃. It was necessary to dilute the sample solution due to its high acidity and its considerable metal content. The solution was diluted 2.5, 5, 10 and 60 times in order to validate the method at different trace Pd and Pt concentrations. The first three dilutions were analysed using calibration curve (a) (Table 4) and the most diluted sample solution was analysed using calibration curve (b) (Table 4). Substantial agreement between the mean results and the certified values were obtained with discrepancies of less than 9% for Pd and 8% for Pt (Table 5).

The on-line preconcentration and separation system developed in this study has proved suitable for the determination of palladium and platinum in complex matrices such as pellet automobile catalysts. The method is sensitive, accurate, easy to operate, and highly selective in acidic media. In the experimen-

tal conditions employed in this study we obtained enrichment factors of nearly 35 for both metals, which can be improved on through the use of different volume ratios, allowing detection limits of 22 ng L⁻¹ for Pt and 2.5 ng L⁻¹ for Pd, which are considerably lower than those reported for this technique [32]. A further advantage of this method is that it can be performed using a sequential ICP-OES instrument and we optimised the experimental conditions to this end. However, the greatest novelty of this method is that not only does it remove more than 98% of the matrix elements such as Al and Pb but it also manages to overcome the interference of Zr in the determination of Pd, which should be of considerable use in the analysis of car catalysts given the high quantities of this element that they contain.

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Lead post-mortem intake in human bones of ancient populations by ^{109}Cd -based X-ray fluorescence and EDXRF

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Abstract

In this work we measured the lead concentration in human bones of Middle Age by means of a portable X-ray fluorescence system based on ^{109}Cd radioactive source. The detection system consists on a Ge hyperpure detector. This system, conceived for *in vivo* Pb analysis in bone, is portable, non-destructive and is based on lead K lines detection.

The analysed bones are part of two collections of bones both from the end of Middle Age and submitted for some years to a lead polluted burial environment. The bones of one collection were buried initially on the soil in a convent, in Lisbon (Portugal) and further on, kept in a lead coffin for around 100 years. The second collection contains bones buried permanently on the soil around an old church on the south of Portugal. This place became a parking car for around 20 years.

In this work we studied the distribution of Pb in cortical bone, and trabecular regions from the outside surface to the inner part of the bone and the results are compared with the obtained ones by energy dispersive X-ray fluorescence (EDXRF).

The obtained values present a strong contamination of Pb in spongy bones kept in the lead coffin with concentrations ranging from 250 to 350 $\mu\text{g Pb/g}$ bone mineral, and 4 to 7 $\mu\text{g Pb/g}$ bone mineral for bones buried in the soil. Good agreement was observed between the results obtained by the two techniques.

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Keywords: Lead; Bone; Archaeology; X-ray fluorescence; ^{109}Cd -based X-ray fluorescence

1. Introduction

The goal of this work is to demonstrate the suitability of the ^{109}Cd -based X-ray fluorescence in archaeological studies of Pb in bones of ancient populations. Moreover, we intend to demonstrate Pb *post-mortem* contamination in buried bones and the strong dependence on the uptake level and the burial place.

The ^{109}Cd -based system is portable and non-destructive and presents advantages when compared with the more common ones based on L spectra detection, namely the access of a clean spectrum, without overlap of a big number of elements. The analysis of such a K spectrum is much more reliable and easy to handle.

During life, Pb becomes incorporated into the bone matrix hydroxyapatite as a result of exchange with Ca at the bone

structure, from deposition when new bone is formed and radial diffusion throughout the bone.

Bone is one of the hardest structures of the human body, composed by two different kinds of tissue: one compact (cortical), and the other one spongy (trabecular).

It has long been known that the bone is the major storage site of lead, more than 90% of total body lead resides in the bones and its retention in the bone can be described in terms of years or decades. The uptake and fixation of lead into bone is a protective mechanism during acute exposure because it limits its distribution to more sensitive tissues. In this context, the development of methods of measuring lead *in vivo* in bone has provided a tool, which has proved to be apt to the task of increasing information about the long term storage of lead using the ^{109}Cd excited K X-ray fluorescence technique [1–3].

Lead determination in mineralised human remains is important for its relationship to dietary habits and environmental conditions of those populations. This is important for archaeol-

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ogists in the reconstruction of human history and food economy of ancient populations [4–10].

Tooth and bone have been indicated as dose monitors for men elemental exposure.

Bone, due to its open morphological structure with internal channels, makes it much susceptible to post-mortem alteration. Physical and chemical changes can occur in bone during its burial period. As bone is fossilized, the natural process of diagenesis serves to alter the bone composition from its *ante-mortem* state. This post-mortem alteration of buried bones is a problematic factor for archaeological studies [11–13].

Diagenesis includes the processes of dissolution, precipitation, adsorption, mineral replacement and recrystallisation.

Elements introduced by groundwater and soil may become incorporated in bone structure in a number of ways. They may reside in pores, voids or microcracks in the bone matrix, form complexes with the organic component, adsorb onto the surface of hydroxyapatite matrix via ionic exchange. These processes and their rate of reaction depend on the physical and chemical characteristics of the burial environment.

Elemental distributions in archaeological human remains have been studied by AAS [4,5], PIXE [6,10,14] and X-ray fluorescence for quantitative determinations [12,13,15] and nuclear microprobe for elemental distribution [8,9].

The concentration of lead in tibia (cortical bone) and calcaneus (trabecular bone) was measured by an *in vivo* X-ray fluorescence technique in active contemporary workers with a system based on the non-invasive ^{109}Cd excited K X-ray fluorescence technique [2,3,16,17]. Pb in bone of contemporary citizens was also measured by energy dispersive X-ray fluorescence (EDXRF) in post-mortem samples, collected from the temporal fossa of the skull immediately after death [18,19].

In this work we measure the post-mortem Pb accumulation in human bones from two different burial places of Middle Age by ^{109}Cd excited K X-ray fluorescence technique, and by energy dispersive X-ray fluorescence (EDXRF), for comparison. The results in terms of Pb post-mortem uptake from the burial place, and its influence in the Pb concentration are discussed and the Pb distribution inside the bones are analysed.

2. Experimental

2.1. Experimental setup

2.1.1. ^{109}Cd radioactive source

The experimental arrangement used in this work to quantify the Pb consists on a 1.11 GBq ^{109}Cd radioactive source; the emitted γ radiation of 88.035 keV is used as incident radiation to ionize the Pb K-shell of the atoms in the sample. The characteristic radiations of Pb, $\text{K}\alpha$ and $\text{K}\beta$ ($\text{K}\beta_1$ and $\text{K}\beta_3$) are used for quantitative calculations. The geometry is a backscatter one, with the source and the detector coupled co-axially in front of the sample, defining a scattering angle of nearly 153° .

The detector used for measuring lead in bone is a low energy HPGe and modular electronics supplied by Canberra-packard. The detector has a sensitive volume (20 mm thick and 20 cm^2 in cross-sectional active area) behind an entrance window of

0.5 mm beryllium. The detector cryostat geometry is of the horizontal integral type and the measure energy resolution is 360 eV for 5.9 keV and 600 eV for 122 keV.

The radioactive source is inside a capsule constructed of stainless steel and has an external diameter of 3.2 mm, a height of 3.25 mm, and an exit window of 0.2 mm thickness. The cylindrical capsule shield and the collimator, each machined from a tungsten alloy (type HD 18.5, MiTech Metals). The collimator is fitted with a 0.5 mm thick copper filter to absorb the Ag K X-rays associated with the decay of ^{109}Cd . The source capsule within its shield was positioned at the centre and in front of the beryllium window of the HPGe detector by means of the detector mounts [20]. A cylindrical iron sleeve with a closed end of styrene slides over the horizontally mounted detector and is held in place by grub screws. The purpose of this sleeve is to protect the beryllium window. The end of the outer cylinder contains slots into which four styrene spokes can fit. Both ends of each spoke are threaded. One end is screwed into an open ended lucite cylinder that holds the capsule shield as a friction fit. Threaded on to the other end of each spoke is a nut that allows for the adjustment of the position of the capsule shield in the plane parallel to the detector window. A source shutter made from the tungsten alloy is mounted on an arm at the edge of the external iron cylinder [20].

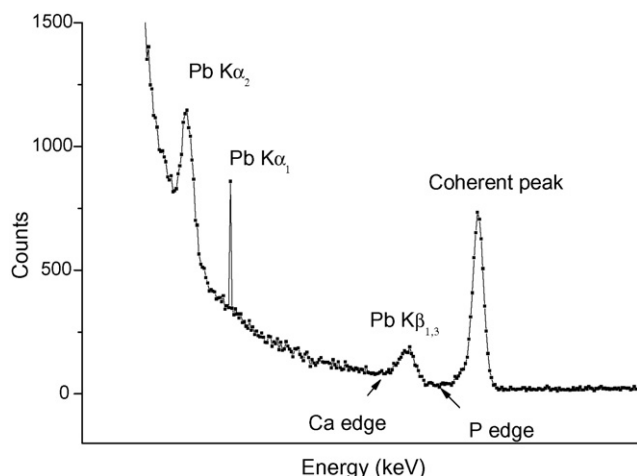
To calibrate the system bone phantoms containing known concentration of lead were constructed from plaster of Paris. The phantoms were cylindrical with an outside diameter of 4 cm and a central medullary canal of diameter 1.6 cm occupied by a bone marrow simulating rod of polyethylene. The obtained results are presented in Table 1.

For either a phantom bone or for each subject the spectrum was accumulated for 2000 s or until the counting error associated with the coherent photopeak was 1% or less. The data for each subject or calibration phantom was fitted by a gradient search least squares procedure. The optimum fit is achieved if a spectrum is divided into three regions, which are examined separately. In all three regions the peaks are fitted to gaussians of fixed width. Region 1 contains the $\text{K}\alpha_1$ and $\text{K}\alpha_2$ X rays with a Compton component that decreases rapidly with increasing energy. This background is best approximated by a double exponential. The background in Region 2, which contains the $\text{K}\beta_1$ and $\text{K}\beta_3$ X-rays, is approximated by a single exponential; the Doppler broadened Compton edge features from calcium and phosphorus or sulphur are also modelled. The background in

Table 1

Comparison of Pb concentrations ($\mu\text{g Pb/g}$ bone mineral) of reference calibrated phantoms obtained in the present work and the certified values

Present work (^{109}Cd source)	Certified values
2.2 ± 0.7	2
6.0 ± 0.8	5
11.6 ± 0.8	11
22 ± 1	22
40 ± 1	40
60 ± 1	60
100 ± 2	100
200 ± 2	200

Fig. 1. Spectrum from a bone obtained by ^{109}Cd .

Region 3 containing the coherent and $\text{K}\beta_2$ peak is also fitted to a single exponential. A common photopeak width in all three regions is used, which allows for a direct comparison of peak amplitudes when deriving lead concentrations, see Fig. 1. The minimum detection limit with this technique is at the level of $1\text{--}2\ \mu\text{g g}^{-1}$.

2.1.2. Energy dispersive system

The EDXRF Spectrometer used in this work to quantify Pb in some of the samples, consists of an X-ray tube equipped with a changeable secondary target, normally in molybdenum. The X-ray tube, the secondary target and the sample are in a triaxial geometry. This allows a decrease in the background, using the advantage of the effect of polarisation of the incident X-ray beam from the tube. The characteristic radiation emitted by the elements present in the sample was detected by a Si(Li) detector, with a 30 mm^2 active area and $8\ \mu\text{m}$ beryllium window. The energy resolution is 135 eV at 5.9 keV and the acquisition system is a Nucleus PCA card. Quantitative calculations are made through the fundamental parameters method [19,21]. Experimental parameters are obtained through calibration, using standard reference materials. The X-ray generator was operated at 50 kV and 20 mA and a typical acquisition time of 1000 s was used [19]. A spectrum of a bone obtained with this equipment is displayed in Fig. 2. The minimum detection limit of this technique is of the order of $1\text{--}2\ \mu\text{g g}^{-1}$.

2.2. Sampling

2.2.1. Sample collection

The bone material consisted on samples from thoracic vertebrae of an adult, collected from a burial place initially on the soil in a Convent in Lisbon, containing bones dated from 1431. The story of these bones is very well documented. The human remains, considered religious relics, were removed from the soil to the main chapel into a mausoleum in 1522. In 1755, after the big earthquake in Lisbon, the vault of the main chapel was destroyed, originating the destruction of the mausoleum which

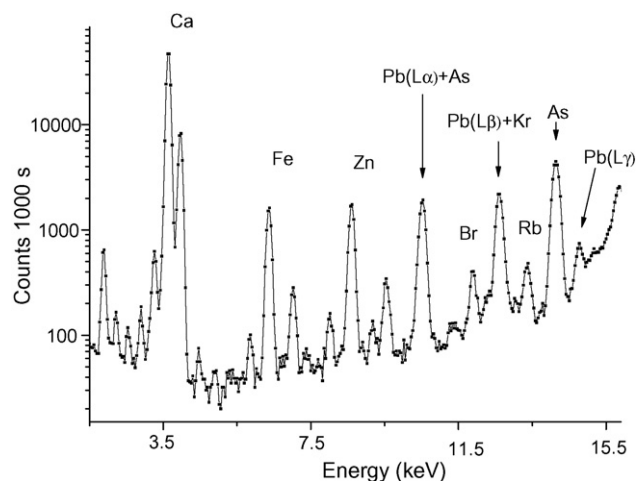


Fig. 2. Spectrum from a bone obtained by EDXRF.

contained the human remains. They were then removed inside a lead coffin into another church until 1836. In this year the bones were removed into a wood coffin and in 1906 the whole human remains started to be identified by archaeologists and doctors in order to be classified.

Furthermore, samples of one femur belonging to an adult person, collected from an archaeological place, in a clay soil, around a catholic church on the south of Portugal, were also studied. This archaeological place was identified as dating from the end of Middle Ages. This area has been a parking car since around the last 20 years. The human skeletons were found close to the surface, unlike the most common in ancient human remains, normally found a few meters deep. The proximity of the surface, and the car exhaustion, together with its weight originated great damage on the human remains, especially at the bone level; these conditions developed a strong lixiviation and most of the bones were completely broken, making impossible the reconstitution of many individuals. Besides the circumstances, approximately 257 individuals were identified. The absence of hypoplasies together with the physical robustness of the individuals might be related to the dietary habits rich in proteins. Mediterranean diet, rich in cereal and figs with high content in carbohydrates, was certainly included in the habits of this population. Life conditions of this population are considered devoid of Pb.

2.2.2. Sample preparation

No sample preparation is necessary for analysis by a ^{109}Cd radioactive source; the sample is placed directly in front of the detector. The several analysed points in the compact region were measured from the external surface in contact with the soil to the inside region by scraping around 1 mm of the compact bone for each analysed point, in a very small area of the studied bone. The used device for scraping was a knife, previously checked for Pb content. The distance between each analysed point was measured by means of a mechanical device measuring the bone diameter with the precision of 0.1 mm . The points in the spongy regions were analysed on a cross-section of each bone, directly in front of the detector.

For EDXRF analysis the samples were obtained by smashing the bone collected from the several regions of the vertebra spongy bone, into a powder, by means of a polyester mill and then pressed into pellets 1.5 cm in diameter without any chemical treatment. Each pellet was glued on a Mylar film, on a sample holder and placed directly on the X-ray beam for elemental determination.

3. Results and discussion

In Fig. 3 we compare the results for Pb concentrations ($\mu\text{g g}^{-1}$) in the vertebra (trabecular bone) collected from the set of bones buried in a lead coffin for 100 years, obtained by a ^{109}Cd radioactive source and EDXRF. The results obtained by the two techniques are in very good agreement. The errors affecting the results are originated by the calculation process of concentrations and takes into account all the errors of the parameters involved in the calculations.

The concentration profiles obtained from the outside of the bone to the inner part, confirm that Pb penetrates into the whole area with higher concentrations in the outer surface in contact with the metal. The diffusion of the metal was from the outside to the inside structure of the trabecular bone. A similar behaviour was found by Boscher-Barre and Trocellier [8] who studied the compact region of a woman's skeleton femur from the 6th century, buried in a Pb sarcophagus. Very high concentration of Pb was found in the external region of cortical bone reaching values of the order of 9% decreasing deeply to the inside region. In this work only the compact region of the bone was studied.

Very high levels of Pb were also found in human femurs of 11th centuries mining silver/lead community [6] buried in the polluted soil close to the mining area. The concentration values for Pb ranged from $2800 \mu\text{g g}^{-1}$ in the outer surface in contact with the soil, to $15 \mu\text{g g}^{-1}$ in the inner compact bone.

The exchange mechanisms between the hydroxyapatite bone matrix and the ions of lead, can explain the accumulation of this

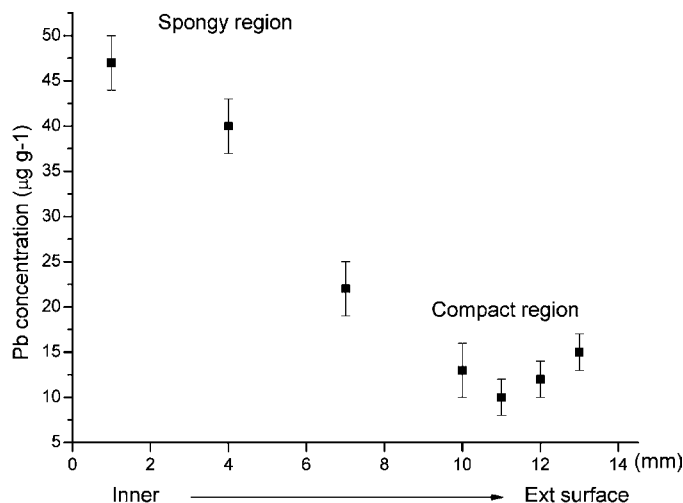


Fig. 4. Distribution of Pb content ($\mu\text{g Pb/g}$ bone mineral) in compact and spongy bone (femur), buried in a contaminated soil, obtained in the present work by ^{109}Cd radioactive source.

element. One possibility is the isovalent replacement of Ca^{2+} ions by Pb^{2+} ions. Pb can also be incorporated as the result of the formation of lead carbonate and lead phosphate within the bone structure. An interaction between Pb and the chemical compounds of the organic bone fraction cannot be excluded. These elevated concentrations are completely different from the results obtained in bones of ancient populations buried in non-contaminated environments.

In Fig. 4 we present the Pb concentrations for a femur, buried in the soil. We can see that in the compact region of the femur, we observe higher concentration in the external area in contact with the soil, decreasing for the internal compact bone and increasing again in the spongy bone region. These values are in agreement with values previously obtained for bones of the same burial place by a different technique [13].

If we compare the results of Fig. 4 with those of Fig. 3, the most evident remarks are the different pattern of Pb concentration profile and the concentration level. The increasing direction of Pb concentrations is reversed. The spongy bone preserved in the Pb coffin presents a strong enrichment at the external surface in contact with the metal, while in the femur buried on the soil the highest levels are in the inner spongy bone. This can be explained taking into consideration that the vertebra is a spongy bone in direct contact with the metal and the uptake starts from the outside to the inside, given that the whole bone has the same structure. The femur contains a thick external surface of compact bone, around 3 mm, in contact with the soil. The compact bone due to its higher hardness is less susceptible to post-mortem alteration, hence to penetration by external elements. Unlike, the spongy bone due to its opened morphology is much more vulnerable to post-mortem effects.

The order of magnitude of Pb concentration is also different in both results due to the composition of the burial place.

Lead content in the inner region of compact bone can be associated to ante-mortem lead accumulation, while the values in spongy bone are tremendously affected by the burial place conditions.

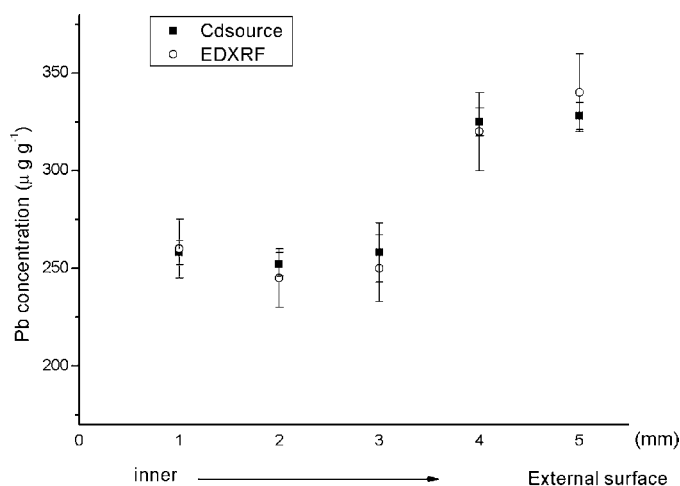


Fig. 3. Comparison of Pb concentrations ($\mu\text{g Pb/g}$ bone mineral) in a spongy bone (vertebra) studied by a ^{109}Cd radioactive source and by EDXRF in the same bone, buried in a lead coffin.

Similar values of Pb concentration in bones of the chalcolithic period, buried in non-polluted Pb soil, were obtained [12] and these values are also similar to the obtained for *in vivo* analysis in compact bone for contemporary citizens not submitted to Pb pollution level: $5 \pm 2 \mu\text{g Pb/g bone mineral}$.

To finalize this study we analysed the Pb content in the soil of the burial place, which was around $65 \mu\text{g g}^{-1}$, the same order of magnitude of the Pb level in trabecular bone of the femur. This value is around three times higher than the corresponding ones in non-polluted soils, which is around $20 \mu\text{g g}^{-1}$ [12,22]. This explains the high values of Pb in trabecular region of the femur buried in the soil.

As final remark we must emphasize that we demonstrate that the Pb in the analysed bones was due to post-mortem contamination from the burial place. Considering the concentration values for contemporary workers professionally exposed to Pb the ratio between concentrations in spongy bone (calcaneus) and compact bone (tibia) is of the order of 2 [1,23], while in the present study for femur that ratio is of the order of 7. On the other side, the maximum value for Pb content in calcaneus bone of exposed workers is $140 \mu\text{g Pb/g bone mineral}$, and in the present study the level in vertebra is around four times higher. Whatever the environmental conditions at the time were, or taking into account contamination from cooking utensils or Pb water pipes, there is no explanation for the high Pb content in the bones except uptake from the burial place.

4. Conclusions

From the whole work we can conclude that the ^{109}Cd -based X-ray fluorescence technique is suitable for post-mortem lead analysis. It is non-destructive and no sample preparation is required. Another advantage of this technique is to be portable and easily transported into museums or excavation places, allowing *in situ* analysis. Moreover, the K spectrum, without overlap of a big number of elements, is more reliable, and easy to handle for quantitative calculations, than the common ones based on the L spectra, in the usual X-ray fluorescence techniques. The drawback of this technique is that only elements of high atomic number are detected with enough efficiency and resolution, while the other technique allows simultaneous detection of most of the sample elements.

Furthermore, this work has highlighted that diagenic alteration in bone depends mostly from the physical and chemical properties of the burial place and the structure of the bone.

The high observed Pb levels in the outer and inner open surfaces in the spongy bone kept in the metallic coffin are surely due to contamination from the container. The Pb concentration

decreases from the external surface in contact with the metal to the inside, while in the femur the highest concentrations are in the inner spongy region. Lead introduced by groundwater and soil was incorporated into the bone structure. The opened morphology of the spongy bone makes it much more susceptible to post-mortem alterations than the compact bone.

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Automated on-line separation preconcentration system for palladium determination by graphite furnace atomic absorption spectrometry and its application to palladium determination in environmental and food samples

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Abstract

A flow injection (FI) system was used to develop an efficient on-line sorbent extraction preconcentration system for palladium by graphite furnace atomic absorption spectrometry (GFAAS). The investigated metal was preconcentrated on a microcolumn packed with 1,5-bis(di-2-pyridyl)methylene thiocarbonylhydrazide immobilized on silica gel (DPTH-gel). The palladium is eluted with 40 μl of HCl 4 M and directly introduced into the graphite furnace. The detection limit for palladium under the optimum conditions was 0.4 ng ml^{-1} . This procedure was employed to determine palladium in different samples.

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Keywords: Palladium; Graphite furnace atomic absorption spectrometry; Preconcentration; Flow injection

1. Introduction

Accumulation of platinum group metals (PGMs) in the environment has been increased over the time. Catalytic converters of modern vehicles are considered to be the main sources of PGM pollution. The available evidence indicates that the PGM, especially Pd, are transported to biological materials through deposition in roots by binding to sulphur-rich low molecular weight species in plants [1–3]. A great number of studies have reported the dispersion and accumulation of precious metal in various environmental compartments. The determination of PGM traces in environmental matrices requires highly sensitive analytical methodology. Platinum is the element that has received the most attention among the PGMs studies, while Pd and Rh have been monitored in more recent research projects. Automobile catalyst emitted particles deposit on the roadside and can be transported to water-bodies through storm-water runoff; the uptake rate in plants and animals is highest for Pd, followed by Rh and Pt; several studies suggest an environmental mobility and uptake gradient of $\text{Pd} > \text{Rh} > \text{Pt}$.

Palladium is emitted in a particulate form together with a relatively high soluble fraction [4,5], and may deposit to accumulate on the road surface. Further contamination of aquatic systems may result from the transport of these particles through storm-water. Under appropriate pH and redox potential conditions, it is assumed that Pd undergoes methylation reactions in the aquatic environment and could be concentrated along the food chain [6].

For the determination of palladium traces highly sensitive and selective techniques including GFAAS, inductively coupled plasma atomic emission spectrometry (ICP-AES), laser atomic fluorescence spectroscopy (LAFS), instrumental neutron activation analysis (INAA), stripping voltammetry (SV) and inductively coupled plasma mass spectrometry (ICP-MS) has been used [7]. Unfortunately, palladium detection using the most sensitive detection methods such ICP-MS, INAA and LAFS suffers from strong spectral interference [8–10]. The application of these techniques is therefore restricted to samples with little or no interference. In environmental samples, the low concentration of PGMs together with the high concentration of interfering matrix components often requires a preconcentration/enrichment step combined with a matrix separation. In this way, the application of on-line column preconcentration and separation procedures to atomic spectrometry has undergone rapid development in recent years [11–15].

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Until recently, FI techniques were scarcely used in GFAAS procedures relative to the other variants of AAS (flame and hydride generation), seemingly because of the discrete nature of GFAAS operations. The combination of FI on-line separation and preconcentration techniques with GFAAS has attracted considerable interest in the last few years on account of its high potentials for enhancing the relative sensitivity and selectivity of GFAAS. The development has been so great so to justify the event that, for example, in one year three excellent reviews were published about this matter [16–18].

The present work describes an on-line column preconcentration and separation system with GFAAS detection for the reliable determination of trace amounts of palladium in environmental samples. This system has previously been employed for determination of platinum [19]. The accuracy of the whole procedure is demonstrated by analysing the certified reference material SRM-2557 (catalyst). Finally, the new method is applied for the analysis of palladium in diverse environmental samples.

2. Experimental

2.1. Instrumentation

A Perkin-Elmer Zeeman/4100 ZL atomic absorption spectrometer equipped with an AS-70 furnace autosampler was used throughout. Pyrolytic graphite coated tubes with pyrolytic graphite platforms were used in all experiments. The light source was a palladium hollow cathode lamp operated at 25 mA; the selected wavelength was 247.6 nm with a spectral slit width of 0.7 nm.

The graphite furnace temperature program for the determination of palladium is shown in Table 1.

The microcolumn containing the DPTH-gel was a glass tube (3 cm × 3 mm i.d.) packed to a height of 0.5 cm; at both ends of the microcolumn, polyethylene frits were fixed to prevent material loss. On the end of this column was placed a piece of sample capillary of the sampler arm, in imitation of the sample tip of the sampler arm. Thus the sample tip of the sampler arm was replaced with this microcolumn, permitting normal working of the sampler.

A peristaltic pump, P (Gilson Minipuls 3), fitted with a vinyl pump tube (1.65 mm i.d.), was used for loading of the sample. A Rheodyne Type 50 six-port rotary valve was used as a switching valve. Transport lines were made using 0.8 mm i.d. Teflon tubing. The peristaltic pump and the selection valve

were readily controlled electronically via two switches on the autosampler that were actuated when the autosampler arm was down. The process was thus fully automated without altering the software of the AA spectrometer. For sample digestion, a microwave oven, Microdigest 301 controlled by Prolabo TX-32, was used. All glassware used was washed with 10% nitric acid for 24 h and rinsed with de-ionized water immediately before use.

2.2. Reagents and standard solutions

Analytical-reagent grade chemicals were used throughout.

Silica gel functionalised with 1,5-bis(di-2-pyridyl)methylene tiocarbohydrazide (DPTH-gel) was synthesised as described elsewhere [20]. A standard 1000 µg ml⁻¹ Pd(II) solution (CertiPUR, Merck) was used. A pH 7.6 buffer was prepared by mixing 5.5 ml of 0.2 M monobasic sodium phosphate with 43.5 ml of 0.2 M dibasic sodium phosphate and diluting to 100 ml with de-ionized water. HCl (Merck) 4 M was used as eluent.

2.3. FI-enrichment/separation procedure

The FI procedure runs through a cycle of three sequences. A complete cycle of the FI-operation is 109 s. The FI system was operated as follows.

Sample loading period: During the electronically pre-programmed loading time, a 2.8 ml min⁻¹ flow of sample (standard or blank) at pH 7.6, buffered with phosphate solutions, is pumped through the microcolumn (located in the sampler arm), the metal ion is adsorbed on the sorbent microcolumn and the sample matrix is sent to waste.

Wash period: When the loading period finishes, the switching valve is automatically activated, and the peristaltic pump is stopped, thus the sampler arm connection with the external peristaltic pump is changed to AS-70 furnace autosampler pumps, permitting the operation of the autosampler in the normal mode; where a wash step takes place with de-ionized water for cleansing resin of matrix sample.

Elution and measurement period: The sampler arm lowers the sample capillary into an autosampler cup (filled with eluent) aspirating 40 µl HCl 4 mol l⁻¹; then, the sampler arm swings over to the graphite furnace and the tip of the sampler capillary is inserted into the dosing hole of the graphite tube where the eluted Pd(II) is deposited as a drop (the eluent injection system, normal operation of autosampler provokes a flow through the column in both directions, up, when it is aspirated and down, when it is injected into the graphite tube, avoiding the continuous increase in column compactness). The sampler arm then returns to its initial position and the cycle of the furnace operation commences; while the temperature program is running, the switching valve is again turned to start a new loading of the sample (standard or blank); thus, when the spectrometer gives the measurement, the microcolumn is ready for a new injection of eluent.

With this procedure, the FI system and the GFAAS instrument were coupled and operated completely synchronously.

Table 1
Graphite furnace temperature programme ($V_i = 40 \mu\text{l}$)

Step	Temperature (°C)	Ramp time (s)	Hold time (s)	Argon flow rate (ml min ⁻¹)
1	110	1	20	250
2	130	5	30	250
3	900	10	20	250
4	2200	0	5	0
5	2400	1	2	250

Table 2
Working conditions for microwave oven

Sample	Step	Reagent	Volume (ml)	Power (%)	Time (min)
A ^a	1	HNO ₃	10	15	10
	2	H ₂ O ₂	5	15	14
B ^a	1	HNO ₃	10	15	10
	2	HNO ₃	10	30	22
	3	H ₂ O ₂	5	30	5
C ^a	1	HCl	15	50	5
	2	HNO ₃	5	30	10

^a A: lentil; B: bovine liver, vegetation; C: catalyst, SRM 2557.

2.4. Sample pre-treatment

The certified reference material (CRM) analyzed to determine the accuracy of the proposed procedure was National Institute of Standard and Technology (NIST), Standard Reference Material (SRM) 2557 catalyst. The sample was first prepared in accordance with the instruction of the analysis certificate, after which an accurately weighed amount of 0.1 g was subjected to microwave digestion. The working condition of the microwave oven is listed in Table 2. After digestion the sample was diluted to 100 ml with de-ionized water in a calibrated flask.

In view of the application of the method to the determination of palladium in foods and beverages, the ability to recover palladium from different samples spiked with palladium was investigated. For this purpose, standard solutions containing palladium were added to 0.2–0.7 g of different food samples and the resulting material was mineralised by microwave digestion as are listed in Table 2.

Tap water is collected immediately prior to the analysis.

Sea and river water was collected in polypropylene bottles previously cleaned by soaking in 0.1 mol l⁻¹ hydrochloric acid. Samples were filtered by using a membrane of 0.45 µm pore size, acidified to 0.1% (v/v) with concentrated HNO₃ and stored frozen until analysis.

3. Results and discussion

3.1. Optimization of the FI preconcentration and separation procedure

The influence of sample pH on the preconcentration of the palladium was investigated at a sample loading rate of 2.8 ml min⁻¹ with 60 s preconcentration times for 0.050 µg ml⁻¹ Pd (II). The results are shown in Fig. 1; as can be seen, the optimum pH range was around 7.0–8.0. All subsequent studies were carried out at pH 7.6.

The effect of the ionic strength of the medium on the efficiency of the preconcentration was investigated by using different concentrations of buffer, resulting in an optimum buffer concentration of 0.1 M with the best sample-to-blank ratio.

When using GFAAS as the detector, quantitative dissolution of the retained analyte in the smallest possible volume of eluent is important to satisfy the restricted volumetric requirements of the

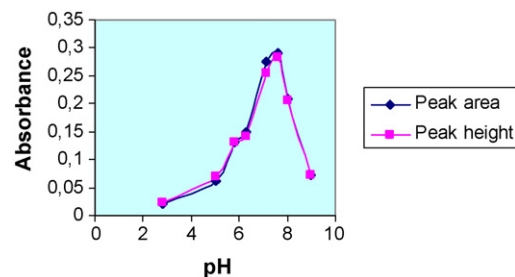


Fig. 1. Influence of pH on the preconcentration of palladium.

graphite tube/platform. As is well known, strong acids are very efficient at liberating metallic ions from their complexes, for this reason HNO₃, HCl and HNO₃ + HCl were tested as eluent. The best results were obtained with HCl 4 mol l⁻¹ that was chosen for subsequent studies.

The influence of volume of eluent used was also studied. As can be seen in Fig. 2, the signal increased as the volume increased up to 30 µl, and then remained constant with further increase in the volume of eluent. An injection volume of eluent of 40 µl was fixed.

Preliminary tests showed that the sample volume was not an important factor when the mass of analyte arriving at the columns was kept constant. The influence of the sample flow rate was studied using a constant volume of injection of eluent of 40 µl. For this purpose, 46 ng of Pd(II) was brought to pH 7.6 and passed through the column at different flow rates (the flow rate was varied by changing the speed of the peristaltic pump). Changes in the flow rates of the sample were studied between 2.0 and 4.6 ml min⁻¹, resulting in an optimum sample flow rate of 2.8 ml min⁻¹ with the best sample-to-blank ratio.

To evaluate the effect of sample loading time on the signal from 2 ng ml⁻¹ Pd(II). A sample flow rate of 2.8 ml min⁻¹ was used. The results obtained show that the signal increased almost linearly for up to 15 min preconcentration time, after which the slope decreased gradually. The sensitivity was increased by increasing the sample loading time; a loading time of 60 s was selected, in order to fit this time into the cycle of the furnace program, thus good sensitivity was achieved with high sampling frequency. Nevertheless, for samples with low concentrations of palladium longer loading times can be employed. The resin located in the microcolumn has a high capacity, its makes possible further lowering the concentration limit by increasing the sample loading time.

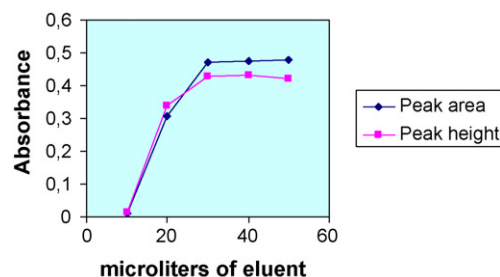


Fig. 2. Influence eluent volume injected (HCl 4 mol l⁻¹).

Table 3
Tolerance of foreign ions in the proposed method

Foreign species	Tolerated ratio (m/m)
Mg ²⁺ , Ni ²⁺ , K ⁺ , Co ²⁺ , SO ₄ ²⁻ , Br ⁻	>4000
Zn ²⁺ , Mn ²⁺ , Cr ³⁺ , SCN ⁻ , HCO ₃ ⁻	3000
Cd ²⁺	1000
Cu ²⁺ , Al ³⁺ , Fe ³⁺ , Ca ²⁺ , Ba ²⁺ , Pb ²⁺	500
Pt ⁴⁺	50
Rh ³⁺	10

As for graphite temperature program: complete dryness of aqueous standards and samples was ensured with two drying steps at 110 and 130 °C; a ramp of 5 s for the second drying temperature at 130 °C avoided spattering of the liquids and resulted in a uniform solid deposit on the surface of the atomizer platform. The rest of the graphite operating conditions was optimised by a univariate form taking the sample-to-blank ratio as election criteria. The optimised furnace temperature program for palladium determination is shown in Table 1.

3.2. Interferences

The effect of the metal ions co-existing with palladium and the some anions, which may be present in sample solutions, were investigated. The results of including significant levels of possible interferents are presented in Table 3. For this study, different amounts of the ionic species tested were added to a 10 ng ml⁻¹ solution of palladium. The starting point was an interferent:palladium ratio of 4000 m/m; if any interference occurred, the ratio was gradually lowered until the interference ceased. The tolerance limits found show that palladium can be determined in the presence of a variety of ions.

3.3. Analytical performance

Under the optimum conditions, with the use of a 60 s pre-concentration time, a sample flow rate of 2.8 ml min⁻¹ and an injection volume of eluent of 40 µl, the characteristic data for the analytical performance of the proposed FI on-line microcolumn separation and preconcentration system for GFAAS determination of palladium are summarized in Table 4, where analytical parameters such as precision, sensitivity, working range, etc

Table 4
Analytical performance of the proposed system

Analytical parameters	Peak area	Peak height
Calibration equation (C _{Pd} in ng ml ⁻¹)	A = 0.0125C _{Pd} + 0.0204	A = 0.098C _{Pd} + 0.0207
Correlation coefficient	0.9939	0.9935
Working concentration range (ng ml ⁻¹)	0–60	0–60
Detection limit (ng ml ⁻¹)	0.4	0.4
Determination limit (ng ml ⁻¹)	2.6	3.2
Precision (% R.S.D., n = 9, C _{Pd} = 10 ng ml ⁻¹)	3.5	3.1
Sampling frequency (h ⁻¹)	33	33

Table 5
Determination of palladium in certified and spiked samples

Sample	Certified value (mg kg ⁻¹)	Found value ^a (mg kg ⁻¹)	Recovery (%)
SRM 2557	233.2 ± 1.9	233.2 ± 2.2	100
Sample	Added (mg kg ⁻¹)	Found ^a (mg kg ⁻¹)	Recovery (%)
Lentil	5	5.3 ± 0.3	106
Bovine liver	5	5.1 ± 0.2	102
Pinus leaves	2	2.1 ± 0.1	105
Sample	Added (ng ml ⁻¹)	Found ^a (ng ml ⁻¹)	Recovery (%)
Tap	5	5.1 ± 0.1	102
water	10	10.0 ± 0.6	100
Sea	5	5.1 ± 0.1	102
water	10	10.1 ± 0.3	101
River	5	4.9 ± 0.3	98
water	10	9.6 ± 0.5	96

^a Mean ± S.D., n = 4.

obtained by peak area and peak height measurements are presented.

In order to test the accuracy and applicability of the proposed method for the analysis of real samples, one reference material was analysed. The result, as the average of the four separate determinations, is shown in Table 5. As can be seen, the palladium concentration determined by the proposed method is in close agreement with the certified value.

The accuracy and applicability of the method was estimated by spiking real samples with a known amount of Pd and determining the recoveries. The recovery studies carried out show recoveries between 96 and 106%, and therefore the suitability of this method is demonstrated.

4. Conclusions

The most frequently stated advantage of GFAAS is its low detection limit. However, in certain instances, the detection limits are still inadequate. In these cases, a preliminary preconcentration and/or separation is required. Conventional off-line procedures for separation and preconcentration, although effective, are usually time-consuming and tedious, and are vulnerable to contamination and analyte loss. FI – on-line column preconcentration – GFAAS has revolutionized ultra-trace element analysis in samples with complicated matrices.

In the present study, we evaluated an analytical procedure for palladium determination based on GFAAS. The FI on-line microcolumn preconcentration and separation system has been shown to be promising for the determination of trace amounts of Pd in samples with complicated and variable matrices as a result of its simplicity, high efficiency and good selectivity and accuracy.

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Short communication

Determination of total water content in inulin using the volumetric Karl Fischer titration

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Abstract

A new sample preparation method for the water content determination of inulin by volumetric Karl Fischer (KF) titration was developed and compared to the usual method of introducing the sample directly in the methanol-based working medium, modified or not by formamide (1:3, v/v) in order to increase sample solubility. In the proposed method, inulins were externally prepared by dissolving them in pure formamide (2.5:7.5, w/w). The time of analysis of the liquid/liquid reaction of the new method between the dissolved sample and the reaction medium is about 1–2 min, while the usual KF method is stopped after the 10 min delay time. The developed method permits the determination of water included in the crystals of the sample, confirmed by analysing both crystalline and amorphous inulin samples. Another advantage of this new method is its applicability for the water content determination of other polysaccharides that are not readily soluble in the working medium. Moreover, water content determination can be done by any type of volumetric KF titrator, as this proposed technique is not dependent on any additional tools such as a built-in homogeniser or a heatable titration beaker.

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Keywords: Inulin; Volumetric Karl Fischer titration; Water content; Water of crystallisation

1. Introduction

Inulin, depicted in Fig. 1, is a natural storage carbohydrate mainly found in chicory roots, Jerusalem artichoke and dahlia tubers. It is a mixture of polysaccharides composed of fructose unit chains (linked by β -(2 $\rightarrow$ 1) D-fructosyl-fructose bonds) of various length, terminated generally by a single glucose unit (linked by an α -D-glucopyranosyl bond). Its composition depends on the plant source, harvesting date but also on extraction and post extraction processes [1]. The use of inulin and derivatives in the food industry are in constant increase, and the main benefits and nutritional interests of these products are broadly discussed in the literature [2].

The most stable form for commercial inulin is the powdered form, which has the advantage of facilitating manipulation, stor-

age and transport. Stored at different relative humidities, powder products can physically changed (i.e. crystallisation), drastically modifying their characteristics, such as caking [3,4]. Because of this, the monitoring of water content of inulin during storage and quality control requires appropriate analytical techniques for accurate determination in order to prevent such negative effects. However, no publication about water content determination particularly in inulin is available and an analytical method for this purpose needed to be developed.

In general, moisture is determined by oven drying and does not correctly represent the water content of a sample. Indeed, high temperatures can induce the formation and the release of volatile substances (water and others), while water included in the crystals might or might not be completely detected. The Karl Fischer (KF) titration is a well-established method for the water content determination [5] based on the Karl Fischer reaction [6]. This technique allows the determination of the total water of a sample and is now the most important direct (primary) method for determining water content in foodstuffs. Details for

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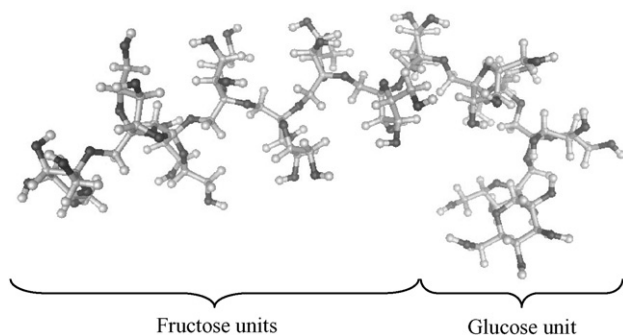


Fig. 1. Chemical structure of inulin. Image obtained by the Qmol version 3.0 software (Gans J.D. from the Cornell University) with a Ball and stick rendering. Courtesy to PhD Nicolas Delsaux from the 'Centre de Biophysique Moléculaire Numérique', Agricultural University of Gembloux.

the KF titration principle were described elsewhere [7]. KF reagents based on methanol are considered toxic. For this reason, Schöffski [8] tested other reagents replacing methanol by ethanol, but they are less adapted for water content determination in polysaccharides. Indeed, to determine the water content of the sample, it must be dissolved in the reaction medium, used as solvent. In some cases, the medium does not completely dissolve the sample. In order to increase solubility, some authors used an internal homogeniser [9–11], worked at elevated temperatures [12–16], modified the polarity of the working medium by adding further solvents to it [17] or pre-extracted the water [18].

These methodologies using the KF titration present some inconveniences as not all titration devices are equipped with an internal homogeniser or a heatable titration vessel, which limits the use of these techniques for wider applications. More so, at high temperature, possible evaporation of sulphur dioxide from the boiling working medium makes a reflux condenser necessary [12]. Pre-extraction of water is not a broadly discussed method in literature. Moreover, the supernatant containing the extracted water does not in all cases include the water of crystallisation, as a residue is still present. The addition of solvent in the reaction cell seems to be the most adequate technique for solubilizing samples in the working medium. However, the combination of some methodologies like the adding of a further solvent (i.e. formamide) in the medium with heating of the system or the utilization of a homogeniser, lead to some side reactions, falsifying the water content determination. Schmitt and Isengard [14] reported that formamide in the medium led to a higher drift because of a side reaction. However, when the drift was taken into account, they found values in accordance with the theoretical value. This phenomenon is known to occur particularly at high temperatures (i.e. 50 °C) and is much less expressed at lower temperatures.

The aim of the present study was to develop a suitable sample preparation for the total determination of water content of inulin, which can be present in an amorphous or crystalline state. For this purpose, inulin samples were analysed by KF titration using the usual method of introducing the powder directly in the methanol base-working medium, modified or not by formamide (1:3, v/v) in order to increase sample solubility. In addition, an

alternative methodology based on the introduction of an inulin solution in pure formamide was developed and compared to the standard KF titration.

2. Experimental

2.1. Materials

2.1.1. Inulin samples

The inulin samples used were two industrial spray dried products extracted from chicory roots, one purchased from Orafiti (inulin 1) and the other kindly supplied by Warcoing (inulin 2) both located in the Walloon Region (Belgium). They were placed into Petri dishes (six replicates) and stored for 3 weeks in hermetically closed containers containing P₂O₅ at 20 °C. This storage was done in order to eliminate the free water.

Determination of the molecular mass distribution of the samples was done by High Performance Anion Exchange Chromatography coupled with Pulse Amperometric Detection (HPAEC-PAD) on a Dionex DX500 chromatographic system operating at 1 ml min^{−1}. About 25 µl of 0.8 g l^{−1} solution were injected. Separation of the various chain lengths was achieved on a Dionex PA100 column as described by Ronkart et al. [4]. The mobile phase consisted of sodium hydroxide (150 mM) elution in isocratic mode, followed by a linear gradient with a solution containing both sodium hydroxide (150 mM) and sodium acetate (1 M). The gradient ended by washing with sodium hydroxide 500 mM. Relative response factors of the various species were taken from Timmermans et al. [19] and Kang [20]. Inulin 1 and 2 had an average degree of polymerisation number (DP_n) and weight (DP_w) of about 26 and 31 and 23 and 30, respectively.

Determination of crystallinity was done by a PW3710 Philips Analytical X-ray B.V. static Wide Angle X-ray Scattering (WAXS) diffractometer, using a Ni filtered Cu Kα radiation, generated by an anode device operating at 40 kV and 30 mA in conjunction with a proportional detector. The patterns were recorded with a fixed time of 0.4 s per step of 0.02° in the 4 < 2θ < 30° range [4,21]. Fig. 2 shows the amorphous state of inulin 1 as only a flat diffractogram is present, while inulin 2 contains several diffraction peaks surimposed on an amorphous halo, hence confirming the semi-crystalline nature of this sample.

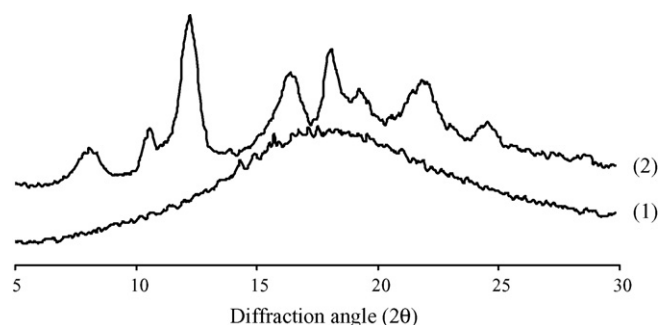


Fig. 2. Static wide angle X-ray scattering (WAXS) diffractograms of inulin samples. (1) inulin 1 and (2) inulin 2.

2.1.2. Karl Fischer titration

The KF titration was carried out with a Karl Fischer titrator DL31 from Mettler Toledo using the two-component technique with Hydra-Point Solvent G and Hydra-Point titrant (5 mg H₂O/ml), both purchased from J.T. Baker (Deventer, Holland). The polarising current for bipotentiometric end-point determination was 20 μ A and the stop voltage 100 mV. The end-point criterion was the drift stabilisation (15 μ gH₂O min⁻¹) or maximum titration time (10 min). The water content was calculated as described by Scaccia [22], and the measurement was corrected for the baseline drift, defined as the residual or penetrating water that the apparatus removes per minute.

For better sample dissolution, the working medium was modified by adding formamide pro analyse min 99% purchased from Merck (Leuven, Belgium).

2.2. Methods

2.2.1. Experimental precaution

Traces of water can penetrate into the titration vessel from the exterior through tubes and joints. For this reason, before starting experiments, 15 ml of titrating agent were washed through. Then, the exact water equivalent of the titrant component was determined by analysing a water standard reference (apura[®] Water standard 1% standard for volumetric Karl Fischer Titration, Merck KGaA, Darmstadt, Germany). Before starting an experiment (drift or water equivalent determinations) or introducing the sample into the working medium for water determination, the cell was titrated to dryness. In the course of time, a slight reagent consumption to keep the titration cell dry is always observed. This so-called drift (expressed in μ g water min⁻¹) is due to water intruding through the tubes and joints. For precise analyses, especially when the determination times were long, consumption due to the drift was subtracted from the result, taking the titration time into account. Sample analyses were started when the drift was lower than 10 μ g water min⁻¹.

2.2.2. KF titration of powdered inulin

About 250 mg of inulin was introduced in the reaction cell. The latter contained either only the methanolic KF working medium or a modified one with additional formamide (1:3, v/v) in order to increase the solubility of these samples.

All mass determinations were carried out by back-weighing, using a Sartorius BP61S balance (± 0.0001 g). Thus, the exact sample weight was determined only after adding the sample into the titration cell. Stirring time in the cell before titration

was fixed at 20 s. Six analyses were performed for each method and sample, and the respective results were averaged.

2.2.3. KF titration of inulin solution in formamide

About 2.5 g of inulin were dissolved in 7.5 g pure formamide in a 15 ml Falcon[®] tube and heated at 45 °C for 30 min, then vortexed until complete dissolution. Then, about 1.5 g of this 25% (w/w) solution was injected in the reaction cell using a syringe.

Beforehand, to determine if inulin was totally dissolved in the formamide solution, a solubility test was performed. Inulin 2 (less soluble in formamide than inulin 1) was dissolved in formamide in the 1–35% (w/w) range. These solutions were then centrifuged at 30,000 $\times$ g for 1 h and the supernatants were checked by HPAEC-PAD as described above. Results have shown a total solubility up to 25% (w/w) and a slight decrease above this concentration. For this reason, the chosen concentration for sample preparation in formamide was 25% (w/w).

3. Results and discussion

Water content calculated for the inulin samples by volumetric KF titration are summarized in Table 1.

3.1. Analysis of powdered inulin by KF titration without formamide in the working medium

The water content found for inulin 1 and 2 was 1.27 and 1.10 g water/100 g anhydrous inulin, respectively. However, the dissolution of the product was not complete, as the working medium was very cloudy, and the analysis was stopped by the imposed maximum titration time of 10 min and not by the drift criterion. So, the water content was underestimated for both samples. This phenomenon can be related with the way the end-point indication is fixed. Indeed, the volumetric KF titration is based on the following two-step reaction:

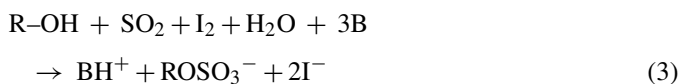
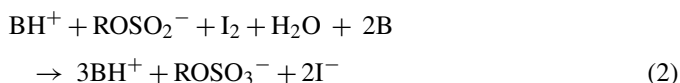
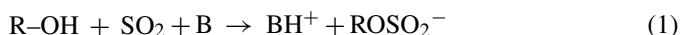


Table 1
Results of the Karl Fischer titration (g water/100 g anhydrous sample) of inulin, by the conventional KF titration (with or without formamide in the working medium) and for the proposed KF method, all performed at 20 °C

Sample	Conventional KF method		Proposed KF method ^a
	Without formamide ^a	With formamide ^a	
Inulin 1	1.3 $\pm$ 0.3 (>10 min)	2.1 $\pm$ 0.1 (4–5 min)	2.1 $\pm$ 0.2 (1–2 min)
Inulin 2	1.1 $\pm$ 0.3 (>10 min)	1.8 $\pm$ 0.3 (>10 min)	2.4 $\pm$ 0.1 (1–2 min)

^a In parentheses, the titration time in min.

In the first step, an alcohol ROH (usually methanol) is esterified with sulphur dioxide. To obtain a quantitative reaction, the ester is neutralised by a base B (usually imidazole) to yield alkyl sulphite (Eq. (1)). In the second step, alkyl sulphite is oxidised by iodine to give alkyl sulphate in a reaction that requires water. The base, again, provides for a quantitative reaction (Eq. (2)) [23].

Two platinum electrodes are submerged in the working medium and are polarized by a constant current. A certain voltage is necessary to maintain this current. When it falls and remains below a certain chosen value for a chosen time, the so-called stop delay time, the analysis is stopped. Another stop criterion is the drift. When the reagent consumption rate reaches the same (or a slightly higher) value as immediately before the start of the analysis, it is assumed that all the water of the sample is detected, and the analysis is stopped. Additionally, it is possible to set a maximum determination time, which must not be exceeded. If the determination is stopped after this maximum time, the analysis is incomplete, since the “real” stop criteria have not been fulfilled. In this case, as the samples were not completely dissolved, water diffusion was low and delayed, so iodine was continually consumed keeping the titration rate above the drift value. Thus, since the measurement was stopped after 10 min and not because water detection was complete, it would have been necessary to increase the maximum titration time to allow the water to reach the working medium completely by diffusion and extraction.

3.2. Analysis of powdered inulin by KF titration with formamide in the working medium

In comparison with the analysis of powdered inulin by KF titration without formamide in the working medium, analysis of inulin 2 was still stopped by the maximum time (10 min), whereas the analysis of inulin 1 was stopped by the drift criterion after 4–5 min. The addition of formamide led to a higher water content estimation. Indeed, 2.07 and 1.83 g water/100 g anhydrous inulin for inulin 1 and 2 were found, instead of 1.27 and 1.10 g water/100 g anhydrous inulin (without formamide). We can correlate this phenomenon with the fact that the addition of formamide to the medium leads to a complete extraction of the water between the amorphous particles of inulin 1, while the extraction of the partially crystalline inulin was not completely achieved.

Although Wünsch and Schöffski [24] claimed that formamide accelerated the iodination of imidazole, leading to slightly over-estimated water content values, we assumed that formamide up to one third by volume did not yield false results. Indeed, the analysis of a 1% water standard before and after the formamide addition in the working medium led to the same mean values even if the addition of formamide to the system delayed the reaching of the drift criterion.

By adding formamide in the methanol-based medium (1:3, v/v), an increase of the water content value is obtained because of a better solubility of the sample.

3.3. Analysis of inulin dissolved in formamide by KF titration

Water content was calculated by subtracting the water content of formamide. The latter was determined before experimentation and was 0.03 g water/100 g formamide.

Inulin 1 analysis led to the same water content determination as the introduction of inulin powder in the titration cell with addition of formamide (2.07 g water/100 g anhydrous inulin), but the analysis time was reduced by at least a factor 2. However, the water content of inulin 2 was higher than that analysed by the introduction of inulin powder in the titration cell. This difference is probably due to the release of water entrapped in the crystals of the semi-crystalline products. So, it can be assumed that the proposed method fully extracts the water content of inulin samples. In addition, the analysis time was only 1–2 min, due to the immediate availability of the water. This shorter analysis time reduces the influence of a possible change of the drift during the analysis. The developed method has a higher precision of the measurements than the methods with incomplete water detection. It is well adapted when the water content of the sample is low.

4. Conclusion

The total water content of inulin has been determined by volumetric Karl Fischer titration, using a complete pre-dissolution of the sample in pure formamide, and was compared to the usual introduction of the sample directly in the methanol-based working medium, modified or not by addition of formamide (1:3, v/v) in order to increase sample solubility. The described method allowed the determination of water included in the crystals, as shown by comparing the results for amorphous and semi-crystalline inulin samples.

The titration time for the proposed method was 1–2 min, while complete water detection was not achieved after 10 min by the standard method. Dissolving the sample beforehand and transferring an aliquot into the titration cell is time consuming, but this is compensated by the shorter titration time. This advantage is even more important, when routine analyses are carried out and several samples can be dissolved simultaneously [18]. Moreover, the proposed method has the advantage of working at ambient temperature and can be applied to every volumetric KF titrator.

This methodology can be applied for the water content determination of other polysaccharides presenting a solubility problem in the working medium.

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Determination of polybrominated diphenyl ethers in soil by ultrasonic assisted extraction and gas chromatography mass spectrometry

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Abstract

An analytical method for the determination of polybrominated diphenyl ethers (PBDEs) in soil was developed. Soil samples were placed in small glass columns and PBDEs extracted from soil, with a low volume of ethyl acetate (5 mL, 2×15 min), assisted by sonication. PBDEs were determined by gas chromatography with electron impact mass spectrometric detection in the selected ion monitoring mode (GC–MS–SIM) and residues were confirmed by their retention times, selected ions and qualifier–target abundance ratios. Recovery studies were performed at 10, 1, 0.1 and 0.05 $\mu\text{g/kg}$ fortification levels, and the recoveries obtained ranged from 81 to 104% with a relative standard deviation between 1 and 9%. The detection limit of the method varied from 2 to 30 pg/g and the quantification limit ranged from 7 to 100 pg/g for the different PBDEs studied. The developed method was linear over the range assayed, 0.01–10 $\mu\text{g/kg}$ with determination coefficients equal or higher than 0.997. The proposed method was used to determine PBDEs levels in soil samples from different areas of Spain and PBDEs were detected in some samples with values ranging from 1.3 to 5.6 $\mu\text{g/kg}$.

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) (Fig. 1) are frequently used as flame retardant additives in a variety of electrical appliances, textiles and building materials. Technical PBDEs are commercially produced by brominating diphenyl ether under catalytic conditions resulting in mixtures of brominated diphenyl ethers with varying degrees of bromination [1]. Due to their ubiquitous appearance in the ecosystem, various PBDEs formulations have been forbidden by the European Union because of their potential bioaccumulation in the food chain and toxicity. The PBDEs have been reported as pollutants in various types of environmental samples, like fish [2–4], sewage sludge [5] and milk [6], but few data on their levels in soil have been published. The two congeners which are most frequently reported in biota are BDE 47 (2,4,2',4'-tetra BDE) and BDE 99 (2,4,5,2',4'-penta BDE) [7].

The PBDEs can enter the soil through atmospheric fallout via dry and wet deposition, or through accumulation in vegetation that eventually reaches the ground on falling foliage [8]. The highest concentrations have been found in the vicinity of industrial sites where these compounds have been used [8,9].

There are 209 different PBDE congeners and many are notoriously difficult to detect because they decompose at relatively low temperatures.

Several methods for extracting PBDEs from soil have been published. The classical extraction technique used in the determination of PBDEs in soil samples is the solid–liquid partitioning with organic solvents, followed sometimes by subsequent clean-up of extracts before their gas chromatographic determination [8]. The traditional Soxhlet extraction has been widely employed, using non-polar solvents such as toluene [9,10] or solvent mixtures like hexane–acetone (1:1, w/w) [11]. The drawbacks of these traditional extraction methods are the consumption of large amounts of solvents and glassware and the long time needed for the analysis. These drawbacks can be reduced by using other extraction techniques developed lately, such as the pressurized liquid extraction (PLE), which reduces the time

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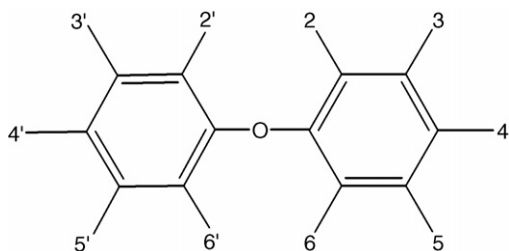


Fig. 1. Chemical structure of PBDEs. See Table 1 for identification.

and amount of organic solvent due the high temperature and pressure used [12,13].

PBDEs are generally determined by capillary gas chromatography [14], using preferably selected ion monitoring–mass spectrometry (GC–MS–SIM), both in electron impact (EI) [8,10] or negative chemical ionization (NCI) [12] mode. The application of an ion trap–tandem mass spectrometry (GC–MS–MS) for trace analysis of PBDEs has been recently reported [11].

The aim of the present paper is the development of a rapid and sensitive method for the determination of PBDEs in soil samples by ultrasonic extraction with a low volume of organic solvent and GC–MS determination. Sample preparation was based on our previously published methods for pesticide analysis in soil by sonication-assisted extraction in small columns (SAESC) [15–18]. The proposed method was validated and applied to the determination of PBDEs in soil samples from different areas of Spain.

2. Experimental

2.1. Materials

2.1.1. Standards

The standard mixture of PBDEs (code BDE-MXD) was supplied by Wellington Laboratories (Guelph, Ont., Canada). This mixture contains B-17 (2,2',4-tri-BDE), B-47 (2,2',4,4'-tetra-BDE), B-66 (2,3',4,4'-tetra-BDE), B-100 (2,2',4,4',6-penta-BDE), B-153 (2,2',4,4',5,5'-hexa-BDE), B-183 (2,2',3,4,4',5',6-hepta-BDE) and BDE 209 (deca-BDE). The decabromodiphenyl ether was not determined because the chromatographic conditions needed are different. The standard mixture in nonane (5 µg/mL) was stored at 4 °C in an amber glass container to prevent photolysis of the brominated compounds.

An intermediate standard solution was prepared by transferring 1 mL from the standard solution to a 10 mL volumetric flask and diluting to volume with hexane to obtain a concentration of 0.5 µg/mL. A set of calibration standard solutions of 10, 1, 0.1 and 0.05 µg/L was prepared by dilution and used to fortify soil samples.

2.1.2. Reagents

Ethyl acetate, dichloromethane and hexane, residue analysis grade, were purchased from Scharlau (Barcelona, Spain) and anhydrous sodium sulfate, reagent grade, was obtained from Merck (Darmstadt, Germany). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to provide

ultrapure water in this study. Florisil (60–100 mesh), research grade, was purchased from Fluka (Chemie, Buchs, Switzerland). Florisil was heated for 24 h at 140 °C before use and employed during the following week.

2.2. Apparatus

2.2.1. GC–MS

GC–MS analysis was performed with an Agilent 6890 (Waldbronn, Germany) gas chromatograph equipped with an automatic split–splitless injector Model HP 7683, and an inert mass spectrometric detector (MSD) Model HP 5973N, which is equipped with an inert ion source. A fused silica capillary column (ZB-5MS), 5% phenyl polysiloxane as non-polar stationary phase (30 m × 0.25 mm, i.d.) and 0.25 µm film thickness, supplied by Phenomenex (Torrance, CA), was employed. Operating conditions were as follows: injector port temperature 290 °C; helium as carrier gas at a flow-rate of 1.0 mL/min; pulsed splitless mode (pulsed pressure 45 psi = 310 kPa for 1.5 min). The column temperature was maintained at 110 °C for 1 min, then programmed at 10 °C/min to 300 °C; and held for 10 min. The total analysis time was 30 min and the equilibration time 2 min. A 2 µL volume was injected splitless, with the split valve closed for 1 min.

The mass spectrometric detector was operated in electron impact ionization mode with an ionizing energy of 70 eV, scanning from m/z 200–800 at 3.62 s/scan. The ion source temperature was 300 °C and the quadrupole temperature 150 °C. The electron multiplier voltage (EM voltage) was maintained 100 V above autotune and a solvent delay of 5 min was employed.

Analysis was performed with selected ion monitoring (SIM) using one target and two qualifier ions. The target and qualifier abundances were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 200 to 800 m/z . The concentration was determined by comparing the peak areas in the sample with those found in the standard of known concentration. Standards were prepared in blank matrix extracts, in order to counteract the matrix effect observed. Table 1 lists the PBDEs studied along with their retention times, the target and qualifier ions and their qualifier to target abundance ratios. The SIM program used to determine and confirm PBDEs in soil has five acquisition windows with an ion dwell time of 100 ms and scan rate of 2.86 and 2.15 cycles/s. PBDEs were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratios. Retention times had to be within ±0.3 min of the expected time and qualifier-to-target ratios had to be within a 20% range for positive confirmation.

2.2.2. Extraction equipment

Glass columns (20 mL) of 10 cm × 20 mm i.d., Afora, Spain, and Whatman No. 1 filter paper circles of 2 cm diameter (Whatman, Maidstone, UK) were used.

An ultrasonic water bath (Raypa, Barcelona, Spain) was used in the extraction step. The generator of this ultrasonic water bath has an output of 150 W and a frequency of 35 kHz. A vacuum

Table 1

Retention times (t_R , min), molecular weight (M_W), target ion (T), qualifier ions (Q_1 , Q_2 , Q_3) and abundance ratios of qualifier ion/target ion (Q_1/T , Q_2/T , Q_3/T)^a

PBDE	Name	IUPAC	t_R	M_W	T	Q_1	Q_2	Q_3	Q_1/T (%)	Q_2/T (%)	Q_3/T (%)
1	2,2',4-tri-BDE	B-17	15.24	406.9	246	406	408		52.6	51.8	
2	2,2',4,4'-tetra-BDE	B-47	17.76	485.8	326	484	486	488	41.0	59.8	39.0
3	2,3',4,4'-tetra-BDE	B-66	18.10	485.8	326	484	486	488	22.9	32.8	21.5
4	2,2',4,4',6-penta-BDE	B-100	19.29	564.7	404	564	566		61.3	58.7	
5	2,2',4,4',5,5'-hexa-BDE	B-153	21.88	643.6	484	641	643		18.8	20.5	
6	2,2',3,4,4',5,6-hepta-BDE	B-183	24.87	722.5	564	721	723		13.2	13.6	

^a Q/T (%) are the results of abundance values of the qualifier ion (Q_1 , Q_2 , Q_3) divided by the abundance of the target ion (T) $\times$ 100.

manifold (Supelco, Visiprep, Madrid) was employed for removing the extraction solvent.

2.3. Soil samples

Soils A and B, used in the recovery assay, were collected from the plough layer (0–10 cm) of experimental plots located in Madrid and Badajoz (Spain), respectively, sieved (2 mm) and stored at room temperature until fortified. The characteristics of these soils were—soil A: pH 7.69, organic matter content 0.97%, sand 44.34%, silt 37.44%, clay 18.22% and field capacity (% at -33 kPa) 14.76; soil B: pH 6.5, organic matter content 0.8%, sand 83.4%, silt 12.2%, clay 4.4% and field capacity (% at -33 kPa) 11.2.

Several soil samples were collected in different areas of Spain. Five soil samples were taken from agricultural fields amended with sludge in Badajoz and two soil samples were collected from an industrial area in Vizcaya. Soil samples were frozen at -18 °C until analyzed.

2.4. Extraction procedure

Two filter paper circles of 2 cm diameter were placed at the end of a glass column (20 mL) and anhydrous sodium sulfate (3 g) was added, then sieved soil (10 g) was placed in the column. For the recovery studies, soil samples were weighed in a weighing funnel (10 mL) and fortified with 1 mL of a mixture of the different PBDEs to reach final concentrations of 10, 1, 0.1 and 0.05 $\mu\text{g/kg}$, allowing 30 min for solvent evaporation. The soil moisture content was adjusted by adding water to study the influence of moisture in the PBDEs recovery. Soil samples were extracted with 5 mL of ethyl acetate for 15 min in an ultrasonic water bath at room temperature. The water level in the bath was adjusted to equal the extraction solvent level inside the columns, which were supported upright in a tube rack and closed with one-way stopcocks. After extraction, the columns were placed on the multipoint vacuum manifold where the solvent was filtered and collected in graduated tubes. Soil samples were extracted again with another 5 mL of ethyl acetate (15 min). The extracting solvent was filtered and soil samples washed with 1 mL of additional solvent. The total extract collected in 10 mL graduated tubes was concentrated with a gentle stream of air to an appropriate volume, 1 mL. A subsequent clean-up step was performed for levels lower than 0.1 $\mu\text{g/kg}$. The extract was loaded in a glass column containing 2 g of Florisil and PBDEs were eluted with 10 mL of hexane–dichloromethane (1:2, v/v). The

eluate was concentrated to 1 mL and stored at 4 °C until analyzed by GC–MS.

3. Results and discussion

3.1. Mass spectrometric determination

PBDEs levels were determined by gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM). Fig. 2 shows representative GC–MS–SIM chromatograms of a blank soil sample and a standard containing 10 $\mu\text{g/L}$ in a fortified blank sample. The analysis of blank samples confirmed the absence co-extracted interferences in extracts from soil samples. The mass spectra of PBDEs have a characteristic fragmentation pattern under impact electron ionization. The $(M - \text{Br}_2)^+$ is the most abundant peak in the spectrum whereas other peaks, as the molecular ion (M^+), are present in a lower relative intensity, Table 1. The target ions selected were the most abundant

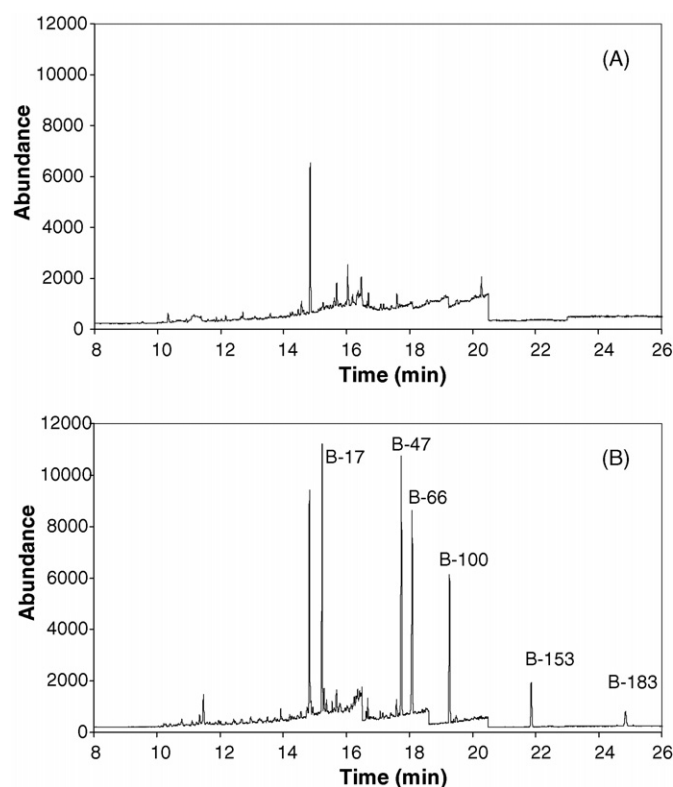


Fig. 2. Chromatograms of (A) a blank soil sample and (B) a standard mixture solution, 10 $\mu\text{g/L}$, in a fortified blank sample.

Table 2

Limits of detection and quantitation (LOD, LOQ), calibration data and repeatability of the proposed method

PBDE	LOD (pg/g)	LOQ (pg/g)	Calibration data		Repeatability (R.S.D., %) ^a	
			Equation	Determination coefficient	Peak area	<i>t_R</i>
B-17	3	10	$y = 1.19 \times 10^{-4}x + 2.53 \times 10^{-4}$	0.997	11	0.03
B-47	2	7	$y = 9.01 \times 10^{-3}x + 1.88 \times 10^{-4}$	0.997	12	0.02
B-66	14	46	$y = 1.02 \times 10^{-4}x + 1.66 \times 10^{-4}$	0.998	12	0.03
B-100	15	50	$y = 4.95 \times 10^{-3}x + 9.90 \times 10^{-3}$	0.997	8	0.02
B-153	14	46	$y = 3.72 \times 10^{-3}x + 8.37 \times 10^{-3}$	0.998	6	0.02
B-183	30	100	$y = 9.53 \times 10^{-2}x + 2.819 \times 10^{-3}$	0.997	12	0.02

^a Repeatability of the chromatographic method. Relative standard deviation of retention times and peak areas (*n* = 10).

Table 3

Recoveries^a of the studied PBDEs in soils A and B

PBDE	Fortification levels (μg/kg)							
	10		1		0.1		0.05	
	A	B	A	B	A	B	A	B
B-17	97 ± 5	97 ± 4	96 ± 5	97 ± 2	95 ± 4	98 ± 7	95 ± 6	97 ± 6
B-47	98 ± 5	100 ± 3	98 ± 6	99 ± 3	104 ± 6	91 ± 3	95 ± 4	96 ± 8
B-66	100 ± 5	100 ± 3	101 ± 3	98 ± 4	102 ± 2	92 ± 4	97 ± 3	85 ± 5
B-100	94 ± 4	98 ± 4	95 ± 4	92 ± 3	104 ± 8	101 ± 2	84 ± 4	81 ± 3
B-153	93 ± 4	99 ± 3	98 ± 2	97 ± 4	90 ± 4	88 ± 4	92 ± 9	88 ± 9
B-183	93 ± 4	101 ± 1	99 ± 2	97 ± 4	91 ± 7	100 ± 2	N.D.	N.D.

N.D.: not determined, concentration below the quantitation limit.

^a Results are the mean of four replicates ± standard deviation.

ions of ion clusters obtained by the loss of Br₂ from molecular ions. These ions have the highest intensity in the mass spectra of the brominated diphenyl ethers. The qualifier ions were the respective molecular ions.

3.2. Method validation

3.2.1. Linearity

The linearity of all PBDEs was determined in the range from 0.01 to 10 ng/g. Fortified blank samples were used as standards because a matrix effect was observed in the chromatographic analysis. The MS response for all PBDEs was linear in the concentration range assayed with determination coefficients equal or higher than 0.997 for all PBDEs. Table 2 summarizes the calibration data of the studied PBDEs.

3.2.2. Repeatability

The repeatability of our chromatographic method was determined by performing the analysis of a sample spiked at 5 μg/kg. The sample was injected 10 times with an automatic injector and the relative standard deviation (R.S.D.) values obtained for the retention times ranged from 0.02 to 0.03%, whereas for peak areas the values ranged from 6.0 to 12.0% (Table 2). The repeatability of the whole analytical method was also determined by replicate analysis of a fortified sample during different days. The repeatability of the method, expressed as relative standard deviation (R.S.D.), was lower than 15% for all compounds.

3.2.3. Specificity

The specificity of the proposed method was assessed by analyzing blank soil samples. The absence of background peaks,

Table 4

Recoveries^a of the studied PBDEs at different soil moisture content (1 and 10%)

PBDE	Moisture content (10%)		Moisture content (1%)	
	A	B	A	B
B-17	97 ± 4	98 ± 5	100 ± 1	99 ± 3
B-47	97 ± 4	99 ± 4	100 ± 1	98 ± 2
B-66	96 ± 4	99 ± 3	97 ± 3	95 ± 2
B-100	95 ± 6	96 ± 5	93 ± 1	93 ± 2
B-153	95 ± 6	97 ± 6	95 ± 4	100 ± 2
B-183	100 ± 4	90 ± 4	98 ± 1	100 ± 2

^a Results are the mean of five replicates ± standard deviation.

above a signal-to-noise ratio of 3, at the retention times of target PBDEs, showed that no interferences occurred.

3.2.4. Recovery

Table 3 shows the recovery results obtained. No detectable levels of PBDEs were found in the blank soils used. The samples were fortified at 10, 1, 0.1 and 0.05 $\mu\text{g/kg}$ before extraction by adding 1 mL volume of the appropriate working standard solution. The fortified samples were allowed to stand for 30 min to let the solvent evaporate before extraction. Four sample replicates spiked at each fortification level were analyzed. The recoveries obtained ranged from 81 to 104%. The precision of the method, obtained as the relative standard deviations (R.S.D.s) of analyte recoveries, was good, lower than 10%. The ions used for quantification are shown in Table 1.

To test the influence of the soil moisture content on the recovery, soil samples were fortified with 5 $\mu\text{g/kg}$, the soil moisture content was adjusted to 10 or 1% and PBDEs extracted with ethyl acetate following the procedure described above. Table 4 shows the results obtained. Good recoveries, higher than 90% and similar to the recovery values previously shown, were obtained for both soil moisture contents and all the compounds studied. These results indicate the adequacy of the method to determine PBDEs in soil samples with different moisture content.

3.2.5. Detection and quantification limits

The limits of detection (LOD) of the proposed method were determined by considering a value 3 times the background noise obtained for blank samples, whereas the limits of quantification (LOQ) were determining considering a value 10 times the background noise. Table 2 summarizes the detection and quan-

tification limits obtained for the PBDEs in soil. The LODs varied from 2 to 30 pg/g and are similar to those reported by other authors that were in the range from 4 to 33 pg/g [8] or from 1 to 46 pg/g [12], although they are somewhat lower than those recently reported which varied from 13 to 250 pg/g . [11].

3.2.6. Application to real samples

The developed method was applied to the determination of PBDEs in soil samples collected in different areas of Spain. No detectable levels of PBDEs were found in soils from agricultural fields amended with sludge. Soil samples from an industrial area showed various levels of PBDEs, B-47 (4.6 ng/g), B-100 (1.3 ng/g), B-153 (2.65 ng/g) and B-183 (5.6 ng/g). B-17 and B-66 were not detected in these soil samples. A representative chromatogram is depicted in Fig. 3 showing the mass spectra with selected ions. These results are in the range of values reported in a published paper on PBDEs levels in surface soils from United Kingdom and Norway [8].

4. Conclusion

A rapid and sensitive method, based on the sonication-assisted extraction of samples placed in small columns, has been developed for the determination of PBDEs in soil by GC–MS–SIM. In the proposed analytical method, the extraction of samples is performed with a low volume of ethyl acetate, high recoveries through the method were obtained, without being affected by the soil moisture content and good reproducibility and low detection and quantification limits were achieved. This method was applied to the analysis of PBDEs in soil samples collected from various agricultural areas of Spain, where PBDEs were not detected, and from an industrial area where various PBDEs were detected in the range from 1.3 to 5.6 $\mu\text{g/kg}$.

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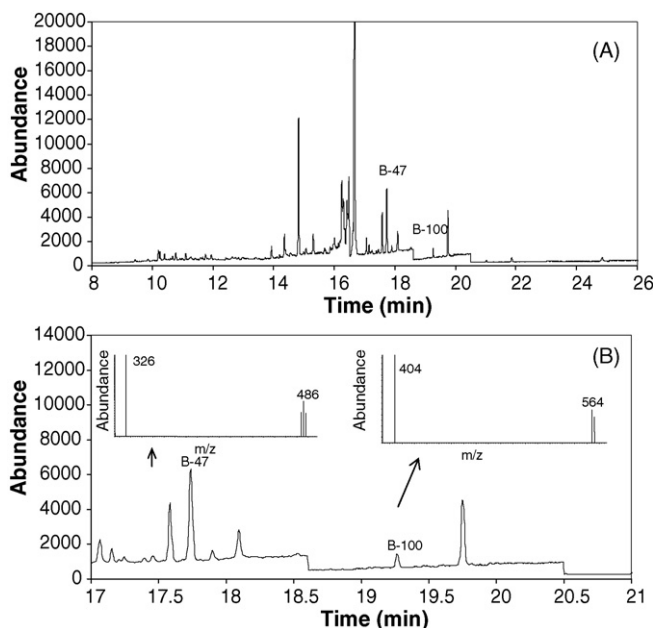


Fig. 3. Chromatograms of a soil sample collected in an industrial area containing B-47 (4.6 ng/g) and B-100 (1.3 ng/g): (A) a GC–MS chromatogram showing the presence of PBDEs B-47 and B-100 and (B) a partial view of the SIM chromatogram with the corresponding mass spectra.

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XRF study of archaeological and metallurgical material from an ancient copper-smelting site near Ein-Yahav, Israel[☆]

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Abstract

A movable XRF instrument (a bench-top designed machine modified to work in the field for archaeological purposes) was used for analysis of artifacts and sediments during the field study (01–07 April 2003) of a small copper-smelting site in the Negev (about 30 km west of the Feinan complex of ancient copper mines and smelting sites in Jordan). The site consists of a relatively small hill with blackened slopes, covered mainly by crushed copper slag. The surface collection of datable objects (i.e. pottery shards and stone tools) indicated that the site had functioned at the end of the Early Bronze Age and during the Roman/Byzantine time.

The analysis of Cu concentration in the ash and in the ground as well as the analysis of ore and slag, were used as markers for the identification of the operation centers and for locating the remains of the smelting devices used at the end of the Early Bronze Age for smelting copper. The ore in use typically contained 35–45% Cu, up to 1% Mn and up to several percent Fe. The slag contained 13–20% Mn and 1–5% Cu and Fe. From these results we are able to estimate the Cu production scale during the first and most active period on this site, at the end of the Early Bronze Age. © 2006 Elsevier B.V. All rights reserved.

Keywords: Archaeometallurgy; Copper; XRF; Slag; Ore

1. Introduction

Among the many sites of ancient metallurgical activity in the Near East, Feinan (Jordan) stands out. According to the results of the detailed investigations conducted at this site by German, Jordanian and American scientists [1,2] the process of Early Bronze Age Cu smelting of the rich local ore was reconstructed as based on natural wind, without the need to use men operated bellows. The location of the furnaces remains, is in a row close to the top of the hill and the slopes are covered by the slag debris. The chemical characteristic of the Feinan slags from Bronze and Iron Ages is the relatively high content of manganese (up to 15–20% Mn).

West of Feinan in the Israeli Arava, several sites of ancient metallurgical activity on hills covered by slag debris, similar to

Feinan, were discovered [3]. This paper is aimed at presenting the results of a study of the chemical composition of slags and copper ore from a site near Ein Yahav (Negev). These results will be compared to the known and published data from the nearby site of Feinan for identifying process similarities. In this paper we are concentrating on the preliminary analytical results of the chemical composition of the slags. The overall archeological results are detailed in a separate paper [4].

2. The experiment

2.1. General background

The research focuses on a small copper-smelting site situated in the central 'Arva', near the Sapir regional center, 1.5 km southeast of the spring of 'Ein Yahav' (today incorporated into *Agam Sapir*—a pool within a small local park), and ca. 30 km west of the Feinan copper mines ('Ein Yahav'; Fig. 1).

The site consists of a crescent shaped chalk hill jutting out of the surrounding flat landscape. The hill is especially

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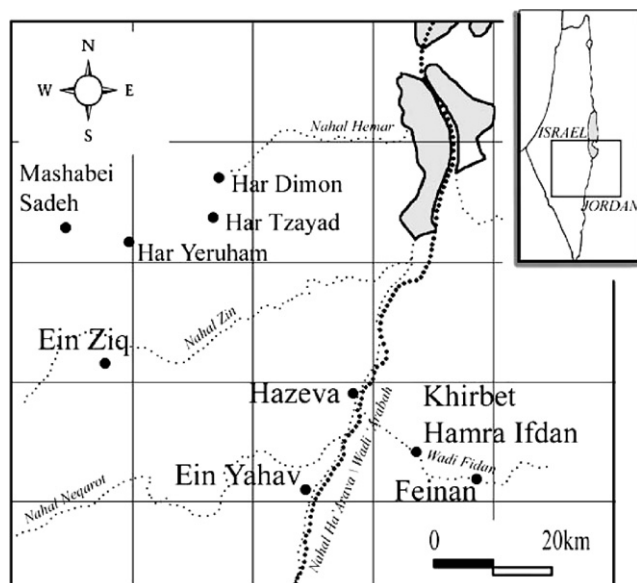


Fig. 1. Regional map and sites mentioned in the text.

prominent within the scenery due to its blackened northwestern slope, apparently a result of a spill of crushed copper slag (Fig. 2).

Preliminary visits to the site revealed, in addition to the copper slag, an ash layer, fragments of furnaces, pottery sherds and stone implements. The surface collection of datable objects indicated that the site had functioned during two periods: the Early Bronze Age and the Roman Period [4].

2.2. Experimental technique

In the pilot season we operated a prototype field XRF machine based on a small bench top model (EX-310LC, Jordan Valley, Israel) with Rh X-ray tube and Peltier cooled silicon PIN-photodiode as detector. This instrument was converted for use on site with a car battery as a power source. For the slag and ore analysis we were using the voltage of 20 kV. This relatively low voltage was found to be most suitable for measuring the content of Mn, Fe and Cu in the slag and ore. For analyses of metal

artifacts we were using higher voltage and special filtering for better contrasting elements such as Sn or Ag.

During preliminary tests the sensitivity limit for determination of the metal content (Cu, Fe, Mn) in ground or stones was established at about 0.01% in weight. For quantitative

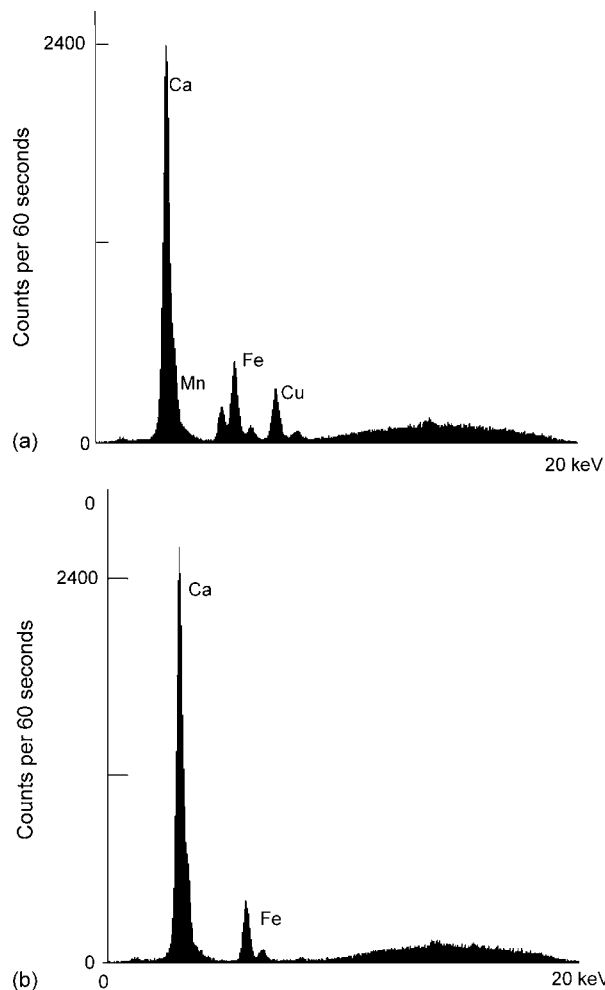


Fig. 3. The spectra of ash enriched by Cu (0.2%) and by Mn (0.15%) (a) and of “pure” ash (b). The content of Fe is about 0.5% in both cases.



Fig. 2. The site. A view from north, looking south.

determinations we used standards made of mixtures of ground or pure sand with a definite amount of additional metal in a concentration region up to about 1%. The accuracy of such technique (several relative percents) was confirmed by comparison with the direct chemical analysis. This normalization could be used for small metal content. For determining large concentrations of metals the samples were diluted in the laboratory by adding pure sand up to less than 1% in weight. The real content was then calculated from the measured dilution ratio.

3. Results and discussion

During the first field season we identified, plotted and studied two major archaeological remains on the site: slag and ash that cover the hill's natural sediment. We identified changes in the copper concentration in the ash. Increase of Cu and Mn content at some places is obvious (Fig. 3a) in comparison with other locations (Fig. 3b). This could be used later as a marker for identifying the centers of operation as opposed to peripheral distribution of archaeological sediment. Assuming that most of the ash derived from the massive burning of wood and/or charcoal for heating the crushed copper ore during the smelting operation, the changes in the concentration of copper identified qualita-

tively in the ash could guide us to the zones where smelting was conducted leaving behind ash 'stained' with higher levels of copper. This assumption is going to be checked for its validity in future field research of the site by excavating the high copper 'stained' areas and comparing them to randomly excavated locations.

Focusing here on the slag samples, the preliminary field and later laboratory XRF analyses have indicated a high percentage of Mn besides Ca, Fe, Cu, and traces of Ti, V, Pb and Sr (typical spectrum of slag is depicted on Fig. 4a). These results were compared in the lab to spectra of compact and powdered samples of the same slag and ore and were found to be practically identical.

The Cu/Mn ratio varies within these samples from 0.05 to 0.2, and the Cu/Fe ratio ranges from 0.1 to 2. It should be noted that this type of Mn enriched slag (in which the Mn comprises 10–20% of the total weight and the averaged Mn content is equal 13%, and Cu content of up to 1% with the averaged Cu content of 0.8% is well known at the Early Bronze Age and Iron Age copper production complex of Feinan [2]. From our measurements using the dilution procedure on several samples the averaged content of Mn for slag samples from Ein Yahav is 16% (3) and the averaged content of Cu is 0.8% (0.3), where the values of the average deviations are given in parenthesis.

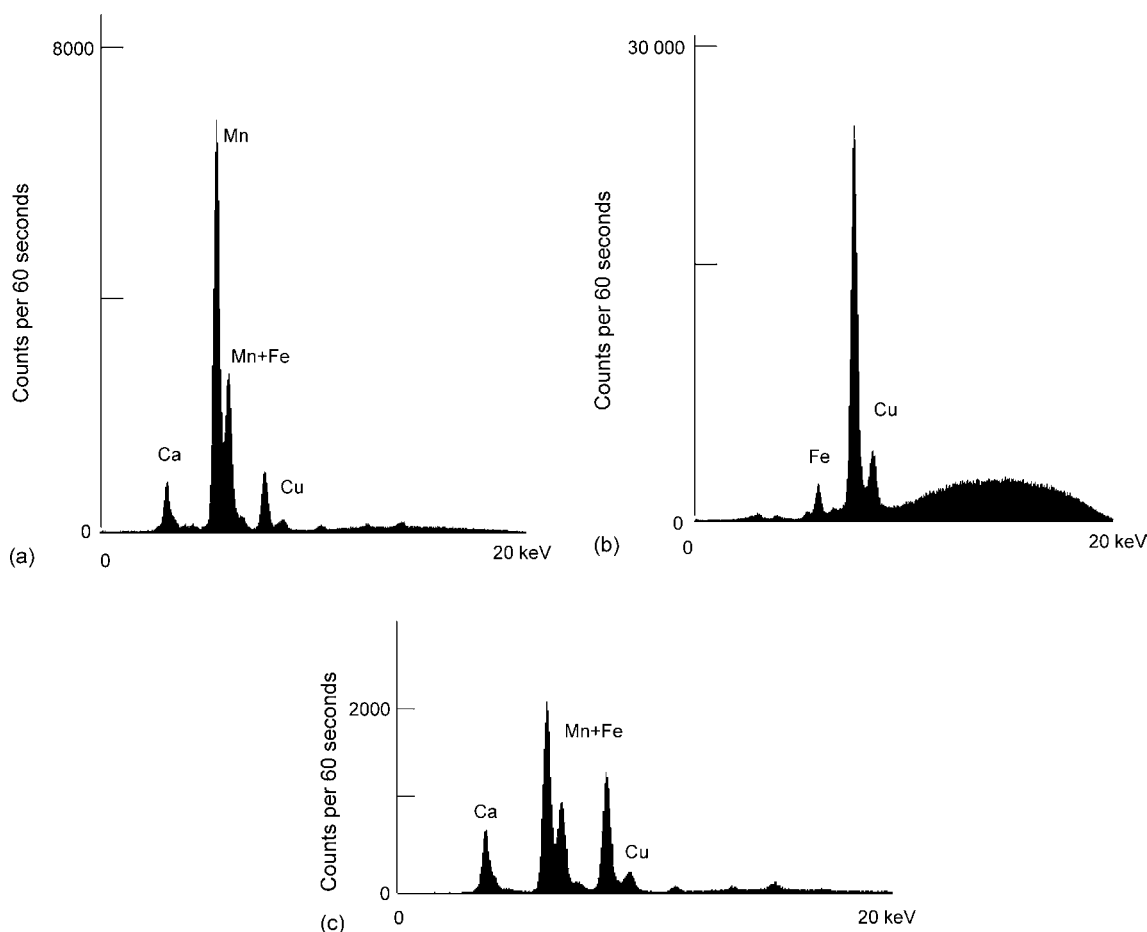


Fig. 4. XRF spectra of: (a) a typical slag containing 13% Mn, 1% Cu and about 2% Fe (determined by dilution procedure); (b) ore: 34% Cu, 6% Fe, 0.8% Mn (determined by dilution procedure) and (c) atypical slag: circa 1–5% Mn and Cu (traces of Pb and Sr).

This data is consistent with the above-mentioned results of the German group in Feinan.

In addition to these slag samples, our tests revealed a group of chemically distinctive samples, which visually however do not appear so uniform: some look like an un-smelted natural ore mineral, while others resemble slag and therefore could be ore that underwent some heat treatment that affected its appearance but has not yet affected its composition. The common characteristics of this group of specimens designate them as rich copper ore containing 30–40% Cu; a Cu line that is much stronger than the Mn impurity line; and an Fe intensity line significantly higher than the Mn one (Fig. 4b). From our measurements these ore samples contain on average 40 (3)% of Cu, 6 (1)% of Fe and 0.9 (0.4)% of Mn. It is worth mentioning here that Hauptmann [2, p. 52] also identified rich copper ores with such impurities as Mn and Fe at Feinan.

A third category of samples is comprised of several specimens that look like a typical slag (smelted and black) but have produced an intermediate spectra (Fig. 4c) between that of the normal slag samples (Fig. 4a) and the ore spectra (Fig. 4b).

In light of these results and on the basis of the Cu/Mn and Cu/Fe intensities' ratios, the samples might be divided into three groups:

1. Typical slag highly enriched by Mn, containing impurities of Cu and Fe, and a significant concentration of Ca (the data of the intensity lines of the main components is shown as crossed circles in Fig. 5).
2. Ore, rich in Cu, containing impurities of Mn and Fe, with no Ca (black circles in Fig. 5).
3. Atypical slag, in which the Cu/Mn and Cu/Fe lines ratios are not far from 1 and the significant Ca fluorescent lines are detected on the spectra (black triangles in Fig. 5).

It is necessary to note here that the most indicative is the value of $I(\text{Cu})/I(\text{Mn})$. This ratio varies from the typical slag to the ore about 1000 times. In such situation the influence of the possible

non-proportionality between intensities and concentrations on the above described classification scheme cannot be significant.

The results of the latter tests enable a reevaluation of the common procedures for estimating the copper production volumes for archaeometallurgical installations. The relevant literature suggests that the amount of slag found at a smelting site might serve as a basis for approximation of the amount of copper produced at that place. It is commonly assumed that a 10:1 ratio exists between the slag mass and the final produced metal quantity (i.e. [2:161]). Accordingly, if S tons of slag were found, the estimated amount of produced copper would equal $0.1S$:

$$C_0 = 0.1S \quad (1)$$

We shall attempt below to estimate the quantities of Cu and Mn before the melting process (in the ore) and after (in the slag), based on the ratio we found in our Ein Yahav slag and ore analysis. This calculation will show that our data questions the validity of the 10:1 ratio, and therefore could lead to a new estimation of the amounts of copper produced on the site.

To best illustrate our claim we shall discuss a situation where 0.1 ton of Cu is produced, generating, according to the common estimate, $S = 1$ ton of slag.

For the simplicity of the calculation, and based on our results, it is assumed that the ore contains 40% of $\text{Cu} = c(\text{Cu})_{\text{ore}}$ and 1% of $\text{Mn} = c(\text{Mn})_{\text{ore}}$, while the slag contains 15% of $\text{Mn} = c(\text{Mn})_{\text{slag}}$ and 1% of $\text{Cu} = c(\text{Cu})_{\text{slag}}$.

Cu amounts will be considered first. Under the above circumstances the amount of Cu in the slag would be $S \times c(\text{Cu})_{\text{slag}} = 1 \times 0.01 = 0.01$ tons. Adding this to the amount of produced Cu (0.1 ton) indicates that the full amount of Cu in the original ore brought into the process was 0.11 tons. Knowing the original Cu content in the ore (40%), the ore mass at the beginning of the melting should have been $0.11/c(\text{Cu})_{\text{ore}} = 0.11/0.4 \approx 0.27$ tons.

At this point Mn contents should be brought into consideration. On the basis of the Mn content in the ore it can be determined that our case had begun with $0.27c(\text{Mn})_{\text{ore}} = 0.27 \times 0.01 \approx 0.003$ tons of Mn. However, an evaluation of the Mn content according to the slag volume provides other results. In the 1 ton of slag the actual amount of Mn is $1c(\text{Mn})_{\text{slag}} = 1 \times 0.15 = 0.15$ tons.

How did the 0.003 tons of Mn in the ore turn into 0.15 tons in the slag? What was the source of such an enormous addition of Mn?

One possible interpretation is that manganese ore was introduced into the smelting concurrently with copper ore. However, if this was indeed the case, then more manganese than copper ore would have been needed for the process, and, of course, such an option raises the question why was not even one piece of manganese ore found during our field sampling, while slag and copper ore are abundant?

We believe that the described anomaly might be explained somewhat differently. The high Mn content in the slag could have been reached, without addition of manganese ore, by a recurring use of the Mn contained slag during repeated smelting processes.

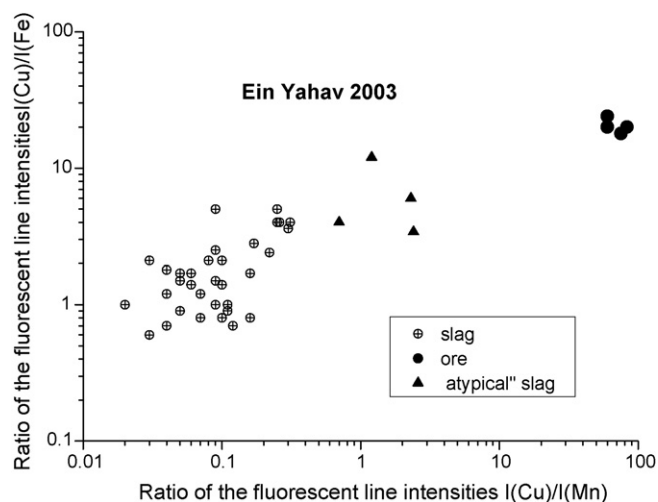


Fig. 5. Ore, typical and atypical slag (according to the intensities' ratios of the major components).

This explanation has far-reaching implications, which will be explained on the basis of the case described above. The 1 ton of slag in our calculation contains 0.01 ton of Cu and 0.15 ton of Mn. If this Mn originated only from the copper ore (through multi sets of smelting whereby copper was extracted), and since Mn comprises 1% of the copper ore, it can be determined that for the production of 0.15 tons of Mn, 15 tons of copper ore should have been processed, assuming that no Mn flux was added separately.

Since 15 tons of copper ore contain 6 tons of copper, and bearing in mind that only 0.01 ton of them were left, it appears that the 1 ton slag sample (of 15% Mn) testifies to the production of 5.99 tons of copper—almost 60 times more than the 0.1 ton “traditional” estimate:

$$C_n \approx 6S \quad (2)$$

This more realistic new estimation of copper production scale C_n is much more than is accepted traditionally. It also seems that at ‘Ein Yahav’ the 10:1 slag/produced-copper ratio is invalid if no manganese was added to the smelt.

Some additional comments are required:

1. This working hypothesis is based on the fact that currently no Mn ore was found on the site. If further investigations in the field will reveal the use of Mn as additional separate flux then our current calculations will have to be altered and calibrated accordingly.
2. If the Mn content in the slag is not exactly 15 times more than its content in the ore, then the “new” estimate of produced copper would change however the difference from the “traditional” estimation will still be very large.
3. The concept of reusing slag for new smelting processes could explain the origin of the so-called “atypical slag”. These could have been slags that were not used enough times to

gain the high Mn content, and therefore they are intermediate not only in the sense of their content, but also in the aspect of their history.

The use of the XRF method in the field during excavations is well known already and is not novel at all [1], but with a modern version of the instruments the preliminary information about the different archaeological artifacts and sediments is available within seconds. It is, therefore, highly useful in many archaeological cases. An additional advantage of this method is the possibility to analyze the archaeological material without any preparation.

4. Conclusion

The chemical content of the slag samples from Ein Yahav is practically the same as in Feinan. Therefore it is very probable that the processes used in both sites were analogous. For the interpretation of the high Mn content in slag we assumed that Mn ore was added to the process and/or slag was used repeatedly for fluxing in Ein Yahav and Feinan.

If Mn ore was not added to the smelt, as currently seems to be the case in Ein Yahav, we can estimate the scale of Cu production in such cases, to be several tens of times more than is traditionally accepted.

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Prostatic Zn determination for prostate cancer diagnosis[☆]

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Abstract

We present our studies of prostatic Zn concentration measurements, carried out in the light of a novel prostate cancer (CAP) diagnosis method proposed by us. The method is based on *in vivo* prostatic Zn mapping by XRF *trans*-rectal probe.

We report on the extensive clinical studies, intended to assess the validity of the novel proposed diagnostic method. Zn content was measured *in vitro* in needle-biopsy samples from several hundreds of patients, and was correlated with histological findings and other patient parameters. For this purpose, a technique of absolute Zn content determination in $\sim 1\text{ mm}^3$ fresh tissue samples by XRF was developed. The experimental details and the main clinical-evaluation results are presented.

We further outline the suggested design of the XRF *trans*-rectal probe for an efficient *in vivo* detection and mapping of the Zn fluorescence radiation from the prostate through the rectal wall. Laboratory phantom studies, a preliminary design concept and its expected performance are also reported.

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Keywords: Prostate; Cancer diagnosis; Zinc; XRF; Biopsy

1. Introduction

The diagnostic process for prostate cancer (CAP) usually starts with positive findings in digital rectal examination and/or in elevated prostate-specific antigen (PSA) serum levels, and ends with *trans*-rectal ultrasound-guided needle biopsy, in which the histological examination is the decisive diagnostic factor. This diagnostic process is costly and not very efficient, due the inadequate specificity and sensitivity of both digital rectal examination and PSA initial tests. In this context it is noteworthy that the needle-biopsy analysis, in addition to side effects, has a non-negligible false-negative rate. It is thus understandable that new

methods for CAP diagnosis are constantly being proposed and their efficacy investigated.

In a previous article [1] we discussed the possibility of exploiting the Zn content in the prostate posterior part (peripheral zone) as a sensitive indicator for CAP development in this region. Many works were published in the past [2,3], reporting on high concentrations of Zn, of about 100–150 $\mu\text{g/g}$ wet weight (ppm) in normal human prostate tissue; this is several times higher than the Zn content in most other human tissues [4]. Furthermore, numerous *in vitro* studies indicated that the average Zn content in the prostate is reduced by about 2–5 times in cancerous tissue as compared to benign one [5–9]. The high Zn content in normal prostate tissue and the reported significant decrease in cancerous one can be measured using the XRF technique, which has adequate sensitivity of a few ppm and has been implemented on humans [10]. If the Zn content decrease is indeed proven to be a sufficiently sensitive and specific indica-

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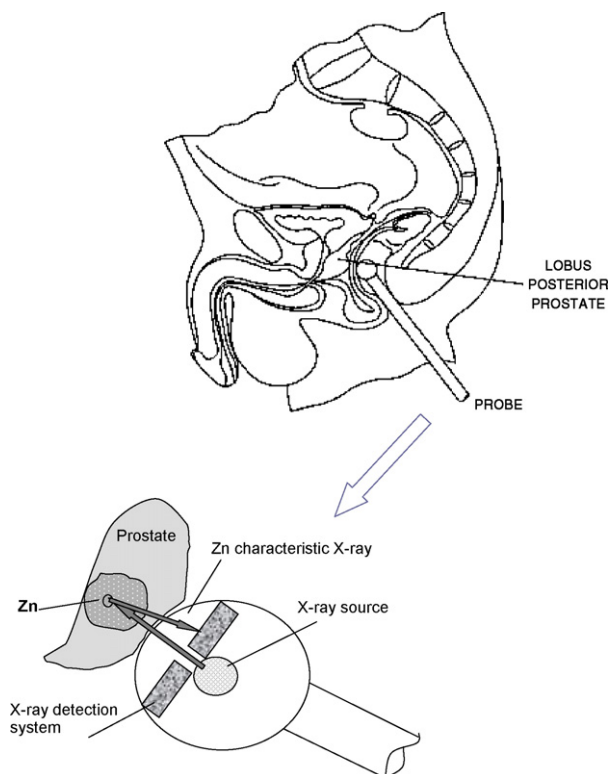


Fig. 1. The proposed XRF *trans*-rectal probe concept for prostate cancer diagnosis.

tor for CAP diagnosis, then the development of a Zn detection method would be perfectly adequate and highly attractive. We have proposed to do this using an XRF *trans*-rectal probe capable of mapping the absolute prostatic Zn content in the peripheral zone, behind the rectal wall (see Fig. 1).

In this article we present two main aspects of the studies we have carried out to confirm this postulate:

First, we describe an extensive clinical study on the relevance of prostatic Zn for CAP diagnosis, performed *in vitro* on needle-biopsy samples taken from several hundreds of patients. This unprecedentedly extensive study is essential for a reliable validation of the relation of Zn content to CAP. This study required the development of techniques for fresh tissue sample handling and absolute determination of the Zn content in very small samples, of $\sim 1 \text{ mm}^3$.

Second, we present our conceptual design of a *trans*-rectal XRF probe for non-invasive *in vivo* mapping of prostatic Zn and, in particular, we report on the laboratory feasibility study of its detection through the rectal wall, with acceptable radiation exposures.

2. Clinical study of prostatic Zn content using needle-biopsy samples

We have launched a systematic clinical study that aims at the evaluation of the diagnostic value of prostatic Zn. This was necessary since existing data fail to provide a clear picture of the spatial and temporal correlation between the malignancy process and the Zn content variation. Moreover, due to the large spread

in the Zn content data in both malignant and non-malignant patient groups [1,5,9], it is clear that a large body of data is required to permit drawing statistically sound conclusions. Our extensive study has been carried out on several hundreds of patients undergoing needle-biopsy examination. This study is the first reported in the literature to encompass such a large number of patients (previously published works usually cite a few tens of cases [1,5–8]) and its statistical validity is thus unprecedented. The approach of using needle-biopsy samples rather than extracted organs allowed access to a large number of patients from all of whom the samples are systematically extracted according to a fixed protocol. It also permitted precise correlation of the Zn content data with histological examination and other patient data. Moreover, since for each patient typically about six or eight samples were measured, and in about half of them the samples were further scanned along the core, we are able to portray a broad picture of the Zn distribution within the prostate.

2.1. The XRF systems for needle-biopsy sample analysis

The measurements of Zn content in prostate needle-biopsy samples were carried out, in parallel, in two medical centers, with the help of two tabletop XRF systems.

The first system, used at the Kaplan Medical center (Rehovot, Israel), was a locally assembled system based on a commercially available air-cooled X-ray generator (XTF5011/75 TH Oxford Instruments, UK), and a 25 mm^2 Peltier-cooled X-ray PIN diode detector (XR-1-CR Amptek). The X-ray generator incorporated a Mo target and the primary X-ray beam was filtered by a $50 \mu\text{m}$ Mo foil. The PIN diode had a 3 mm in diameter entrance aperture made of pure aluminum.

Up to six needle-biopsy samples per patient, each being a 0.5 mm in diameter and 15–20 mm long tissue cylinders folded in two, were placed at 8 mm distance on the sample-table; the latter was made of a $2.5 \mu\text{m}$ Mylar foil stretched on a rectangular plastic frame. Wet sponges and water drops were placed at the table edges; the samples were covered with an identical Mylar foil (see Fig. 2a). This procedure was found to ensure minimal scattering from the table constituents; it also prevented sample drying during the measurement (measured to be $\cong 1\%$). The sample-table was linearly translated manually, introducing one sample at a time into the measurement site. The irradiated area on the sample-table was $4 \text{ mm} \times 9 \text{ mm}$, defined by a rectangular aperture on the primary beam. Signals from the X-ray detector, after proper amplification and time-shaping, were analyzed by a PC-borne multi-channel analyzer; the stored spectra were then analyzed and the intensities of the Zn fluorescence peak as well as the Compton-scattering peak were recorded, which permitted deriving the absolute Zn content as explained below. The measurements, carried out over a fixed time duration of 120 s per sample, yielded the average Zn content per sample with a precision (defined by the number of counts within the peaks) ranging from 30% to 5%, for 30 and 500 ppm, respectively. Due to the manual handling of sample displacement and data acquisition, and the relatively long measurement time per sample (dictated

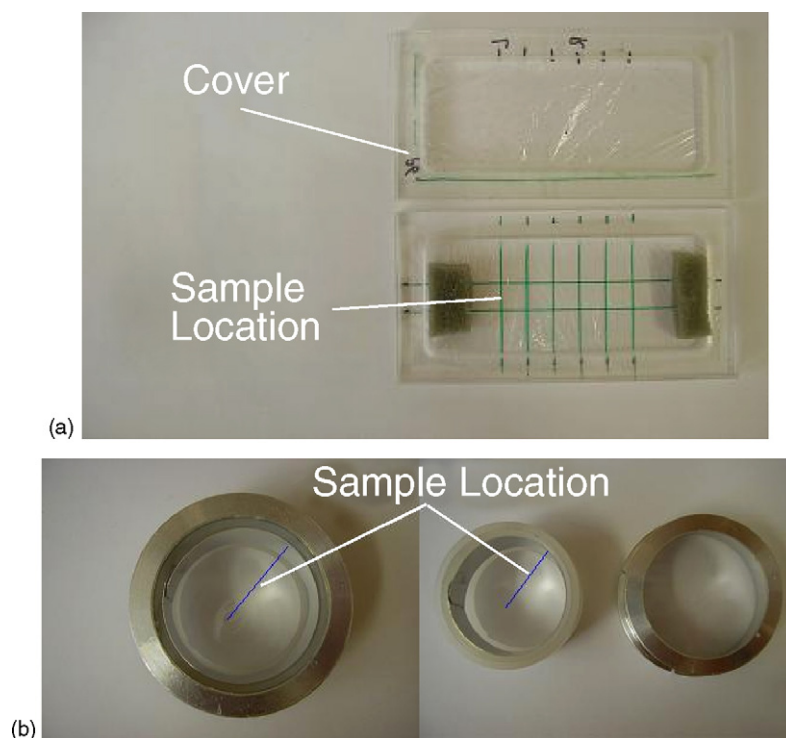


Fig. 2. The needle-biopsy sample support used with the home assembled (a) and the commercial (b) XRF systems. In (a) wet sponges are included next to the six samples to avoid drying; in (b) each sample is secured between the two foils closing the plastic and metal cups (right), when these are tightened together (left).

by the limited X-ray generator flux), each patient measurement took 20–30 min.

The second system, used at the Sheba Medical Center (Tel-Hashomer, Israel), was a commercial unit (EX-2600 bench top XRF analyzer manufactured by Jordan Valley Applied Radiation, Israel), custom-modified for our application. With its optimized assembly geometry, collimators and filters (150 μm Mo filter on the primary X-ray beam from a Mo target), it had about 20-fold higher X-ray flux and superior spectrum quality compared to the XRF instrument described above.

In this system, up to eight needle-biopsy samples, aligned in the tangential direction, were mounted on a rotating table. Each sample was placed on a 2.5 μm Mylar foil fixed on standard plastic cups (inner diameter 24 mm) which, in turn, were tightly fitted into the Al cups placed on the instrument table; the Al cups were sealed by an identical Mylar foil. (To eliminate contribution from traces of Zn in the plastic cups material, a thin Al layer was inserted in these cups, see Fig. 2b.) Thus each sample was individually sandwiched between the two Mylar foils, which prevented them from drying. The sample loading procedure took only a couple of minutes per patient; the table movement, the data acquisition and the analysis were performed automatically. This XRF system was designed to scan the samples and measure the Zn content within four different segments, each 5 mm long, along the sample. For this purpose, the irradiated area was limited to 2 mm $\times$ 5 mm and four different spectra were acquired and analyzed for each sample. The duration of each acquisition point lasted 20 s, with a total of 90 s per sample and typically 12 min per patient. The resulting precision was 15% to 5% for levels of 50 to 200 ppm, respectively.

2.2. Absolute Zn content and calibration standards

The goal of these measurements was to determine the absolute Zn concentration in the needle-biopsy samples. We assumed that the Zn amount is proportional to the intensity of the Zn $K\alpha$ line, $I(\text{Zn})_{\text{sample}}$, which we obtained from the X-ray spectra by integrating the intensity under the peak and subtracting the background. The sample mass is unknown and could not be directly measured; instead, we used the Compton-scattering intensity, $I(\text{Cs})$, obtained from the same X-ray spectrum by integrating the intensity under the Compton Mo $K\alpha$ peak (see Fig. 3). From this quantity we subtracted the contribution $I(\text{Cs})_{\text{empty}}$ from the empty table or empty cup (typically amounting to less than 30% of $I(\text{Cs})$), and calculated $I(\text{Cs})_{\text{sample}} = I(\text{Cs}) - I(\text{Cs})_{\text{empty}}$. The relation between $I(\text{Cs})_{\text{sample}}$ and the actual sample weight was derived from calibration standards made for this purpose. The calibration standard is a sample having a known Zn_{st} (ppm) concentration and designed to have a geometry and composition similar to that of a tissue sample. A measurement of the calibration standard yielded the respective $I(\text{Zn})_{\text{st}}$ and $I(\text{Cs})_{\text{st}}$ intensities (the latter is the value after correction for the empty table or cup), from which the calibration coefficient K was calculated:

$$K = \frac{I(\text{Cs})_{\text{st}} \times \text{Zn}_{\text{st}} (\text{ppm})}{I(\text{Zn})_{\text{st}}} \quad (1)$$

The Zn concentration in the sample is given by

$$\text{Zn}_{\text{sample}} (\text{ppm}) = \frac{K \times I(\text{Zn})_{\text{sample}}}{I(\text{Cs})_{\text{sample}}} \quad (2)$$

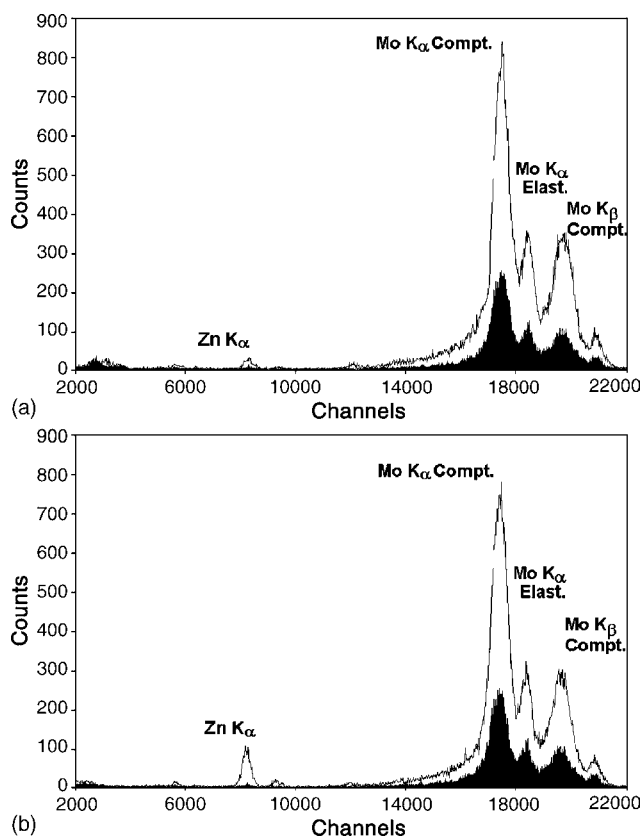


Fig. 3. XRF spectra recorded from an empty cup and biopsy samples at Sheba Medical Center. (a) An empty cup and a sample with 48 ppm Zn; (b) an empty cup and a sample with 233 ppm Zn.

The calibration coefficient K was determined from measurements performed with various standard samples. It is most important for the standard sample to have a similar composition to the tissue, because both the self-absorption effect and the Compton scattering are very strongly dependent on the composition. In our geometry the scattering strongly depends also on sample shape and size; we therefore prepared standards of similar dimensions to those of the tissue samples. We have used samples extracted with a needle-biopsy instrument, from a hard-boiled egg-white substance, previously loaded with a known amount of Zn. Only very short measurements could be performed on the egg samples, which tend to dry rapidly. A more practical standard sample was made of a 0.5 mm inner diameter and ~ 0.25 mm thick wall polyethylene tube, filled with tissue-equivalent solution of known Zn concentration. In this case the $I(Cs)_{\text{empty}}$ was measured with an empty tube, to account for the tube wall contribution to the Compton peak, and the wall attenuation correction was measured independently from the X-ray fluorescence of a thin Cu wire placed inside this plastic tube. Another very convenient standard sample was made of Vaseline loaded with a known Zn concentration, and placed on the sample holder foil with a syringe to obtain the correct size and shape. Sandwiched between two foils, this standard was stable over many months; it is routinely used to check the system calibration stability, together with a thin Cu wire used to verify the energy-calibration stability. The calibration factor K was derived from

the average results of all calibration measurements. Another stable standard was produced from a 0.5 mm thick rectangular Lucite bar, on which a drop of Zn-loaded varnish was deposited; it was used to inter-calibrate both XRF systems, ensuring that absolute Zn concentration values were consistent in both sets of data. Examples of X-ray spectra measured at Sheba Medical Center from biopsy samples and from an empty cup are shown in Fig. 3.

2.3. Measurements in clinic

The above-described tabletop XRF systems were located at the ultrasound-guided biopsy clinics in both medical centers. The patient-extracted samples were immediately placed on their respective supports and covered with a temporary, water-loaded cover, while waiting to be measured. Following the measurement described above, the rectal end of all samples was marked with a black tissue-ink (Black India, No. 4418, Item # 44204 by Stanford, USA) and they were stored in Formaldehyde, in separate tubes; track was kept of the extraction prostate site. The samples were then processed in a routine way at the pathology departments: embedding in paraffin wax, slicing into 4 μm thick slices, and staining with hematoxylin and eosin. The slides were examined by pathologists to provide the diagnosis and the %gland, namely the fraction of surface occupied by the glandular tissue. At Sheba Medical Center this analysis was done in the four sections along the sample, to match the four sections Zn measurement. The diagnosis is adenocarcinoma (CAP), benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), atypical small acinar proliferation (ASAP) or granulomatous inflammation (GRAN) [11–13]. The measured Zn concentration (e.g. per measurement point, averaged per sample or averaged per patient) was correlated with various parameters from the histological examination and from the patient's files. Some of the results are presented below.

2.4. Results

The currently ongoing clinical study in both medical centers has so far resulted in samples examined from 310 patients. As explained above, the data can be analyzed to show various correlations of measured Zn levels with patient's examination parameters.

In the data analysis we applied three categories of Zn content averaging:

- I. *no averaging*, using single measurement points (SMP) at Sheba Medical Center (i.e. one quarter of a full needle-biopsy core);
- II. *per-sample* Zn concentration data (averaging four SMPs at Sheba Medical Center or using per-sample data at Kaplan Medical Center);
- III. *per-patient* Zn concentration data (averaging all samples of a given patient).

A general conclusion from our analysis is that category III data (the *per-patient* data) has limited value for cancer diagno-

Table 1
Zinc, %glands and diagnosis from 31 single measurement points of a single patient

Sample no.	Fourth quarter			Third quarter			Second quarter			First quarter (rectal end)			Average zinc/sample
	Zinc (ppm)	Diagnosis	%gland	Zinc (ppm)	Diagnosis	%gland	Zinc (ppm)	Diagnosis	%gland	Zinc (ppm)	Diagnosis	%gland	
L1	35	CAP	60	45	CAP	40	39	CAP	20	40	CAP	20	40
L2	39	CAP	30	41	CAP	30	43	CAP	20	58	CAP	10	45
L3	16	CAP	10	17	CAP	15	20	CAP	15	25	CAP	5	19
L4	29	CAP	80	21	CAP	80	19	CAP	80	26	CAP	10	24
R5	190	BPH	5	140	BPH	10	145	BPH	15	129	BPH	15	151
R6				302	BPH	10	225	BPH	10	195	BPH	10	241
R7	342	BPH	10	194	BPH	10	215	BPH	15	230	BPH	15	245
R8	343	BPH	10	269	BPH	10	256	BPH	10	248	BPH	10	279

This case presents a perfect match between low Zn and CAP.

sis, with low prospects of yielding better diagnosis than PSA. On the other hand, category I (the single measurement point) Zn concentration data exhibit very good prospects for yielding a diagnostic tool that is much superior to PSA, with diagnostic power approaching that of biopsy (see below). Category II data yielded an intermediate result. This indicates a local Zn-decrease process (at least in the first stage of the malignancy development), the effect of which is diluted when averaged over a large tissue volume. This conclusion is, of course, very important for the design of the *trans*-rectal XRF probe, discussed below.

Table 1 provides the measured Zn concentration and %gland values as well as the histological diagnosis in 32 SMPs from a single patient. In this particular case, an exceptionally clear match is exhibited between low Zn concentration values and the occurrence of cancer. Such clear-match cases are however, rare. In the majority of cases, as is typical for biological data, the fluctuations in Zn concentration are large, and the clinical value of the Zn level may only be evident statistically.

This is well demonstrated in Fig. 4: it is based on SMPs and shows the normalized distribution of CAP and BPH diagnosis rate versus zinc concentration. It is obvious from this figure that the two groups have different distributions, peaking at different

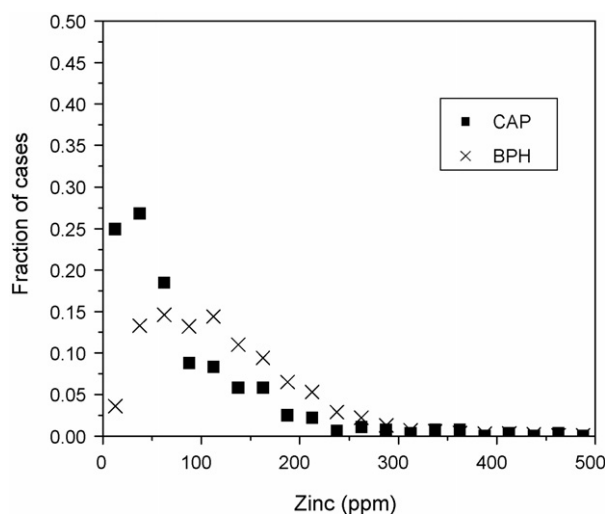


Fig. 4. Normalized distributions of BPH and CAP diagnosis vs. zinc concentration, based on ~5000 single measurement point data. The two distributions peak at different Zn values but they are both broad and largely overlap.

Zn values; however, they are both broad and have significant overlap.

Fig. 5a shows the average zinc content in SMPs diagnosed as cancer or benign, selected by their location along the sample;

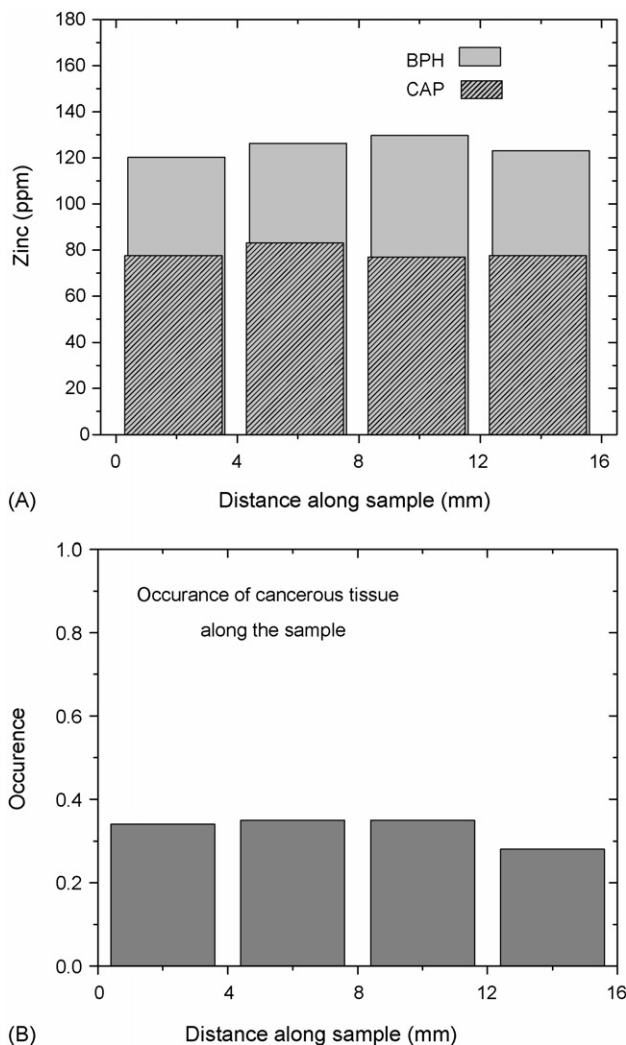


Fig. 5. (a) The average zinc concentration, based on ~5000 single measurement point data, in the four sections along the sample core, for CAP and BPH. The distribution is flat. (b) The occurrence of cancerous tissue samples in the four sections along the sample core.

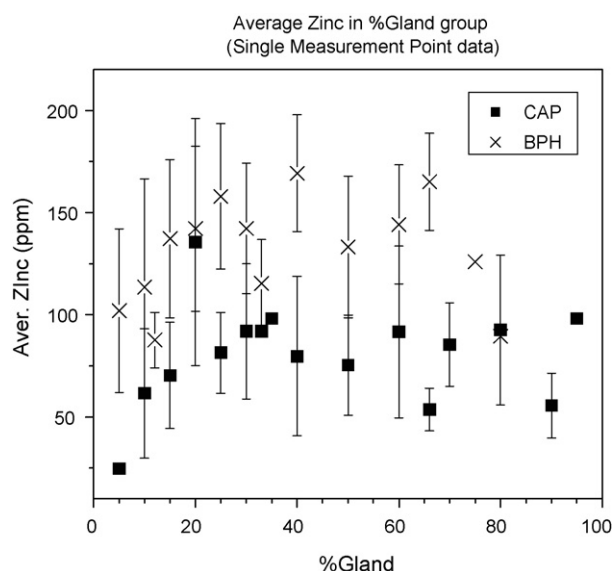


Fig. 6. The average zinc concentration vs. %gland, based on ~5000 single measurement point data of Zn level, %glands and diagnosis. The error bars represent the standard deviation of the data in each data group. In spite of the large spread in the Zn values, it is possible to delineate the two diagnosis groups within clearly distinct limits.

the response is flat, in the sense that there is no dependence on the location along the sample. Fig. 5b is similarly based on SMPs and presents the relative occurrence of CAP-diagnosed tissue sections selected by their location along the sample; there is no dependence on location except for a slight drop at the most distant location.

The %glands obtained from histology carries further information on the malignant process, as it significantly increases due to the excessive multiplication of epithelial cells. The malignant process involves the loss of cells' ability to accumulate zinc and a change in their metabolism. The average zinc concentration versus %gland, based on ~5000 SMPs data, is plotted in Fig. 6. The error bars represent the standard deviation of the data in each data group. In spite of the large spread in the Zn values, it is possible to delineate the two diagnosis groups within distinct limits.

The diagnostic power of a given criterion (i.e. the value of a given parameter or set of parameters) is best judged by the curve depicting the relation between true positive to false positive rate variation with this criterion; the larger the surface under the curve the higher its diagnostic power. In Fig. 7 we show such curves for PSA, Zn and Zn content normalized to %glands, based on SMP data. Judging by the area under the curves, Zn content analysis has diagnostic value superior to PSA, whereas Zn content normalized to %glands is far better than either. The detailed discussion of these results will be given elsewhere.

3. Feasibility study of *in vivo* measurement of prostatic Zn content

3.1. Laboratory phantom studies

The general scheme of the proposed apparatus for CAP diagnosis by detection of Zn content loss is shown on Fig. 1: A

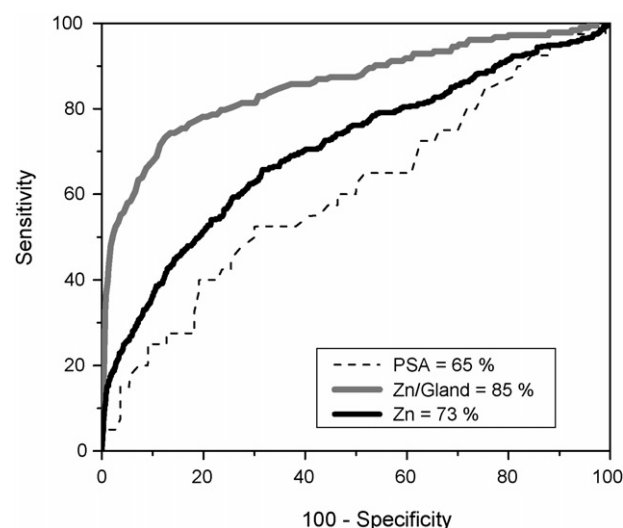


Fig. 7. Curves of sensitivity vs. specificity for PSA, Zn (SMP) and Zn normalized to %glands (single measurement points). An ideal diagnostic tool will have 100% sensitivity and 100% specificity, namely a step function with area 100%.

trans-rectal probe containing an X-ray source, or X-ray guiding system connected to an X-ray generator, irradiates the prostate through the rectal wall. An array of X-ray detectors generates the spectrum of the scattered and fluorescence radiation emitted from the tissue. The most severe impediments to designing such a probe are the low penetrability of the characteristic ~8.64 keV Zn X-rays, and the necessity of obtaining precise quantitative data under conditions of appreciable scattering from the surrounding tissue. Moreover, it is absolutely mandatory to eliminate any contribution (Zn fluorescence and scattering) from the rectal wall tissue, situated between the probe and the examined tissue. The wall is typically 2–3 mm thick, and unless special precaution is taken, the scattered and fluorescence radiation from this tissue will dominate the spectrum.

To study this problem we have used a laboratory phantom, made of two compartments, a thin one representing the rectal wall and a thick one representing the prostate, filled with tissue-equivalent solutions loaded with various concentrations of Zn. We have demonstrated [14] that the above-discussed impediments may be surmounted by using a collimation scheme that strictly limits the solid angle of both *primary* and *fluorescence + scattered* X-ray beams with an appropriate collimator. We have shown that the Zn concentration can be reliably measured within the focal volume defined by the intersection of the two collimated beams, and when this focal volume is placed in the “prostate compartment” of the phantom, it provides data free of contributions from the “rectal wall compartment”. This method should permit eliminating the spectral contributions from the rectal wall tissue, reliably measuring the Zn concentration in the prostate tissue at depths up to several millimeters.

The rectal wall will nevertheless cause considerable attenuation of the primary and fluorescence beams, and give rise to some scattering into the detector. Consequently, the main factor limiting the radiation intensity is the dose absorbed in the

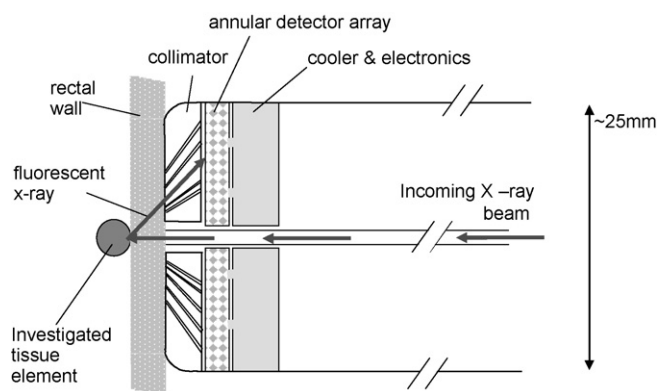


Fig. 8. A conceptual scheme of the probe: an array of confocal thin collimators, drilled in an 8 mm metal plate, is placed between the tissue and the annular, cooled detector. It protects the detector from scattered radiation and defines the examined tissue element.

rectal wall. Using the laboratory phantom data, we have calculated the integrated fluence of incident radiation required to obtain evaluation of the Zn content in the prostate. Assuming an annular X-ray detector (see Figs. 1 and 8) of area $\sim 200 \text{ mm}^2$ and including a 3 mm thick rectal wall attenuation effect, we have determined the fluence required to measure Zn levels of 200–50 ppm to a precision of 10%. This resulted in an incident fluence of 4×10^9 to 2×10^{10} photons/cm², for the 200 and 50 ppm, respectively. These fluences are equivalent to radiation exposures of 0.8–4 R. These exposures are of the same order of magnitude as the typical skin entrance exposure values in mammography, which range between 0.5 and 5 R for thin and thick breasts, respectively [15]. Calculations of the absorbed dose using Monte Carlo simulations are in progress at present.

A large detection solid angle is beneficial for improving the data statistics and reducing the dose.

3.2. A probe scheme

The main components in the proposed probe are the X-ray source, of a narrow energy spectrum centered at $\sim 17.4 \text{ keV}$ energy, an annular-shape $\sim 20 \text{ mm}$ diameter array of spectroscopic X-ray detectors, possibly cooled, and a multi-collimator system, arranged to have the focal volume within the examined tissue. The latter is realized with a few mm thick metallic steel or aluminum plate, perforated with confocal holes of small diameter (0.1–0.2 mm), pointing at one focal volume within the prostate; the holes may be cylindrical or of a slightly conical profile (Fig. 8). The primary beam, focused into the gland, passes through a central collimating aperture in the multi-collimator plate, and through a corresponding opening in the annular-shaped X-ray detector, placed next to the collimator plate. The intersection of the primary beam with the focal volume of the multi-collimator plate defines the target volume within the prostate; the fluorescence radiation from this volume will be detected by the annular detector that subtends a large solid angle. In this design the detector will be well shielded from scattered radiation.

4. Summary

We have presented the idea of a novel prostate cancer diagnosis tool, based on *in vivo* mapping of the Zn concentration in the posterior prostate tissue, where most malignant tumors develop. The Zn examination is non-invasive and will be performed on patients prior to the biopsy examination, to classify them better prior to this invasive and costly test.

To this end we launched a clinical study aiming at evaluating the diagnostic value of the prostatic Zn content measurement. The study, currently ongoing in two medical centers, is based on Zn concentration measurements in needle-biopsy samples from several hundreds of patients; the data analysis is performed in correlation with the patient's histological diagnosis and with other patient parameters. Two tabletop XRF systems were developed and adapted for this study and special care was taken to develop tools and protocols for system calibration and for handling of the XRF measurement on such fragile, small-volume samples. These new XRF measurement protocols, performed in between the biopsy procedure and the histological analysis, were shown not to interfere with standard clinical procedures and not damage the biopsy samples. The unprecedentedly large body of data on prostatic Zn content, accumulated within the framework of this clinical study, is important for evaluating the sensitivity and specificity of the newly proposed prostate cancer diagnosis concept.

We have presented some data showing the clear difference in Zn levels in BPH (benign) and CAP (cancer) tissue, and the problem of overlap in the distributions from these two patient groups. The sensitivity vs. specificity curves based on single measurement points (SMP) data accumulated so far summarize the diagnostic power and show that Zn concentration is indeed a more indicative parameter than PSA: for example, for detecting 80% of cancer points the specificity of PSA is $\sim 25\%$ and the specificity of Zn is 45% (almost a factor of 2!). It indicates that as a pre-biopsy criterion Zn could be much superior to PSA and using it may reduce the rate of non-malignant cases that are currently referred to biopsy. (This rate today is typically 75–78% worldwide.) Such a procedure will render the costly biopsy test more efficient and cost effective, and will permit extending the biopsy test to a broader population, e.g. younger age-group, lower PSA, etc. Moreover, the proposed CAP diagnostic method involves mapping of the Zn concentration, namely providing detailed information on the spatial distribution of Zn; this will provide further possibilities for distinguishing between random local fluctuation in Zn content (noise) or a systematic Zn reduction over an extended region, supposedly related to cancer lesion. The aspect of spatial correlations between Zn and CAP is the subject of another study, to be reported elsewhere.

In view of the promising preliminary results from the clinical study, and the conclusion that Zn should be measured locally and not averaged over a large tissue volume, we have developed the concept of a *trans*-rectal XRF probe, which measures and maps the prostatic Zn content through the rectal wall. A conceptual probe design was outlined, and the results of some laboratory tests with phantoms were reported. It is claimed that, with an appropriate collimation of the primary and flu-

orescence + scattered beams, it should be possible to reliably measure the Zn content in a small volume of the prostate tissue, *in vivo*, without the interference of the rectal wall tissue. The proposed probe is estimated to yield the prostatic Zn content within 10–20 s per measuring point, and within a permissible absorbed dose in the rectal wall, of 0.13–0.5 mGy.

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Biosorption of heavy metals on *Aspergillus fumigatus* immobilized Diaion HP-2MG resin for their atomic absorption spectrometric determinations

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Abstract

A solid phase extraction procedure based on biosorption of copper(II), lead(II), zinc(II), iron(III), nickel(II) and cobalt(II) ions on *Aspergillus fumigatus* immobilized Diaion HP-2MG has been investigated. The analytical conditions including amounts of *A. fumigatus*, eluent type, flow rates of sample and eluent solutions were examined. Good recoveries were obtained to the spiked natural waters. The influences of the concomitant ions on the retentions of the analytes were also examined. The detection limits (3sigma, $N=11$) were $0.30 \mu\text{g l}^{-1}$ for copper, $0.32 \mu\text{g l}^{-1}$ for iron, $0.41 \mu\text{g l}^{-1}$ for zinc, $0.52 \mu\text{g l}^{-1}$ for lead, $0.59 \mu\text{g l}^{-1}$ for nickel and $0.72 \mu\text{g l}^{-1}$ for cobalt. The relative standard deviations of the procedure were below 7%. The validation of the presented procedure is performed by the analysis of three standard reference materials (NRCC-SLRS 4 Riverine Water, SRM 1515 Apple leaves and GBW 07605 Tea). The procedure was successfully applied for the determination of analyte ions in natural waters microwave digested samples including street dust, tomato paste, black tea, etc.

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Keywords: *Aspergillus fumigatus*; Diaion HP-2MG; Biosorption; Preconcentration; Trace metal; Atomic absorption spectrometry

1. Introduction

Due to heavy metal pollution in the environment including soils, foods and waters is a problem for human being [1–4], accurate determination of the heavy metal ions in the environmental samples by instrumental techniques like graphite or flame atomic absorption spectroscopy and inductively coupled plasma optical emission spectroscopy is an important place in the field of analytical chemistry. Lower levels of the heavy metals than the quantitation limits of the instrumental techniques and higher levels of the concomitant ions in the real samples are two big and important problems for metal determinations in environmental samples by using these techniques [5–9]. In order to solve these problems, separation and preconcentration techniques including solvent extraction, membrane filtration, cloud point extraction, electrodeposition, ion-exchange, solvent sub-

lation and solid phase extraction are used for traces metal ions [10–17].

Heavy metal ions could be adsorbed on the higher organisms including mosses, bacteria and algae [18–21]. The uptake of metals by biomass can take place actively, by means of a metabolic activity-dependent process bioaccumulation or by means of a passive and usually rapid (several minutes) metabolism-independent process called biosorption [19]. Biosorption is exclusively responsible for metal concentration by non-living biomass owing to the absence of metabolic activity necessary for intracellular metal accumulation [19]. This point is used by the researchers on the preconcentration–separation of the heavy metals at trace level in the environment. The system is based on biosorption of the heavy metals and desorption of these metals from the organisms. Biosorption of trace metals by microorganisms can be realized in batch and continuous modes [19]. An important part of the studies on biosorption is based on the immobilization of the organisms on the natural or synthetic polymeric materials [19–24]. Microorganisms immobilized natural and synthetic adsorbents have been used for heavy metal separa-

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Table 1

Comparative data from some recent studies on biosorption of heavy metals on microorganism immobilized on adsorbents

Elements	Media	Detection system	PF	DL ($\mu\text{g l}^{-1}$)	R.S.D. (%)	Reference
Cr(III), Cr(VI)	<i>Saccharomyces cerevisiae</i> immobilized on sepiolite	FAAS	75	94	<5	[21]
Fe ³⁺ , Co ²⁺ , Mn ²⁺ , Cr ³⁺	<i>Agrobacterium tumefaciens</i> immobilized on Amberlite XAD-4	FAAS	25	2.8–3.6	<10	[22]
Fe ³⁺ , Co ²⁺ , Cr ³⁺	<i>Saccharomyces carlsbergensis</i> immobilized on Amberlite XAD-4	FAAS	–	2.8–7.4	<5	[23]
Mn ²⁺	<i>Saccharomyces carlsbergensis</i> immobilized on Amberlite XAD-4	FAAS	–	60	<5	[24]
Cr(III), Cr(VI)	<i>Funaria hygrometrica</i> immobilized in a polysilicate matrix	ICP-MS, FAAS	20	0.15 for ICP-MS and 145 for FAAS	<10	[25]
Fe ³⁺ , Co ²⁺ , Mn ²⁺ , Cr ³⁺	<i>Escherichia coli</i> immobilized on Amberlite XAD-4	FAAS	25	1.3–3.8	<5	[26]
Cu ²⁺ , Zn ²⁺ , Fe ³⁺ , Ni ²⁺ , Cd ²⁺	<i>Escherichia coli</i> immobilized on sepiolite	FAAS	50	67–95	<10	[27]
Cu ²⁺ , Zn ²⁺ , Fe ³⁺ , Ni ²⁺ , Cd ²⁺	<i>Aspergillus niger</i> immobilized on sepiolite	FAAS	50	–	<5	[28]
Cu ²⁺ , Zn ²⁺ , Cd ²⁺	<i>Saccharomyces cerevisiae</i> immobilized on sepiolite	FAAS	–	–	–	[29]
Fe ³⁺ , Ni ²⁺	<i>Saccharomyces cerevisiae</i> immobilized on sepiolite	FAAS	50	6.5–8.7	<8	[30]
Pd ²⁺	<i>Saccharomyces cerevisiae</i> immobilized in calcium alginate beads	GFAAS	–	7 ng g ⁻¹	–	[31]
Cr(III), Cr(VI)	<i>Saccharomyces cerevisiae</i> immobilized on controlled pore glass	ICP-OES	12	0.45–1.5	–	[32]
Pt ²⁺ , Pd ²⁺	<i>Saccharomyces cerevisiae</i> and <i>Chlorella vulgaris</i> immobilized on silica gel	GFAAS	–	0.4–0.8	<5	[33]
Cu ²⁺ , Pb ²⁺ , Zn ²⁺ , Fe ³⁺ , Ni ²⁺ , Co ²⁺	<i>Aspergillus fumigatus</i> immobilized on Diaion HP-2MG	FAAS	50	0.30–0.72	<7	Present work

tion and preconcentration from various media with successfully analytical results. Some comparative data about biosorption is summarized in Table 1. *Aspergillus fumigatus* is a microorganism. It causes more infections worldwide than any other mould. Four percent of all patients dying in tertiary care hospitals have invasive aspergillosis. The fungus causes allergic diseases in asthmatics and patients suffering from cystic fibrosis. Invasive aspergillosis can occur in individuals with cavities caused by tuberculosis or other cystic lung diseases [34]. According to our literature survey, *A. fumigatus* is not used on the biosorption of traces heavy metal ions prior to atomic absorption spectrometric determination of them.

In this work, a separation–preconcentration procedure was presented for the enrichment of copper(II), lead(II), zinc(II), iron(III), nickel(II) and cobalt(II) ions on *A. fumigatus* immobilized Diaion HP-2MG. The analytical conditions for the quantitative recoveries of the analytes including pH of solutions, sample volume, etc., were investigated.

2. Experimental

2.1. Instrument

A Perkin-Elmer AAnalyst 700 atomic absorption spectrometer with deuterium background corrector was used. All measurements were carried out in an air/acetylene flame. A 10 cm long slot-burner head, a lamp and an air–acetylene flame were

used. The operating parameters for working elements were set as recommended by the manufacturer.

A pH meter, Sartorius pp-15 Model glass-electrode was employed for measuring pH values in the aqueous phase. Milestone Ethos D closed vessel microwave system (maximum pressure 1450 psi; maximum temperature 300 °C) was used. Digestion conditions for microwave system for SRM 1515 Apple leaves, GBW 07605 Tea, street dust and black tea samples were applied as 6 min for 250 W, 6 min for 400 W, 6 min for 550 W, 6 min for 250 W and vent: 8 min. For fish and tomato paste, digestion conditions for microwave system were applied as 2 min for 250 W, 2 min for 0 W, 6 min for 250 W, 5 min for 400 W, 8 min for 550 W and vent: 8 min [35].

2.2. Reagents and solution

All chemicals used in this work, were of analytical reagent grade and were used without further purification. Deionized water (Milli-Q Millipore 18.2 M Ω cm⁻¹ resistivity) was used for all dilutions. All the plastic and glassware were cleaned by soaking in dilute HNO₃ (1 + 9) and were rinsed with distilled water prior to use. The element standard solutions used for calibration were produced by diluting a stock solution of 1000 mg l⁻¹ of the given element supplied by Sigma and Aldrich. Stock solutions of diverse elements were prepared from high purity compounds. The calibration standards were not submitted to the preconcentration procedure.

Sodium phosphate buffer (0.1 M) was prepared by adding an appropriate amount of phosphoric acid to sodium dihydrogen phosphate solution to result in a solution of pH 2. Ammonium acetate buffers (0.1 M) were prepared by adding an appropriate amount of acetic acid to ammonium acetate solutions to result in solutions of pH 4–6. For pH 7, sodium borate (0.1 M) buffer solution was used. Ammonium chloride buffer solutions (0.1 M) were prepared by adding an appropriate amount of ammonia to ammonium chloride solutions to result in solutions of pH 8–10.

Standard reference materials (NRCC-SLRS 4 Riverine Water, SRM 1515 Apple leaves and GBW 07605 Tea) were used in the experiment.

Diaion HP-2MG is methacrylic ester copolymer, and has relatively hydrophilic nature. Its surface area, mesh size and pore size are $500 \text{ m}^2 \text{ g}^{-1}$, 25–50 mesh and $170 \text{ \AA}$, respectively. Diaion HP-2MG is purchased from Supelco (no. 1-3601). In order to remove organic and inorganic contaminants, the resin was washed successively with methanol, distilled water, 1 M HNO_3 in acetone, distilled water, 1 M NaOH and distilled water.

2.3. Preparation of *A. fumigatus* immobilized Diaion HP-2MG

A. fumigatus immobilized Diaion HP-2MG resin was prepared the procedure given by Baytak and Turker [22]. The immobilization of *A. fumigatus* on the substrate was performed as follows: 150 mg of dry and dead bacteria powder was mixed with 1 g of Diaion HP-2MG. The mixture was wetted with 2 ml of doubly distilled water and thoroughly mixed. After mixing, the paste was heated in an oven at about 105°C for 1 h to dry the mixture. The wetting and drying step were repeated to maximize the contact between *A. fumigatus* and Diaion HP-2MG, thereby improving the immobilization efficiency. Then, the product obtained was ground to get original size (20–40 mesh) and used as an adsorbent.

2.4. Test procedure

The biosorption procedure presented was tested with model solutions. Forty to 50 ml of solution containing $2.5\text{--}20 \mu\text{g}$ of the metal ions was added 10 ml of buffer solution (desired pH between 2 and 10). Then *A. fumigatus* immobilized Diaion HP-2MG column was preconditioned by passing buffer solution. The buffered metal solution was passed the column at a flow rate of 5 ml min^{-1} . The sample solution was permitted to flow through the column under gravity. After passing of this solution completely, the column was rinsed with twice 10 ml of water. The sorbed metal ions on the column were eluted with 8–10 ml portion of 1 M HCl. The residue is diluted to 10.0 ml with 1.0 M HCl. The eluent was analysed for the determinations of metal concentrations by flame atomic absorption spectrometer.

2.5. Analysis of the real samples

Tea (GBW 07605) and apple (SRM 1515) leaves standard reference materials (250 mg) and black tea sample (1.0 g) from

East Blacksea region of Turkey were digested with 6 ml of HNO_3 (65%), 2 ml of H_2O_2 (30%) in microwave digestion system and diluted to 50 ml with deionized water [35–37]. A blank digest was carried out in the same way. Then the preconcentration procedure given above was applied to the final solutions.

For the microwave digestion of canned fish from Tokat City, tomato paste from Tokat City, street dust from Tokat City, 1.0 g of sample from Tokat was digested with 6 ml of concentrated HNO_3 and 2 ml of concentrated H_2O_2 in microwave system. After digestion the samples, the volume of the digested sample was made up to 25.0 ml with distilled water [35–37]. Blanks were prepared in the same way as the sample, but omitting the sample. The preconcentration procedure given above was applied to the samples.

NRCC-SLRS 4 Riverine Water, tap from Tokat City, river water from Greenriver-Tokat, seawater from Black Sea and spring water from Sivas City analyzed were filtered through a cellulose membrane filter (Millipore) of $0.45 \mu\text{m}$ pore size. The pH of the samples was adjusted to 8.0 with buffer solution. The sample was passed through the column. The metal adsorbed on *A. fumigatus* immobilized Diaion HP-2MG column were eluted with 1 mol l^{-1} HCl. The levels of analyte ions in the samples were determined by flame atomic absorption spectrometry.

3. Results and discussion

3.1. Influences of pH

On the biosorption of the metal ions on microorganisms, pH is the one of the important factor for the retentions of traces metal ions. Because of this point, the influences of pH of the aqueous solution on the retentions of the analyte ions on *A. fumigatus* immobilized Diaion HP-2MG resin were investigated by using model solutions containing analyte ions. The recovery values were given in Fig. 1. Copper(II), lead(II), zinc(II), iron(III), nickel(II) and cobalt(II) ions were quantitatively ($\geq 95\%$) recovered at the pH range of 8–9 for copper, nickel, zinc, cobalt and 8–10 for lead and iron. The all-further works were performed at pH 8.0 by using ammonia/ammonium buffer solution.

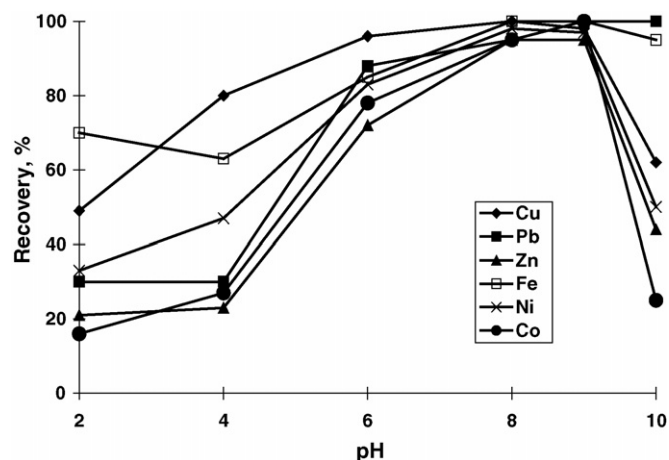


Fig. 1. The effects of pH on the recoveries of metal ions on *Aspergillus fumigatus* immobilized Diaion HP-2MG.

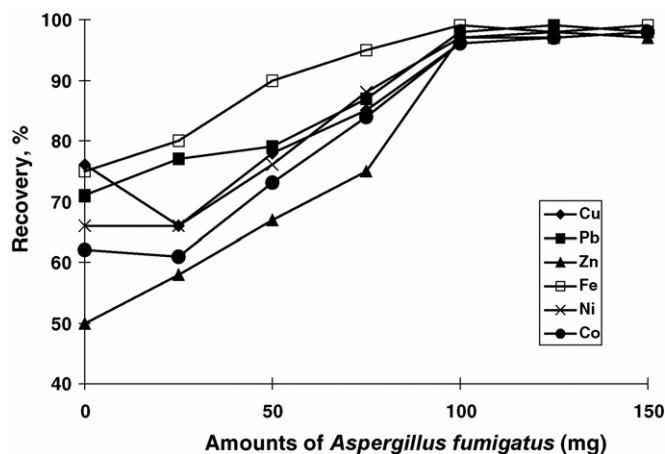


Fig. 2. The influences of amounts of *Aspergillus fumigatus* immobilized on Diaion HP-2MG.

The recovery values for all analyte ions on the column filled with Diaion HP-2MG without *A. fumigatus* at pH range of 8–10 were below 80%. The recoveries for analytes on the column filled 150 mg of *A. fumigatus* without Diaion HP-2MG at pH range of 8–10 were below 60%. These points show that for the quantitative recoveries of analyte ions, it is necessary that the combination of *A. fumigatus* and Diaion HP-2MG. These points are agreed with the literature values [20–22].

3.2. Effect of amounts of *A. fumigatus* on the recoveries of analytes

In order to observe the influences of the amounts of *A. fumigatus* on the retentions of analyte ions, the procedure given in Section 2.4 was performed with different amounts of *A. fumigatus* and then the preconcentration procedure given in Section 2.5 was applied. The results were depicted in Fig. 2. The recoveries of analytes were increased with the increased amounts of *A. fumigatus* that immobilized on Diaion HP-2MG resin. Quantitative recovery values for analytes were obtained after 125 mg of *A. fumigatus*. In all subsequent works, 150 mg of *A. fumigatus* was immobilized on 0.5 g of Diaion HP-2MG.

3.3. Eluent type and its volume

The elution of biosorbed metal ions from the *A. fumigatus* immobilized Diaion HP-2MG was also studied by using HCl and HNO₃ at various concentrations. Quantitative recoveries were obtained by using 1 M HCl for copper(II), lead(II), zinc(II), iron(III), nickel(II) and cobalt(II). By using 0.5 and 1 M HNO₃, except lead and nickel, other analyte ions were quantitatively recovered.

Effect of volume of 1 M HCl as eluent was also examined on the recoveries of analytes. All the analytes were quantitatively recovered from *A. fumigatus* immobilized Diaion HP-2MG at 8–10 ml of 1 M HCl.

3.4. Flow rates of sample and eluent solutions

The influences of the sample and eluent flow rates on the retentions and recoveries of analyte ions on *A. fumigatus* im-

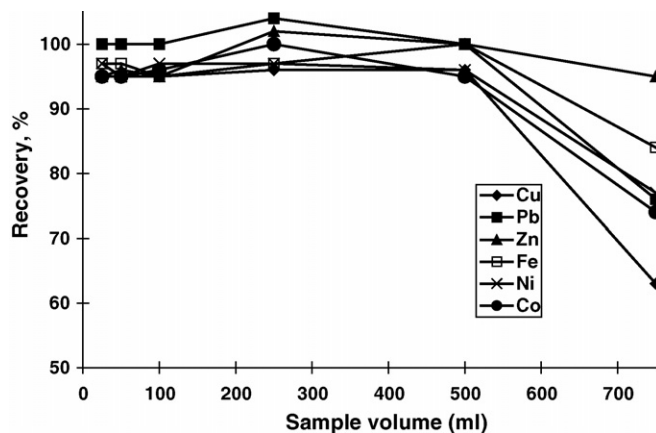


Fig. 3. Effects of sample volume on the retentions of analytes on *Aspergillus fumigatus* immobilized Diaion HP-2MG ($N=3$).

bilized Diaion HP-2MG were also examined in the flow rate range of 2–10 ml min⁻¹. All the analyte ions were quantitatively retained and recovered in the sample and eluent flow range of 2–6 ml min⁻¹. In the all-further works, 5 ml min⁻¹ was selected as sample and eluent flow rate.

3.5. Effect of sample volume

To obtain high preconcentration factor, sample volume is one of the important analytical factor for solid phase extraction studies for metal ions. The influences of sample volume on the recoveries of analyte ions on *A. fumigatus* immobilized Diaion HP-2MG were investigated in the range of 25–750 ml range. The results are depicted in Fig. 3. Analyte ions were quantitatively ($\geq 95\%$) recovered till 500 ml. After 500 ml of sample volume, recovery values not quantitative. The preconcentration factor was 50 when eluent volume is 10 ml.

3.6. Effect of divers ions

As pointed in Section 1, one of the main problem in the atomic absorption spectrometric determination of the heavy metal ions is interference from the matrix. In the presented work, the influences of the some ions which are known as interferic ions in the AAS determination were investigated on *A. fumigatus* immobilized Diaion HP-2MG. The interference concentration choice was based on literature values found for separation and preconcentration studies [38–42]. The results for this study are given in Table 2. The tolerance limit of foreign ions was taken as that value which caused an error of not more than $\pm 5\%$ in the absorbance. The ions normally present in water do not interfere under the experimental conditions used. Also, some of the transition metals at mg l⁻¹ levels were not interfered on the recoveries of the analyte ions. This results show that the proposed preconcentration/separation method could be applied to the highly saline samples and the samples that contains some transition metals at the tolerable levels given in Table 2. The samples analyzed in the presented work contain alkaline and earth alkaline ions at mg l⁻¹ and transition metals at $\mu\text{g l}^{-1}$ levels [43–45].

Table 2

Tolerable limits of some divers ions on the retentions of metal ions on *Aspergillus fumigatus* immobilized Diaion HP-2MG (sample volume: 50 ml, pH 8, $N=4$)

Ion	Added as	Concentration (mg l ⁻¹)	Recovery (%)					
			Cu	Pb	Zn	Fe	Ni	Co
Na ⁺	NaCl	20000	98 ± 3	97 ± 2	96 ± 3	100 ± 1	95 ± 2	95 ± 2
K ⁺	KCl	5000	95 ± 2	97 ± 3	95 ± 3	98 ± 2	95 ± 3	95 ± 2
Ca ²⁺	CaCl ₂	2000	95 ± 4	96 ± 2	99 ± 2	97 ± 3	95 ± 2	96 ± 2
Mg ²⁺	MgCl ₂	2000	95 ± 3	95 ± 3	97 ± 1	99 ± 2	95 ± 3	95 ± 3
Cl ⁻	NaCl	25000	96 ± 2	96 ± 2	95 ± 2	100 ± 1	95 ± 3	96 ± 2
F ⁻	NaF	2000	97 ± 3	96 ± 2	99 ± 2	96 ± 3	95 ± 2	96 ± 2
NO ₃ ⁻	KNO ₃	2000	96 ± 2	95 ± 2	99 ± 3	98 ± 2	95 ± 3	95 ± 2
SO ₄ ²⁻	Na ₂ SO ₄	2000	97 ± 3	95 ± 3	95 ± 2	97 ± 3	95 ± 1	95 ± 3
PO ₄ ³⁻	Na ₃ PO ₄	1000	100 ± 1	95 ± 2	98 ± 3	99 ± 2	97 ± 2	95 ± 2
Al ³⁺	Al ₂ (SO ₄) ₃	10	96 ± 2	95 ± 3	96 ± 3	95 ± 3	97 ± 2	96 ± 3
Ag ⁺	AgNO ₃	25	95 ± 3	96 ± 2	100 ± 3	98 ± 3	97 ± 4	95 ± 3
Mn ²⁺	MnSO ₄	25	97 ± 2	96 ± 4	97 ± 4	96 ± 3	95 ± 2	96 ± 1
Cd ²⁺	CdSO ₄	25	95 ± 2	95 ± 3	96 ± 4	97 ± 3	95 ± 2	96 ± 3
Cr ³⁺	Cr(NO ₃) ₃	10	98 ± 3	95 ± 3	96 ± 3	97 ± 2	97 ± 2	96 ± 2

3.7. Adsorption capacity

In order to study the adsorptive capacity of *A. fumigatus* immobilized Diaion HP-2MG, batch method was used. To 0.1 g resin was added 50 ml of solution containing 1.0 mg of metal ion at pH 8.0. After shaking for 1 h, the mixture was filtered. Ten millilitres of the supernatant solution was diluted to 100 ml and determined by flame atomic absorption spectrometry. This procedure was repeated for each analyte ions separately. The capacity of sorbent for analytes were found as—Cu: 5.7 mg g⁻¹, Pb: 7.1 mg g⁻¹, Zn: 8.5 mg g⁻¹, Fe: 4.9 mg g⁻¹, Ni: 6.8 mg g⁻¹ and Co: 4.4 mg g⁻¹.

3.8. Analytical performance

The relative standard deviations for flame atomic absorption spectrometric determinations for analytes are between 1.0 and 9.0%. The detection limits, defined as the concentration equivalent to three times the standard deviation ($N=11$) of the reagent blank were found as—Cu: 0.30 µg l⁻¹, Pb: 0.52 µg l⁻¹, Zn: 0.41 µg l⁻¹, Fe: 0.32 µg l⁻¹, Ni: 0.59 µg l⁻¹ and Co: 0.72 µg l⁻¹.

To estimate the accuracy of the procedure, different amounts of the investigated metal ions were spiked in artificial seawater prepared according to literature [46] and a hot spring water sample. The resulting solutions were submitted to the presented procedure given in Section 2. The results were given in Table 3. Good agreement was obtained between the added and found analyte content. The recovery values for the analyte ions were generally in the range of 95–103%. It shows that the presented solid phase extraction method can be applied for the separation/preconcentration of analyte ions in the real samples which have high salt content.

3.9. Application

The validation of the presented procedure is performed by the analysis of three standard reference materials (NRCC-SLRS

4 Riverine Water, SRM 1515 Apple leaves, GBW 07605 Tea). The certified and observed values for NRCC-SLRS 4 Riverine Water, SRM 1515 Apple leaves and GBW 07605 Tea were given in Table 4. The results found were in good agreement with the certified values of SRM's.

Application of the presented method were performed various natural water samples. The results were given in Table 5. The procedure for analyte ions was also applied to various microwave

Table 3

Analysis of two waters spiked analyte ions (sample volume 50 ml, $N=4$)

Element	Added (µg)	Hot spring water		Artificial sea water	
		Found (µg)	Recovery (%)	Found (µg)	Recovery (%)
Cu	0	ND	—	ND	—
	5	4.8 ± 0.3	96	4.8 ± 0.4	96
	10	9.9 ± 0.4	99	9.8 ± 0.4	98
	20	20.2 ± 0.5	101	19.8 ± 0.6	98
Pb	0	ND	—	ND	—
	10	9.8 ± 0.3	98	9.8 ± 0.4	98
	20	19.6 ± 0.5	98	19.2 ± 0.5	96
	40	40.8 ± 0.7	102	41.2 ± 0.6	103
Zn	0	ND	—	ND	—
	2.5	2.4 ± 0.2	96	2.5 ± 0.2	100
	5	4.8 ± 0.4	96	4.9 ± 0.3	98
	10	10.1 ± 0.6	101	9.9 ± 0.6	99
Fe	0	ND	—	ND	—
	5	4.9 ± 0.4	98	4.8 ± 0.3	96
	10	10.1 ± 0.5	101	9.7 ± 0.5	97
	20	19.6 ± 0.6	98	19.8 ± 0.7	99
Ni	0	ND	—	ND	—
	5	4.8 ± 0.3	96	4.9 ± 0.4	98
	10	9.8 ± 0.5	98	9.9 ± 0.4	99
	20	20.2 ± 0.7	101	19.6 ± 0.5	98
Co	0	ND	—	ND	—
	5	5.1 ± 0.3	102	4.8 ± 0.3	96
	10	10.1 ± 0.5	101	9.7 ± 0.5	97
	20	20.2 ± 0.7	101	19.6 ± 0.6	98

ND: not detected.

Table 4

The results for reference standard materials ($N=4$)

Element	NRCC-SLRS 4 Riverine Water ($\mu\text{g l}^{-1}$)		SRM 1515 Apple leaves ($\mu\text{g g}^{-1}$)		GBW 07605 Tea ($\mu\text{g g}^{-1}$)	
	Certified value	Our value	Certified value	Our value	Certified value	Our value
Cu	1.81	1.8 ± 0.1	5.64	5.6 ± 0.4	17.3	17.5 ± 0.5
Pb	0.086	BDL	0.47	0.46 ± 0.04	4.4	4.3 ± 0.3
Zn	0.93	0.95 ± 0.06	12.5	11.5 ± 0.9	26.3	25.9 ± 2.1
Fe	103	102.2 ± 0.9	(83)	80.2 ± 4.65	264	257.5 ± 20.1
Ni	0.67	BDL	0.91	0.92 ± 0.10	4.6	4.7 ± 0.2
Co	0.033	BDL	(0.09)	0.10 ± 0.01	0.18	0.17 ± 0.01

BDL: below the detection limit. The values in the parentheses are not certified.

Table 5

The application of the presented method in natural water samples for contents of analyte ions ($N=4$)

Element	Tap water ($\mu\text{g l}^{-1}$)	River water ($\mu\text{g l}^{-1}$)	Sea water ($\mu\text{g l}^{-1}$)	Spring water ($\mu\text{g l}^{-1}$)
Cu	4.8 ± 0.3	6.6 ± 0.5	5.2 ± 0.3	3.1 ± 0.2
Pb	3.7 ± 0.2	BDL	BDL	12 ± 1
Zn	27 ± 1	52 ± 5	42 ± 4	6.1 ± 0.4
Fe	35 ± 4	12 ± 1	51 ± 4	11 ± 1
Ni	8.3 ± 0.5	3.6 ± 0.3	4.1 ± 0.2	12 ± 1
Co	1.6 ± 0.2	BDL	1.1 ± 0.1	4.7 ± 0.3

BDL: below the detection limit.

Table 6

Levels of analyte ions in microwave digested various samples ($N=4$)

Element	Canned fish ($\mu\text{g g}^{-1}$)	Black tea ($\mu\text{g g}^{-1}$)	Tomato paste ($\mu\text{g g}^{-1}$)	Street dust ($\mu\text{g g}^{-1}$)
Cu	3.2 ± 0.1	15 ± 1	1.7 ± 0.1	49 ± 3
Pb	4.3 ± 0.3	3.1 ± 0.3	BDL	33 ± 3
Zn	42 ± 3	9.9 ± 0.5	1.8 ± 0.1	60 ± 1
Fe	8.5 ± 0.5	132 ± 10	0.6 ± 0.1	1223 ± 85
Ni	5.4 ± 0.3	3.8 ± 0.2	3.1 ± 0.2	66 ± 4
Co	3.3 ± 0.2	1.4 ± 0.1	BDL	15 ± 1

BDL: below the detection limit.

assisted digested environmental samples. These samples were digested by closed microwave system, and then preconcentration/separation procedure given in Section 2 was applied. The results are given in Table 6.

4. Conclusion

The presented procedure is based on the immobilization of *A. fumigatus* on Diaion HP-2MG and biosorption of copper(II), lead(II), zinc(II), iron(III), nickel(II) and cobalt(II) ions on these resin. The procedure is simple, economic and fast. The sorption capacity of the *A. fumigatus* immobilized Diaion HP-2MG is higher than many of the sorption capacity of the resins functionalized with chelating reagents [5–7,35,37,40]. Also preparation of the *A. fumigatus* immobilized Diaion HP-2MG resin is simple than the preparation of chelating resins. The reusability of *A. fumigatus* immobilized Diaion HP-2MG was as high as greater than 50 cycles without any loss in its sorption behavior. The system was also successful in preconcentrating analytes from large sample volume (500 ml). The matrix effects were reasonably tolerable. The method is relatively rapid as compared with previously reported solid phase extraction procedures for the enrichment of metal ions at trace levels. The preconcentration

factor and detection limits of the proposed method are comparable with the preconcentration methods based on biosorption of metals [21–33]. Comparable results were obtained from other biosorption studies in literature (Table 1). The procedure was successfully applied to analyte ions contents of various environmental samples including natural waters.

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Effect of insignificant bias and its uncertainty on the coverage probability of uncertainty intervals

Part 1. Evaluation for a given value of the true bias

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Abstract

This paper investigates the coverage probability of the uncertainty intervals determined in compliance with the GUM and EURACHEM Guide, which are defined by expanded uncertainty U about the results uncorrected with the insignificant biases and corrected with the significant biases. This coverage probability can significantly fall below the chosen level of confidence in some cases as Maroto et al. discovered by using the Monte Carlo method. Their numerical results obtained provided that only the β errors have occurred in the test significance and findings that the coverage reduction depends on the mutual proportions of the magnitudes of the systematic error, overall uncertainty and bias uncertainty are confirmed in this paper by using probability calculus and numerical integration. This problem is also studied when all possible experimental biases, both significant and insignificant, are considered. From this point of view, the reduction of the coverage probability turns out to be less severe than from the previous one. The coverage probability is also investigated for some uncertainty intervals computed in different ways than the above mentioned documents recommend. The intervals defined by U about the results corrected with both significant and insignificant bias give always the same coverage probability equalling the chosen level of confidence. The intervals with some uncertainties modified or enlarged with the insignificant biases remove or moderate the coverage reduction.

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1. Introduction

In the ISO “Guide to the Expression of Uncertainty in Measurement” (GUM) [1], the known method for evaluating and expressing measurement uncertainty was established and it was adapted to analytical measurements in the EURACHEM/CITAC document *Quantifying Uncertainty in Analytical Measurement* (EURACHEM Guide) [2]. In terms of these documents, the uncertainties arising from random effects and those arising from corrections for systematic effects are combined in the overall standard uncertainty using the uncertainty propagation principles and no distinction is made between them; the GUM claims that there is no inherent difference between these types of uncertainty components.

In case of the random effects, the repeated results of a sample observation are dispersed by these effects and the standard uncertainty is the standard deviation of the probability distribution of the observed results. In case of the systematic effects, these effects bring about the same, firm bias of the observed results. The uncertainty in such a case is the uncertainty, with which the experimental bias, having been determined, estimates the value of the true bias. Then the standard uncertainty is the standard deviation of the probability distribution of all possible experimental biases estimating the given true bias. According to the GUM [1], there is a difference in the grasp of probability in both cases. In the former case the probability indicates the “relative frequency” with which an event will occur. In the latter case, the probability indicates the “degree of believe” that an event will occur.

The GUM [1] concept of uncertainty considers that the results of a measurement have been corrected for all recognized significant systematic effects and that every effort has been made

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to identify such effects. The significant effect is characterised: “significant in size relatively to the required accuracy of the measurement”. However, this document does not establish how to prove this significance (I have not managed to find it there). According to the EURACHEM Guide [2] and also many papers, e.g. [3–5], the experimental bias determined in a trueness study is significant provided that it differs significantly from zero with respect to its standard uncertainty. This significance is tested by the t -test. When the experimental bias is found significant, it should be applied as a correction to compensate all future results of routine samples for the systematic effect. In the opposite case (insignificant experimental bias), the correction is not necessary. However, the bias uncertainty should be included in the overall combined uncertainty in both cases [1,2].

Due to the β error (type II error) in this significance test, there is always a probability that the experimental bias is found insignificant, though in fact the evaluated systematic error is so large that it significantly affects the results of routine samples. In such cases, which can occur with a non-negligible probability, the uncertainty interval about the uncorrected result can encompass the true value of the measurand with a considerably lower probability than the chosen level of confidence is (usually 95%).

These consequences of the β error were disclosed and investigated by Maroto et al. [6,7]. These authors showed the conditions under which the insignificant biases significantly decreased the coverage probability of uncertainty intervals. They recommended avoiding this underestimation of the uncertainty intervals by including of such insignificant biases in the uncertainty budget. This paper is concerned with the consequences of β error too, but it applies a different mathematical approach to this problem and in addition a different evaluating view, which has been used in paper by O'Donnell and Hibbert [8].

2. The β error consequences determined by Maroto et al.

Maroto et al. investigated the β error consequences by using the Monte Carlo method; they assumed both constant errors [6] and proportional errors [7]. In the former paper they inquired three cases of models with a few uncertainty components arising from both random and systematic effects and with different values of those components. These components were considered constant in each investigated case. The authors studied how the magnitude of the true bias – true systematic error (denoted by δ) of a hypothetical analytical method would affect the coverage probability of the uncertainty intervals of the results obtained by this method. They took some selected values of the true bias δ and for each given δ they obtained possible values of its estimate – experimental bias (denoted by b). These values of b originated from a normal distribution with a mean of δ and a standard deviation equal to the uncertainty of the experimental bias determination (denoted by $u(b)$); this distribution can be denoted by $N[\delta, u(b)]$. The values of b were computed from results generated by the Monte Carlo method (simulated results obtained by the biased analytical method on a certified reference material (CRM) and simulated certified values of the CRM). The

obtained experimental biases were tested on significance

$$t = \frac{b}{u(b)} \quad (1)$$

When a found value of t was 1.96 or smaller, the experimental bias was assumed insignificant with a significance level of 0.05. For each given δ , the frequency of such cases was recorded; it indicated the probability of the β error. When the bias was found significant, a new value of b was again simulated. In the case of an insignificant bias, the authors simulated a result (denoted by c) obtained with the biased method for a routine sample. Its value was drawn from a normal distribution. The mean of this distribution was $(\mu + \delta)$, where μ denoted the true concentration of the determined sample (the applied denotation is simpler than the origin one). The standard deviation of the distribution (denoted by σ) equalled the standard uncertainty combined from the uncertainty components reflecting all random errors of the applied hypothetical method. The obtained result was not corrected because the experimental bias was insignificant. Its overall standard uncertainty was

$$u = \sqrt{u^2(b) + \sigma^2} \quad (2)$$

and its overall expanded uncertainty was

$$U = k \cdot u \quad (3)$$

where the applied coverage factor, k , was 1.96 so that the coverage probability was exactly 95%.

In the end the authors checked whether the true concentration μ was contained in the uncertainty interval defined by U about the result c and recorded the frequency of such cases. Since the numbers of these simulations were huge the relative frequency gave the coverage probability of the uncertainty interval under the given value of δ .

Using tables and plots, the authors documented that when the values of δ increased, the β error probability decreased admittedly and the coverage probability decreased too. They showed that this coverage depression depended on the relative size of the bias uncertainty $u(b)$ in comparison with the overall standard uncertainty u (ratio $u(b)/u$). When $u(b)$ was higher than 30% of u , the coverage probability might fall too low (in extreme cases lower than 80%). The authors made analogous conclusions when they investigated proportional bias (more exactly recovery) and relative uncertainty [7]. They meant that in such cases, the uncertainty interval defined by the common expanded uncertainty U was too underestimated and consequently they recommended applying different uncertainty intervals, which were modified with b . Particularly they proposed to calculate uncertainty intervals obtained by so-called SUMU method or $U(\text{bias})$ method [6,7], see below.

3. The coverage probabilities of the intervals defined by U in case of the β error—probability calculus

This chapter shows that the β error probability and the coverage probability can be obtained by using classic statistic computations. (The standard uncertainties are assumed

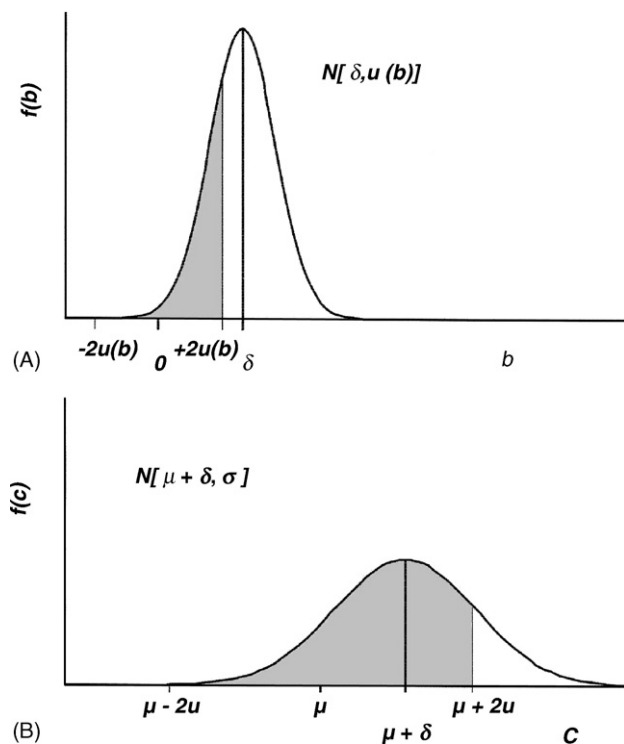


Fig. 1. (A) The normal distribution of all possible experimental biases b , which could be determined for a biased analytical method having the true systematic error δ (it is unknown in reality) and the bias uncertainty $u(b)$. The shaded area indicates the β error probability, i.e. the percentage of the insignificant biases. (B) The normal distribution of all possible results c for a sample with the true analyte concentration μ (it is unknown in reality); the applied measuring method given in (A) has the overall combined uncertainty u and the combined uncertainty of all random effects σ . The shaded area indicates the coverage probability in case of an insignificant bias, i.e. probability that μ is encompassed in the interval defined by $\pm 2u$ about an uncorrected measured result.

to be determined with high degrees of freedom and consequently the standard normal distribution is applied instead of the t -distribution. These computations are precise and therefore the precise value of the 95th percentile is preferred, i.e. 1.96. The usual round value, 2, is used in the legends of graphs.)

An example of the studied problem is shown in Fig. 1 where two probability density functions are plotted. The plot in Fig. 1A represents the distribution of possible experimental biases b ; distribution $N[\delta, u(b)]$, both δ and $u(b)$ are assumed to be independent on the analyte level. The β error probability, i.e. the probability, P , that the occurred value of b will be one from the possible insignificant biases (i.e. from the interval $0 \pm 1.96u(b)$), is indicated by the shaded region. It can be computed by using the cumulative distribution function of the standard normal distribution, $\Phi(z)$, after the standardization of the interval bounds; e.g. the standard score, z , for the right bound of $1.96u(b)$

$$z = \frac{1.96u(b) - \delta}{u(b)} = 1.96 - \delta/u(b) \quad (4)$$

$$P(-1.96u(b) \leq b \leq 1.96u(b)) \\ = \Phi(1.96 - \delta/u(b)) - \Phi(-1.96 - \delta/u(b)) \quad (5)$$

It can be seen that the β error probability depends on only one parameter (not statistical)— $\delta/u(b)$. The results computed in this way for some selected values of this parameter are given in Table 1 (last column).

The second density function (Fig. 1B) represents the distribution of possible future results c , which can be obtained with the biased method for a routine sample— $N[\mu + \delta, \sigma]$. The randomly occurred results are not corrected because the experimental bias is insignificant. Once such an experimental bias has occurred, it is not at all involved in the computation of the uncertainty intervals: $c \pm 1.96u$. Fig. 1B shows that when the value of δ is higher, the interval of $\mu \pm 1.96u$ defines a considerably smaller area under the distribution curve than 95%. If the systematic error were low enough, than that area would be higher than 95%, since the standard deviation of that distribution σ is smaller than the overall standard uncertainty u . It is easy to calculate the defined area representing the value of coverage probability, CP,

$$\begin{aligned} \text{CP} &= P(c - 1.96u \leq \mu \leq c + 1.96u) \\ &= P(\mu - 1.96u \leq c \leq \mu + 1.96u) \\ &= \Phi\left(\frac{\mu + 1.96u - (\mu + \delta)}{\sigma}\right) \\ &\quad - \Phi\left(\frac{\mu - 1.96u - (\mu + \delta)}{\sigma}\right) \\ &= \Phi\left(\frac{-\delta + 1.96u}{\sigma}\right) - \Phi\left(\frac{-\delta - 1.96u}{\sigma}\right) \end{aligned} \quad (6)$$

It can be seen that the coverage probability depends only on three quantities, δ , u and σ , hence it may be expressed as a function of two parameters. Overall uncertainty u is combined from σ and $u(b)$ (see Eq. (2)), therefore uncertainty σ can be expressed by using u and $u(b)$. Then the coverage probability may be computed as a function of two parameters: e.g. $\delta/u(b)$ (Maroto et al. [6] applied the β error probability instead) and $u(b)/u$. The coverage probabilities for some selected values of these parameters are given in Table 1. Line 1 in Fig. 2 shows

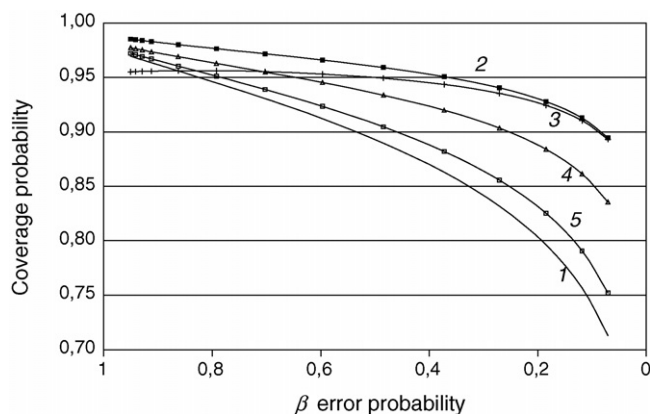


Fig. 2. The mean coverage probability of the uncertainty intervals computed with different methods vs. the β error probability (uncertainties for Case 1); only the cases of the insignificant biases were taken into account. The uncertainty intervals were defined: 1, U about uncorrected results; 2, $U(b)$ method; 3, SUMU method and U about corrected results; 4, RSSu method; 5, RSSU method.

Table 1

Coverage probability of the uncertainty intervals defined by $1.96u$ about uncorrected results obtained by a biased analytical method; dependence on the method characteristics (the computations consider only the insignificant values of experimental biases)

$\delta/u(b)$	$u(b)/u$							β error probability
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	
0.0	0.9511	0.9545	0.9601	0.9675	0.9764	0.9857	0.9939	0.9500
0.2	0.9511	0.9544	0.9597	0.9669	0.9754	0.9846	0.9930	0.9454
0.4	0.9510	0.9538	0.9585	0.9649	0.9726	0.9812	0.9898	0.9315
0.6	0.9507	0.9529	0.9565	0.9615	0.9678	0.9754	0.9840	0.9078
0.8	0.9504	0.9517	0.9538	0.9568	0.9610	0.9667	0.9748	0.8741
1.0	0.9500	0.9500	0.9502	0.9506	0.9518	0.9547	0.9611	0.8299
1.2	0.9495	0.9480	0.9457	0.9429	0.9403	0.9390	0.9415	0.7756
1.4	0.9489	0.9457	0.9405	0.9337	0.9261	0.9190	0.9150	0.7119
1.6	0.9482	0.9429	0.9343	0.9228	0.9091	0.8942	0.8802	0.6404
1.8	0.9474	0.9398	0.9273	0.9102	0.8890	0.8643	0.8365	0.5635
2.0	0.9466	0.9363	0.9194	0.8959	0.8659	0.8289	0.7835	0.4840
2.2	0.9456	0.9324	0.9105	0.8797	0.8394	0.7881	0.7218	0.4051
2.4	0.9445	0.9282	0.9007	0.8617	0.8098	0.7421	0.6525	0.3299
2.6	0.9434	0.9235	0.8899	0.8417	0.7769	0.6914	0.5777	0.2611
2.8	0.9422	0.9184	0.8781	0.8199	0.7410	0.6368	0.5000	0.2004
3.0	0.9408	0.9129	0.8654	0.7962	0.7023	0.5792	0.4223	0.1492
3.2	0.9394	0.9071	0.8516	0.7707	0.6611	0.5199	0.3475	0.1075
3.4	0.9378	0.9008	0.8369	0.7435	0.6180	0.4602	0.2782	0.0749
3.6	0.9362	0.8940	0.8211	0.7146	0.5733	0.4013	0.2165	0.0505
$u(b)/\sigma$								
	0.101	0.204	0.314	0.436	0.577	0.750	0.980	

(δ) True systematic error, ($u(b)$) experimental bias uncertainty, (u) overall combined uncertainty, (σ) combined uncertainty of all random effects.

the results obtained with some selected values of the β error probability and the uncertainties applied in Case 1 of the paper [6]; i.e. $u(b) = 0.70$, $\sigma = 1.5$, $u = 1.655$ (its value of 1.64 given in Ref. [6] is a little incorrect). These results are the same as the ones obtained by the Monte Carlo method [6].

4. The coverage probabilities of the modified uncertainty intervals—probability calculus

As stated above, Maroto et al. [6] found out that when the experimental biases were insignificant and their uncertainties were higher, the uncertainty intervals defined by U about the uncorrected results c were underestimated, i.e. their coverage probabilities were too low. The authors sampled four methods modifying the uncertainty intervals about the uncorrected results in order to get higher coverage probabilities. These modified uncertainty intervals are defined (they are termed by their specific denomination applied in literature [6–10]):

$$U(\text{bias}) = U + |b| \quad (7)$$

$$U(\text{RSSu}) = k\sqrt{(u^2 + b^2)} \quad (8)$$

SUMU with two different sides

$$U_+ = U - b \text{ and } U_- = U + b, \text{ provided } |b| \leq U \quad (9)$$

$$U(\text{RSSU}) = \sqrt{U^2 + b^2} \quad (10)$$

Maroto et al. [6] studied the coverage probability of these modified intervals also by using the Monte Carlo method.

To calculate the coverage probabilities of these modified uncertainty intervals in the cases of β error is a bit more difficult matter than the previous application of probability calculus. The possible future results c have the distribution $N[\mu + \delta, \sigma]$. We need the probability, with which an uncertainty interval ranging from $c - U_{e-}$ to $c + U_{e+}$ encompasses μ , or inversely the probability that a future result c belongs between $\mu - U_{e+}$ and $\mu + U_{e-}$. U_{e-} and U_{e+} are general denotations of the sides of the modified uncertainty intervals; the widths of the left and right sides can be different.

$$\begin{aligned} P(c - U_{e-} \leq \mu \leq c + U_{e+}) &= P(\mu - U_{e+} \leq c \leq \mu + U_{e-}) \\ &= \Phi\left(\frac{\mu + U_{e-} - (\mu + \delta)}{\sigma}\right) - \Phi\left(\frac{\mu - U_{e+} - (\mu + \delta)}{\sigma}\right) \\ &= \Phi\left(\frac{-\delta + U_{e-}}{\sigma}\right) - \Phi\left(\frac{-\delta - U_{e+}}{\sigma}\right) \end{aligned} \quad (11)$$

Since both U_{e+} and U_{e-} depend on the found value of b , the probability changes according to this value (conditional probability). Consequently, the coverage probability can be assumed invariable only for values of the experimental bias, which are from an interval between b and $b + db$, where db is an infinitesimal difference of b . The values of b occur in conformity with the probability density function, $f(b)$, of the distribution $N[\delta, u(b)]$. The probability that a found value of b belongs to that infinitesimal

mal interval equals $f(b) \cdot db$. The coverage probabilities given by Eq. (11), which are individual for each infinitesimal interval of b , are used in the computation of the mean coverage probability for the whole range of the insignificant experimental biases (from $-1.96u(b)$ to $1.96u(b)$). Since each individual coverage probability, ICP, occurs with its own probability, this mean must be computed as a weighted mean. The weights are those probabilities $f(b) \cdot db$ and their sum equals the total probability of those insignificant b values, i.e. the β error probability (this probability can also be computed according to Eq. (5)). This mean coverage probability, MCP, is computed over the whole range of the insignificant experimental biases for given values of δ and $u(b)$ and certain type of the uncertainty modified with insignificant bias

$$\begin{aligned} \text{MCP} &= \frac{\int_{-1.96u(b)}^{1.96u(b)} \text{ICP} \cdot f(b) \cdot db}{\int_{-1.96u(b)}^{1.96u(b)} f(b) \cdot db} \\ &= \frac{\int_{-1.96u(b)}^{1.96u(b)} \left[\Phi\left(\frac{-\delta+U_{e-}}{\sigma}\right) - \Phi\left(\frac{-\delta-U_{e+}}{\sigma}\right) \right] \cdot f(b) \cdot db}{\int_{-1.96u(b)}^{1.96u(b)} f(b) \cdot db} \end{aligned} \quad (12)$$

The general uncertainties U_{e-} and U_{e+} in Eq. (12) can be replaced by those particular modified uncertainties defined by Eqs. (7)–(10). The definite integrals obtained in this way were solved numerically. The resulting mean coverage probabilities computed with the uncertainties of Case 1 from the article by Maroto et al. [6] were plotted in Fig. 2.

As stated above, in paper [7], Maroto et al. also studied the problem of coverage probability in case of proportional errors of the measured results. It means that they assumed the magnitudes of random and systematic errors to increase with the concentration level of the analyte. Consequently, they replaced the absolute uncertainties changing in this instance with the invariable relative standard uncertainties, i.e. with coefficients of variation (relative standard deviations) and the changing true biases with the invariable true recoveries. (This problem was not studied by the probability calculus in this paper. I think this problem to be mathematically very similar to the previous one.) When the authors applied the methods defying those special uncertainty intervals modified or enlarged with the insignificant uncorrected experimental biases they distinguished between the mathematical method of uncertainty calculation and the model of errors [6,7]. It means that the same mathematical method applied in combination with the two different error models (either constant or proportional) was always taken as the only method, though both combinations would give different uncertainty intervals being applied on the same measured results (of course, only one error model could fit a set of observed results). Also paper [10] grasps a “method defining uncertainty intervals” in this way. However, O'Donnell and Hibbert [8] take the same mathematical method combined with the two error models as two different methods of uncertainty computations (e.g. they distinguish the RSSu method and the method denoted by names of its authors Barwick and Ellison [11]).

5. Evaluation of coverage probability considering only the β error cases

The plot in Fig. 2 depicts the relationships between the β error probabilities and the coverage probabilities of the uncertainty intervals defined by all the mentioned methods about uncorrected results. This plot is identical with the one given in paper [6] though the depicted results in both plots were obtained by two completely different methods. It shows that the Monte Carlo method and probability calculus give the same results and also confirms the disturbing results published by Maroto et al. [6,7]. These results indicate that in case of the insignificant experimental biases, which are not applied in the corrections of results, the coverage probabilities of the uncertainty intervals defined by expanded uncertainty U can be very low. Table 1 shows two conditions for it: (1) the true bias δ must be high enough in comparison with the uncertainty of the experimental bias $u(b)$ (parameter $\delta/u(b)$) and (2) the $u(b)$ must be high enough in comparison with the overall uncertainties u (parameter $u(b)/u$). The table also says that a higher ratio $\delta/u(b)$ brings about a lower probability that an insignificant experimental bias (β error probability) will occur and thereby also a lower probability of such a high depression of the coverage probability. When the coverage probability of the computed uncertainty intervals is to be close to the chosen level of confidence in spite of the fact that the parameters $\delta/u(b)$ and $u(b)/u$ of a given analytical method are high, those uncertainty intervals modified with the experimental biases should be computed (see Fig. 2). It means that it is necessary to direct the uncertainty computations according to the parameter $u(b)/u$, which depends on the ratio of both types of uncertainty ($u(b)/\sigma$; see Table 1). This conclusion seems to cast doubt upon the thesis given in the GUM [1] that there is no inherent difference between the uncertainty components arising from random effects and from systematic effects.

6. The coverage probabilities of uncertainty intervals for the whole distribution of b

The investigation of coverage probability in the previous chapters considered only the β error cases. It is true that (1) when δ differs from zero, each experimental bias insignificant by the t -test is a case of the β error and also that (2) practically each analytical method has its own systematic error δ , which of course can be negligible and undiscovered. From this point of view such a narrow base of the coverage probability evaluation seems to be justifiable. Nevertheless, I think that this evaluation should take into account all possible results obtained for the whole distribution of experimental biases b conditioned by a given value of the parameter $\delta/u(b)$. This distribution includes both significant biases applied in the correction and insignificant biases not applied in it. Consequently besides those underestimated uncertainty intervals defined by U about uncorrected results there always must be some uncertainty intervals defined by U about corrected results, which can be properly estimated or maybe even overestimated. In addition the uncertainty intervals with those problematically low coverage probabilities are less probable because they appear only under high values of δ

when the insignificant experimental biases not applied in the correction are infrequent. Therefore, it is questionable to evaluate the coverage probabilities only on account of the β error cases (only for the insignificant experimental biases), which are detached from all possible cases. Such an approach considering the whole distribution of the experimental biases was applied by O'Donnell and Hibbert [8], who also studied the effect of the bias and its uncertainty on the coverage probability of uncertainty intervals by using the Monte Carlo method.

The whole distribution of experimental biases b ranges from $-\infty$ to ∞ and it is supposed normal with a mean of the true bias δ and standard deviation of $u(b)$. Any value from it can randomly occur in the trueness study. The probability, that the found value will be contained in an infinitesimal interval between b and $b + db$, equals $f(b) \cdot db$ (see above). The value of b does not influence the expanded uncertainty U of future results ($U = 1.96u$), but it determines whether the future results for routine samples will be corrected or not. The coverage probabilities for the uncertainty intervals defined by expanded uncertainty U about the corrected results depend on the values of b and δ (on their difference). For each found value of b an individual coverage probability exists

$$\begin{aligned}
 \text{ICP} &= P(c - b - U \leq \mu \leq c - b + U) \\
 &= P(\mu + b - U \leq c \leq \mu + b + U) \\
 &= \Phi\left(\frac{\mu + b + U - (\mu + \delta)}{\sigma}\right) \\
 &\quad - \Phi\left(\frac{\mu + b - U - (\mu + \delta)}{\sigma}\right) \\
 &= \Phi\left(\frac{b - \delta + U}{\sigma}\right) - \Phi\left(\frac{b - \delta - U}{\sigma}\right) \quad (13)
 \end{aligned}$$

the distribution of c is $N(\mu + \delta, \sigma)$. On the other hand, the coverage probabilities for those uncertainty intervals defined by expanded uncertainty U about the uncorrected results do not depend on the value of b (see Eq. (6)). It is obvious that there are uncertainty intervals with different individual coverage probabilities that can be both higher than 0.95 and lower than 0.95. It implies that the total coverage probability for all possible values of b , under a given value of δ , must be computed again as the weighted mean from all these individual coverage probabilities. The weights are the probabilities, with which the corresponding values of b occur in the followed infinitesimal intervals. The total sum of these weights equals the probability that any value of b occurs, i.e. 1 (the resulting equation to compute this weighted mean is identical with so-called total probability law [12,13], see appendix A).

7. Calculations considering the whole distribution of b and the obtained results

This chapter investigates four cases of uncertainty calculations. All four cases presuppose that when an experimental bias has been found significant the results measured on routine samples are corrected with it and the uncertainty intervals

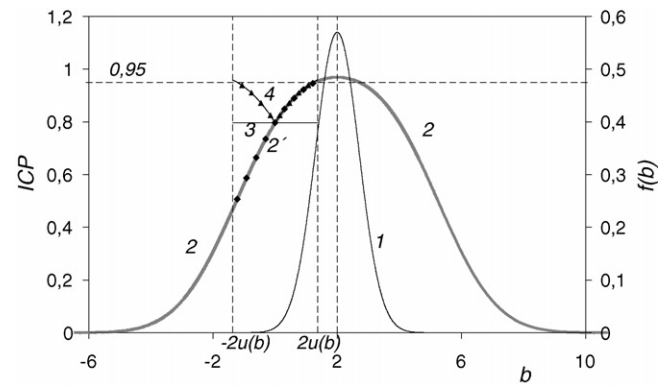


Fig. 3. The individual coverage probability of the uncertainty intervals obtained by different computation methods for all possible experimental biases b vs. the given value of b and the experimental bias distribution (1); conditions $\delta=2$, uncertainties for Case 1. The uncertainty intervals were defined in the region of the significant b by U about corrected results (2); in the region of the insignificant b , i.e. from $-2u(b)$ to $2u(b)$), by the SUMU method and also by U about corrected results (2'), by U about uncorrected results (3), by the $U(\text{bias})$ method (4).

are defined by the expanded uncertainty U . The cases differ only in the part solving the insignificant experimental biases (this part was studied in the previous chapters). In three studied instances the measured results are not corrected and three different types of uncertainty intervals are assumed: defined (1) by U , (2) by $U(\text{bias})$ and (3) by SUMU methods. The last (4) case presupposes that the measured results are corrected with the insignificant biases (though they should not be) and then the normal expanded uncertainty U is used. These cases were studied with the uncertainties given in Case 1 [6] ($u = 1.655$, $u(b)/u = 0.423$).

Fig. 3 illustrates the situation when $\delta = 2$ (i.e. $\delta/u(b) = 2.857$). The plot there depicts the probability density function $f(b)$ of experimental biases (line 1: $N(\delta, u(b))$) and a few lines (2–4) representing the relationships between the individual coverage probabilities and values of b for all the investigated cases. Line 2 is common and expresses the ICP of the uncertainty intervals defined by U about the corrected results (intervals in case of significant biases). This line is interrupted by the range of insignificant experimental biases (from $-1.96u(b)$ to $1.96u(b)$). (This part of the diagram shows the area investigated by Maroto et al. [6], i.e. the area of the β errors.) In this range of b , there are three lines expressing ICP. Line 2' (the continuation of the outside line 2) gives the ICP for the uncertainty intervals defined by U about the results corrected with the insignificant experimental biases b . This line shows also the ICP for the uncertainty intervals determined by SUMU method about the uncorrected results (both methods give the same intervals). Line 3 (the horizontal line expressing a constant ICP of 0.796) is for the uncertainty intervals defined by expanded uncertainty U about the uncorrected results. Line 4 (its right part is nearly identical with line 2') represents the ICP for the uncertainty intervals defined by $U(\text{bias})$ about the uncorrected results. Fig. 4 illustrates the same relationships when $\delta = 0$. The interval from $-1.96u(b)$ to $1.96u(b)$ shows the probability 95% that an insignificant experimental bias will occur (the area delimited with this interval below line 1). It is the probability of the right t -test decisions,

Table 2

Coverage probability of the uncertainty intervals defined by $1.96u$ about uncorrected results obtained by a biased analytical method; dependence on the method characteristics (the computations consider only the insignificant values of experimental biases)

$\delta/u(b)$	$u(b)/u$							β error probability
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	
0.0	0.9508	0.9533	0.9572	0.9624	0.9683	0.9740	0.9777	0.9500
0.2	0.9508	0.9531	0.9567	0.9616	0.9672	0.9727	0.9765	0.9454
0.4	0.9506	0.9524	0.9554	0.9594	0.9640	0.9689	0.9729	0.9315
0.6	0.9504	0.9515	0.9533	0.9558	0.9590	0.9626	0.9667	0.9078
0.8	0.9501	0.9503	0.9506	0.9512	0.9523	0.9542	0.9579	0.8741
1.0	0.9497	0.9489	0.9475	0.9459	0.9445	0.9440	0.9463	0.8299
1.2	0.9493	0.9474	0.9443	0.9403	0.9361	0.9326	0.9321	0.7756
1.4	0.9490	0.9460	0.9412	0.9349	0.9277	0.9207	0.9160	0.7119
1.6	0.9487	0.9448	0.9386	0.9302	0.9201	0.9093	0.8991	0.6404
1.8	0.9485	0.9440	0.9366	0.9265	0.9139	0.8994	0.8832	0.5635
2.0	0.9484	0.9434	0.9353	0.9241	0.9097	0.8919	0.8701	0.4840
2.2	0.9483	0.9433	0.9349	0.9231	0.9077	0.8877	0.8616	0.4051
2.4	0.9484	0.9435	0.9353	0.9236	0.9080	0.8872	0.8590	0.3299
2.6	0.9485	0.9440	0.9364	0.9254	0.9104	0.8902	0.8625	0.2611
2.8	0.9487	0.9447	0.9380	0.9281	0.9145	0.8961	0.8712	0.2004
3.0	0.9489	0.9456	0.9398	0.9314	0.9197	0.9040	0.8835	0.1492
3.2	0.9491	0.9464	0.9418	0.9349	0.9253	0.9128	0.8973	0.1075
3.4	0.9493	0.9472	0.9436	0.9382	0.9309	0.9216	0.9109	0.0749
3.6	0.9495	0.9480	0.9452	0.9412	0.9359	0.9294	0.9228	0.0505
3.8	0.9496	0.9485	0.9466	0.9438	0.9401	0.9359	0.9323	0.0329
4.0	0.9498	0.9490	0.9477	0.9458	0.9433	0.9408	0.9391	0.0207
5.0	0.9500	0.9499	0.9498	0.9497	0.9496	0.9495	0.9496	0.0012
$u(b)/\sigma$								
	0.101	0.204	0.314	0.436	0.577	0.750	0.980	

(δ) True systematic error, ($u(b)$) experimental bias uncertainty, (u) overall combined uncertainty, (σ) combined uncertainty of all random effects.

but it is calculated as the β error probability (see Table 2). The significant biases, lying outside this interval, are the cases of α error (type I error) in the significance test.

It is evident that the equations to calculate the mean coverage probability from the ICPs must differ only in the parts solving the region of the insignificant experimental biases. The simplest case arises when the intervals defined by expanded uncertainty U about always corrected results are computed. It is identical with the case when for the range of the insignificant biases the

intervals defined by SUMU method about the uncorrected results are computed. The integration is computed continuously along both lines 2 and 2' in Figs. 3 and 4 over the whole range of possible biases

$$\text{MCP} = \int_{-\infty}^{\infty} \left[\Phi \left(\frac{b - \delta + U}{\sigma} \right) - \Phi \left(\frac{b - \delta - U}{\sigma} \right) \right] \cdot f(b) \cdot db \quad (14)$$

In the other cases, it is necessary to integrate by degrees over three ranges (see Figs. 3 and 4): the central range of the insignificant experimental biases (for the followed special uncertainty intervals about the uncorrected results) and the left and right ranges of the significant biases (both for the uncertainty intervals defined by U about the corrected results). When the general denotations U_{e-} and U_{e+} are applied this equation results

$$\begin{aligned} \text{MCP} = & \int_{-\infty}^{-1.96u(b)} \left[\Phi \left(\frac{b - \delta + U}{\sigma} \right) - \Phi \left(\frac{b - \delta - U}{\sigma} \right) \right] \cdot f(b) \cdot db \\ & + \int_{-1.96u(b)}^{1.96u(b)} \left[\Phi \left(\frac{-\delta + U_{e-}}{\sigma} \right) - \Phi \left(\frac{-\delta - U_{e+}}{\sigma} \right) \right] f(b) db \\ & + \int_{1.96u(b)}^{\infty} \left[\Phi \left(\frac{b - \delta + U}{\sigma} \right) - \Phi \left(\frac{b - \delta - U}{\sigma} \right) \right] \cdot f(b) db \quad (15) \end{aligned}$$

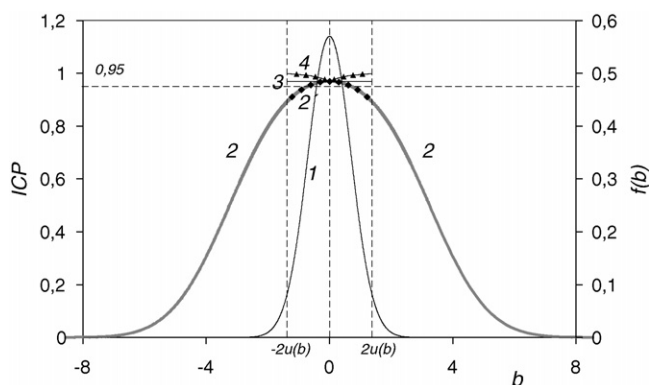


Fig. 4. The individual coverage probability of the uncertainty intervals obtained by different computation methods for all possible experimental biases b vs. the given value of b and the experimental bias distribution (1); conditions $\delta=0$, uncertainties for Case 1. The uncertainty intervals were defined in the region of the significant b by U about corrected results (2); in the region of the insignificant b , i.e. from $-2u(b)$ to $2u(b)$, by the SUMU method and also by U about corrected results (2'), by U about uncorrected results (3), by the $U(\text{bias})$ method (4).

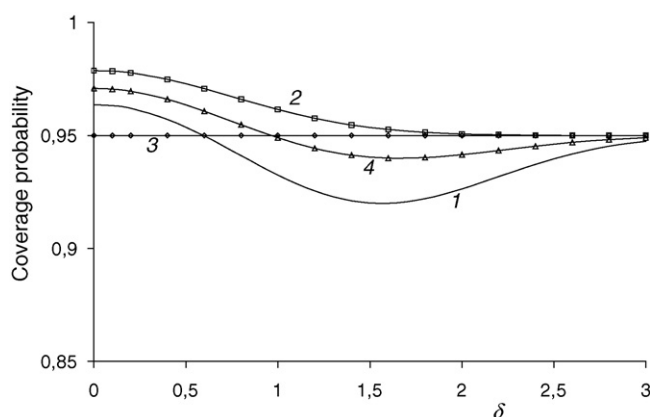


Fig. 5. The mean coverage probability of the uncertainty intervals computed with different methods vs. the true systematic error δ (uncertainties for Case 1); the whole distribution of the experimental biases was taken into account. The uncertainty intervals were defined: for the significant biases by U about corrected results in all cases and for the insignificant biases 1: U about uncorrected results, 2: $U(\text{bias})$ method, 3: SUMU method and U about corrected results, 4: $U_e(95\%)$ method.

The integrals calculating MCP for the given four instances are specified in Appendix B. Their solutions obtained for uncertainties given in Case 1 [6] and some selected values of δ are depicted in Fig. 5. When in the range of the insignificant experimental biases the uncertainty intervals defined by expanded uncertainty U about the uncorrected results are used (line 1), the mean coverage probability is less than 0.95 provided the true bias δ is larger than approximately 0.6. However, it does not fall below 0.92 (the minimum of this line). The mean coverage probability for this way of uncertainty calculation (the EURACHEM Guide method [2]) was investigated in detail. The results for some selected values of parameters $\delta/u(b)$ and $u(b)/u$ (or also for corresponding values of $u(b)/\sigma$ and β error probability) are in Table 2. It is interesting that the dependence of the mean coverage probability on the parameter $\delta/u(b)$ shows minima. When the values of δ are negligible, no correction for them is necessary and therefore the uncertainty intervals U about uncorrected results have excessive individual coverage probabilities (e.g. 0.969 for the case depicted in Fig. 4). The probability, that such intervals will occur, is high since the insignificant experimental biases are very probable and therefore the mean coverage probability over the whole b distribution exceeds 0.95. With medium values of δ (Fig. 3) the most probable biases are the significant, but the insignificant biases are still possible. Consequently, the uncertainty intervals about uncorrected results, which have low ICP under these conditions (e.g. 0.796 in Fig. 3), are probable enough to cause the depression of the mean coverage probability. When the values of δ are high, the probability of the insignificant biases is very low and the significant biases have the leading part. This situation is similar to the case when the results are always corrected and then the mean coverage probability converges to 0.95.

When the $U(\text{bias})$ method is applied in the case of the insignificant experimental biases (line 2 in Fig. 5), the mean coverage probabilities are higher than 0.95, especially for low values of δ when the probability of such insignificant biases is high (line

4 in Fig. 4). It is necessary to remark that for a high coverage probability, e.g. 0.95, its additional elevation, though small, represents a relatively large extension of the uncertainty intervals (see [10]). When in the region of the insignificant experimental biases the SUMU method is applied for the uncorrected results (line 3 in Fig. 5), the obtained mean coverage probabilities are always 0.95. The same values of mean coverage probability are reached when the measured results are always corrected (i.e. with both significant and insignificant experimental biases) and the uncertainty intervals are defined by U . The relationships between mean coverage probability and δ expressed with lines 3 (results are always corrected and U is always applied) and 1 (results are corrected only with the significant experimental biases and U is always applied) in Fig. 5 agree with the results obtained by O'Donnell and Hibbert [8] with the Monte Carlo method.

In that paper, O'Donnell and Hibbert [8] also investigated the mean coverage probability for such cases when the results for routine samples were not corrected with the significant biases but some types of uncertainty intervals modified with the significant experimental biases were computed instead (in case of insignificant biases they calculated the uncertainty intervals defined by U about the uncorrected results). They published results showing the SUMU method as the worst, since this way of uncertainty computations gave lowest mean coverage probabilities. The obtained mean coverage probabilities were lower than for the case when the intervals defined by U about always uncorrected results were computed (if I understand these published results well I am not able to explain them¹).

In this chapter besides the well known methods a recent method incorporating the uncorrected bias in the uncertainty intervals was studied. This method, denoted by $U_e(95\%)$ was proposed in paper [10]. It defines an uncertainty interval, which is constructed symmetrical about the uncorrected results and which is so wide that its coverage probability equals 0.95 (this coverage is computed for the found experimental bias b). This method enlarges the uncertainty intervals like the $U(\text{bias})$ method but applies only a necessary part of b for the coverage probability to be just 0.95

$$U_e(95\%) = U + E|b| \quad (16)$$

The coefficient E depends on b/u and this relationships can be expressed in this way

$$E = -0.02015 + 1.191 \cdot b/u - 0.5738 \cdot (b/u)^2 + 0.08102 \cdot (b/u)^3 + 0.00811 \cdot (b/u)^4 \quad (17)$$

This equation was obtained from the values of E tabulated in [10]. Line 4 in Fig. 5 shows the results obtained by this

¹ The cases when the correction with the significant biases is replaced by the uncertainties modified with them are not studied in this present paper, because I have already studied them in paper [10] but from a bit different viewpoint. Some statisticians seriously object to these types of uncertainty intervals because of theoretical causes. I think these objections not to be valid when the modified uncertainty intervals are applied only in case of the insignificant biases, i.e. in the instances studied by this paper.

method when the insignificant biases occur (in case of significant biases the intervals defined by U about the corrected results are applied). This line expressing the relationship between the mean coverage probability and δ deviates both positively and negatively from the expected value (0.95) like line 1, but the negative deviations are less.

8. Evaluation of coverage probability considering the whole distribution of b

The obtained results confirms when the uncertainty intervals are calculated according to the GUM [1] and the EURACHEM Guide [2], the β error can causes that the mean coverage probability evaluated over the whole distribution of the experimental biases for a given value of δ is lower than 0.95. Table 2 shows that if the bias uncertainty is extremely high (values $u(b)/u$ higher than 0.5) the mean coverage probability can fall below 0.90. This coverage depression is not such dramatic as the previous approach indicates, nevertheless it can be considered too large. The mean coverage probability can be increased by application of those uncertainty intervals modified with the experimental bias in case of the insignificant biases. The SUMU uncertainty is shown as the best because it keeps the mean coverage probability at a level of 0.95, but the obtained intervals are asymmetric. The other studied methods are also acceptable, either $U(\text{bias})$ method, which makes only positive deviations of the mean coverage probability, which might be regarded as too high, or $U_c(95\%)$ method, which moderately deviates the mean coverage probability from 0.95 to both sides. Very important findings, revealed by O'Donnell and Hibbert [8] and confirmed in this paper, are that the uncertainty intervals, which are defined by U about the results always corrected with both significant and insignificant biases, keep the mean coverage probability at a level of 0.95. This finding states that a measured result should be corrected not only when the experimental bias is statistically significant with respect to its $u(b)$ (on the base of the t -test; see Eq. (1)) but also when the experimental bias is too high—really significant. The question is what means ‘really significant’ in this case. Provided that significance is grasped in this way, the idea saying that there is no inherent difference between both types of uncertainty components (from random and systematic effects) is saved. However, only the GUM allows this interpretation of significance since it asks to correct all systematic effects significant in size relatively to the required accuracy of a measurement [1]; the EURACHEM Guide defines significance only by the t -test [2].

9. Conclusions

This paper confirms the results of the coverage probability evaluations obtained by Maroto et al. [6] and also some results published by O'Donnell and Hibbert [8]. It proves that these results made by the Monte Carlo method can also be obtained by probability calculus. It confirms that the coverage probabilities of the uncertainty intervals defined in agreement with GUM [1] and EURACHEM Guide [2] can fall below 0.95 because of the β error in the t -test of the bias signif-

icance and that this coverage depression depends on these three quantities (their relative magnitudes): the true bias δ and the uncertainty of the experimental bias $u(b)$ and the overall uncertainty u (or the combined uncertainty of the components arising from the random errors σ). The performed computations apply only two parameters expressing the relative magnitudes of these quantities: $\delta/u(b)$ and $u(b)/u$. The paper tries to show that the approach by Maroto et al. [6], which took into account only the possible insignificant experimental biases under a given value of the true bias, does not give a complete view. It prefers the approach by O'Donnell and Hibbert [8] evaluating the coverage probabilities of uncertainty intervals from the viewpoint of all possible experimental biases, both significant and insignificant. This viewpoint does not show the coverage probability depression to be as severe as Maroto et al. indicated. The lowest coverage (below 0.9) appears for the parameter $\delta/u(b)$ of a middle size (about 2) and with an extremely high value of $u(b)/u$ (higher than 0.5). When $\delta/u(b)$ is low then the coverage probability is higher than 0.95 and when $\delta/u(b)$ is extremely high the coverage reaches a value of 0.95. When the results are corrected with both significant and insignificant biases the coverage probability equals 0.95 at all values of δ . It indicates that from this point of view besides the t -test an additional criterion of the experimental bias significance should be taken into account. This criterion would discover other experimental biases, which should be applied in the result correction. Then the coverage probability would not need to be increased by calculating some uncertainty intervals modified with the insignificant biases, e.g. by using $U(\text{bias})$ or SUMU methods, as Maroto et al. recommended.

At the end of Part 1 of this work it is necessary to note that there is a shortcoming of the used computations evaluating coverage probability. The coverage probability here is quantified for given values of true bias, which are in fact unknown. The analyst who knows only an experimental bias of the applied analytical method (an estimate of that true value) would need the coverage probability expressed as a function of this known quantity. Then, he could compute the coverage probabilities of various types of uncertainty intervals for his given case and he could make a decision on it. Part 2 of this work tries to solve this problem.

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Appendix A

The mean coverage probabilities in Eqs. (14) and (15) are in fact computed on the base of the total probability law [12,13]. Let $B_1, B_2, \dots, B_n$ be mutually exclusive events whose union is the whole sample space of an experiment. Then for the probability

of an event A , $P(A)$, this formula is valid

$$P(A) = \sum_{i=1}^n P(A|B_i)P(B_i)$$

where $P(B_i)$ denotes the probability of B_i and $P(A|B_i)$ denotes the probability of A given B_i (conditional probability). (It means

$$\begin{aligned} \text{MCP} = & \int_{-\infty}^{-1.96u(b)} \left[\Phi\left(\frac{b-\delta+1.96u}{\sigma}\right) - \Phi\left(\frac{b-\delta-1.96u}{\sigma}\right) \right] \cdot f(b) \cdot db + \int_{-1.96u(b)}^{1.96u(b)} \left[\Phi\left(\frac{-\delta+U_-}{\sigma}\right) - \Phi\left(\frac{-\delta-U_+}{\sigma}\right) \right] \\ & \cdot f(b) \cdot db + \int_{+1.96u(b)}^{+\infty} \left[\Phi\left(\frac{b-\delta+1.96u}{\sigma}\right) - \Phi\left(\frac{b-\delta-1.96u}{\sigma}\right) \right] \cdot f(b) \cdot db \end{aligned}$$

that there are constraints that the events B_i must be disjunctive and $\sum_{i=1}^n P(B_i) = 1$.)

Eqs. (14) and (15) are derived for events B that the found experimental bias will be randomly drawn from the whole distribution $N(\delta, u(b))$ (possible values b are from $-\infty$ to ∞) and its value will belong into a given infinitesimal interval from b to $b+\delta b$, event $B(b)$. The probability of such an event equals $f(b) \cdot db$; the total sum of these probabilities computed by integration equals 1. The event A occurs when the uncertainty interval, which is defined by one of the investigated methods about the result for a routine sample when values of u , $u(b)$ and δ are given, encompasses the true value μ . (The method can combine a certain way of uncertainty computation about the results corrected in the case of significant bias and a different way of computation about the results uncorrected in the case of insignificant bias.) The probability of event A can be supposed for the case that the experimental bias found will be one from all possible values of b , i.e. the total probability $P(A)$ —termed mean coverage probability in the text above. However, the coverage probability can also be supposed only for the case that the found value of b belongs to a given infinitesimal interval db —that the event $B(b)$ has occurred. It is the conditional probability $P(A|B(b))$, termed individual in the text above. Then the total probability law can be expressed like this

$$P(A) = \int_{-\infty}^{\infty} P(A|B(b)) \cdot f(b) \cdot db$$

There is a difference between our case and the classic total probability law. The distribution of our events B is a distribution of continuous random variable while in the classic case it is a distribution of discrete random variable. (The calculations of the coverage probabilities of uncertainty intervals in the case when only the β errors occurred can be also explained as an application of the total probability law.)

Appendix B

The complete relationships to compute the mean coverage probabilities can be derived from Eqs. (12), (14) and (15). This

appendix specifies only the relationships, which can be derived from the most extensive formula expressed by Eq. (15). They compute the MCPs when the whole distribution of b is considered. The obtained equations consist of three integrals; two integrals for the uncertainty intervals defined by U about the results corrected with the significant biases are common for all cases, the third one alternates and it is expressed separately for each studied case. The general equation is

The particular expressions of the middle integral (the symbols U_- and U_+ generally denote the left and right sides of the studied type of uncertainty interval) can be obtained by substitution from Eq. (3)— U about uncorrected results

$$\left[\Phi\left(\frac{-\delta+1.96u}{\sigma}\right) - \Phi\left(\frac{-\delta-1.96u}{\sigma}\right) \right] \cdot \int_{-1.96u(b)}^{1.96u(b)} f(b) \cdot db$$

Eq. (9)—SUMU about uncorrected results (also U about corrected results, see Eq. (14))

$$\int_{-1.96u(b)}^{1.96u(b)} \left[\Phi\left(\frac{-\delta+1.96u+b}{\sigma}\right) - \Phi\left(\frac{-\delta-1.96u+b}{\sigma}\right) \right] \cdot f(b) \cdot db$$

Eq. (7)— U (bias) about uncorrected results

$$\int_{-1.96u(b)}^{1.96u(b)} \left[\Phi\left(\frac{-\delta+1.96u+|b|}{\sigma}\right) - \Phi\left(\frac{-\delta-1.96u-|b|}{\sigma}\right) \right] \cdot f(b) \cdot db$$

Eq. (16)— $U_e(95\%)$ about uncorrected results

$$\int_{-1.96u(b)}^{1.96u(b)} \left[\Phi\left(\frac{-\delta+1.96u+E|b|}{\sigma}\right) - \Phi\left(\frac{-\delta-1.96u-E|b|}{\sigma}\right) \right] \cdot f(b) \cdot db$$

The term $f(b) \cdot db$ in these equations has to be replaced by

$$\frac{1}{\sqrt{2\pi}u^2(b)} \exp\left(-\frac{(b-\delta)^2}{2u^2(b)}\right) db$$

The solutions of these integrals were sought numerically by using the trapezoid rule on PC with program Microsoft Excel.

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On-site analysis of volatile nitrosamines in food model systems by solid-phase microextraction coupled to a direct extraction device

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Abstract

Analysis of nitrosamine (NA) standards in a model system was carried out by extraction using SPME coupled to a direct extraction device (DED) and subsequent GC/MS in selected ion monitoring mode. Gelatine (20%, w/v) systems of a NA standard ($10 \mu\text{g L}^{-1}$) were prepared, in order to mimic food protein matrix systems such as meat and meat products, fish and so on. Different SPME fibre coatings were tested. Both divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) and carboxen/polydimethylsiloxane (CAR/PDMS) fibres coupled to DED satisfactorily extracted all nine NA included in the studied standard (EPA 8270 nitrosamines mix, Sigma–Aldrich) from the gelatine system at 25°C without any sample manipulation. Values of reproducibility, linearity and limit of detection for each type of fibre are reported. SPME-DED appears as a rapid, non-destructive technique for preliminary screening of the presence of toxic substances such as NA in solid foods.

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Keywords: SPME; Direct extraction; Nitrosamines; Gelatine

1. Introduction

The concern about the presence of toxic compounds, such as NA, polycyclic aromatic hydrocarbons, polychlorinated biphenyls or dioxins, in foodstuffs has increased in the last decades since several of these families of compounds have turned out to show mutagenic and carcinogenic effects [1,2]. From the analytical point of view, the development of new methods that are capable of analysing this sort of compounds at the levels found in food samples is essential.

The name nitrosamine is related to compounds that present the NNO characteristic group [3]. NA are mutagenic and carcinogenic with action on the liver, kidney and lung [4,5]. Studies about sources of NA for humans have found this type of compounds in different foodstuffs, such as bacon, ham, frankfurters, sausages, cheese or beer [6–9].

The level of tolerance to exposure for humans ranges from 5 to $10 \mu\text{g kg}^{-1}$ of body weight. In many countries a monitoring program was applied to follow the level of volatile *N*-nitrosamines

in food. For example, USA set the level of $10.0 \mu\text{g kg}^{-1}$ of *N*-nitrosopyrrolidine (NPYR) as the limit permitting to introduce products to market [10]. This demands analytical techniques with the lowest possible detection limits [4,7,11].

Current methodologies for analysis of NA in food systems imply a sampling step that deteriorates the quality of solid foods. Moreover, such analytical techniques are laborious, time consuming and need organic solvents. Solid-phase microextraction (SPME) was introduced by Pawliszyn and co-workers [12]. SPME integrates sampling, extraction, concentration and sample introduction in a simple process, and uses no solvent during extraction. Previous studies have demonstrated the feasibility of SPME for analysing tobacco specific nitrosamines [13] and *N*-nitrosodibutylamine and *N*-nitrosodimethylamine in smoked hams [14]. Nevertheless, a portion of sample is also needed for SPME analysis. In previous papers, we have described the use of a small direct extraction device (DED) [15,16], which enables the introduction of the SPME fibre in the core of solid matrixes, allowing the analysis of volatile compounds from solid foods with no or little deterioration of the product. Such a device (Fig. 1) has an internal chamber in which the SPME fibre is exposed. This small chamber has several holes that allow the communication of the internal headspace with the external solid

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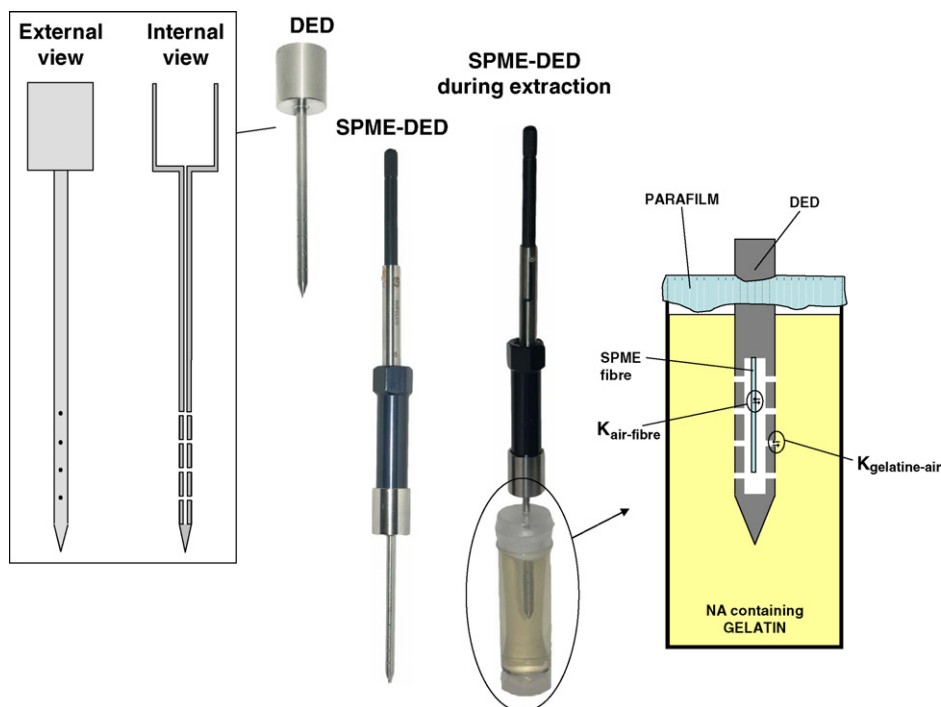


Fig. 1. Scheme of the use of SPME-DED in gelatines mimicking solid foods.

matrix. Extraction of volatile compounds from dry cured hams and paté with only minor or none physical damage of the foodstuffs by SPME couple to DED has been previously reported [15,16].

In the present work, our objective was to study the feasibility of using SPME-DED for extraction of NA from solid matrixes mimicking solid foodstuffs and to compare the efficiency of different types of SPME stationary phases for extraction of these compounds.

2. Experimental

2.1. Reagents and materials

NA standard was supplied by Sigma–Aldrich (EPA 8270 nitrosamines mix, Sigma–Aldrich, St. Louis, USA). This solution contained nine analytes: *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosopiperidine (NPIP), *N*-nitrosodi-*n*-butylamine (NDBA) and *N*-nitrosodiphenylamine (NDPheA).

Methanol was obtained from Scharlau Chemie (Barcelona, Spain) and was HPLC grade. Gelatine (300 bloom) was supplied Sigma–Aldrich (St. Louis, USA).

2.2. Safety considerations

NA are believed to be carcinogenic; please handle accordingly.

2.3. Preparation of NA standards

NA standard stock solution at $10 \mu\text{g mL}^{-1}$ was prepared by diluting $10 \mu\text{L}$ of the NA standard ($2000 \mu\text{g mL}^{-1}$) in 2 mL of methanol. NA standard working gelatines ($0.1\text{--}50 \mu\text{g L}^{-1}$) were prepared by addition of the appropriate volume of the NA stock solution to 30 mL of gelatine solutions (20% in double distilled water) contained in 30 mL polypropylene tubes. Gelatine solutions were prepared by addition of the appropriate amount of gelatine to boiling water and agitation. NA standard stock solution was added when the gelatine was at 60°C . Working gelatines were immediately sealed with polyethylene caps, vortexed for 30 s and stored at $2\text{--}4^\circ\text{C}$ until analysis. Gelatines of each concentration were prepared in duplicate, except for the $10 \mu\text{g L}^{-1}$ ones, used for calculating the reproducibility, which were prepared in quintuplicate. Analysis of NA in gelatines by SPME-DED was carried out within the next 7 days of preparation.

2.4. Preparation of meat patties spiked with NA

Meat patties were prepared with 68% of chicken meat, 30% of distilled water and 3% of sodium chloride. These ingredients were minced until achieving a homogeneous paste. Polypropylene tubes with polyethylene caps were filled with 30 mL of this mix, and subsequently NA standard stock solution was added to obtain the appropriate final concentration of NA ($0.1\text{--}50 \mu\text{g L}^{-1}$). Gelling of meat proteins was achieved by cooking the tubes at 70°C during 15 min . Meat gels were stored at $2\text{--}4^\circ\text{C}$ until analysis.

Table 1

Volatile NA included in the studied standard, together with the selected ion for detection in MS SIM mode, the retention time for each analyte and their molecular weight

Nitrosamines	<i>m/z</i>	Ret. time (min)	MW	log <i>K</i> _{ow}	Water solubility (mg L ⁻¹)	Vapor pressure (mmHg)
<i>N</i> -nitrosodimethylamine (NDMA)	74	8.27	74	-0.57	1000000	2.7
<i>N</i> -nitrosomethylethylamine (NMEA)	88	12.02	88	0.04	300000	2.09
<i>N</i> -nitrosodiethylamine (NDEA)	102	17.24	102	0.48	106000	0.86
<i>N</i> -nitrosopyrrolidine (NPYR)	100	36.29	100	-0.19	1000000	0.06
<i>N</i> -nitrosomorpholine (NMOR)	116	36.40	116	-0.44	861527.5	0.036
<i>N</i> -nitrosodi- <i>n</i> -propylamine (NDPA)	130	36.48	130	1.36	13000	0.389
<i>N</i> -nitrosopiperidine (NPIP)	114	37.45	114	0.36	76480	0.14
<i>N</i> -nitrosodi- <i>n</i> -butylamine (NDBA)	84	39.66	158	2.63	1270	0.0469
<i>N</i> -nitrosodiphenylamine (NDPheA)	169	43.04	198	3.13	35	0.0000697

Polarity expressed as log *K* octanol–water (log *K*_{ow}), water solubility in mg L⁻¹ at 25 °C and vapour pressure at 25 °C for each NA from available literature [38] are also included.

2.5. SPME-DED extraction of NA

NA were directly extracted from gelatines and meat patties by inserting the DED into the core of the gelatine or meat gel and subsequent introduction of the SPME fibre into the DED following the procedure previously described by Ruiz et al. [15]. In order to control the temperature, tubes containing the gelatines or meat patties were kept in a thermostatised water bath at 25 °C. Tubes containing the gelatine systems and meat patties were conditioned at this temperature during 15 min previous to extraction. A diagram of the use of SPME-DED is shown in Fig. 1.

Different SPME stationary phases were tested for extraction of NA from gelatine systems: CAR/PDMS (85 µm), DVB/CAR/PDMS (50 µm/30 µm) and DVB/PDMS (65 µm). Selection of the fibres to be studied was based on available scientific literature and Supelco recommendations. Once carried out the extraction, the SPME fibre was pulled out from the DED and analytes were subsequently desorbed onto the gas chromatography column by inserting the fibre in the injection port, that was set at the appropriate temperature (280 °C for CAR/PDMS, 270 °C for DVB/CAR/PDMS and 260 °C for DVB/PDMS). For extraction of NA from meat gels CAR/PDMS SPME fibres (which had been previously selected based on the results obtained with the gelatine systems) were used.

2.6. Gas chromatography–mass spectrometry

Analyses were performed using an Agilent 6890 series gas chromatograph (Agilent, Avondale, USA) coupled to a mass selective detector (Agilent 5973, Agilent, Avondale, USA). Analytes were separated using a 5% phenyl–methyl silicone (HP-5) bonded phase fused silica capillary column (Hewlett-Packard, 50 m × 0.32 mm i.d., film thickness 1.05 µm), operating at 6 psi of column head pressure, resulting in a flow of 1.3 ml min⁻¹ at 40 °C. The SPME fibres were desorbed and maintained in the injection port at the appropriate temperature during the whole chromatographic run. The injection port was in splitless mode. The temperature program for NA was 40 °C of initial temperature, raised to 80 °C at a rate of 5 °C min⁻¹, maintained at 80 °C for 27 min, and then raised to 250 °C at a rate of 6 °C min⁻¹, and held for six additional minutes.

The transfer line to the mass spectrometer (MS) was maintained at 280 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1756 V and collecting data at a rate of 1 scan s⁻¹. Detection of compounds was carried out in selected ion monitoring (SIM) mode. Selected ions chosen for each compound and retention times are shown in Table 1. In order to establish the retention time for each analyte, stock solutions were previously injected under the same chromatographic conditions as those for detection of NA in SIM mode but using the mass selective detector in scan mode over the *m/z* range of 30–500.

3. Results and discussion

3.1. Extraction of NA from gelatine model systems

As indicated in the introduction, the main goals of our study were to check the feasibility of using SPME-DED for analysing NA in solid samples, and to select the SPME fibre coating with a better performance for such purpose. Given that it would be very difficult to achieve a homogeneous concentration of NA in those real food samples for which SPME-DED was developed (mainly whole piece meat and fish products), we tested the analytical method on model systems made with gelatine, which allows a correct dilution of NA while the gelatine is still liquid, while showing similar characteristics to muscle foods when it is solid, since it is quite elastic and the matrix is constituted by proteins. Of course, some other components of the foodstuff could influence extraction (i.e. fat, salt, additives, ...) [17] but as a first and general approach we considered gelatine as a good model system, better than other gels mainly constituted by carbohydrates.

Fig. 1 shows the DED, the way in which it is coupled to the SPME, and how SPME-DED is used to extract NA from tubes containing gelatines. DED basically consists in a hollow and narrow tube with holes in the area in which the SPME fibre is exposed. These holes permit the communication between the headspace inside the device and the surface of the solid sample, enabling the extraction of volatile substances with the SPME fibre. The end of the device is pointed in order to facilitate its penetration in the solid.

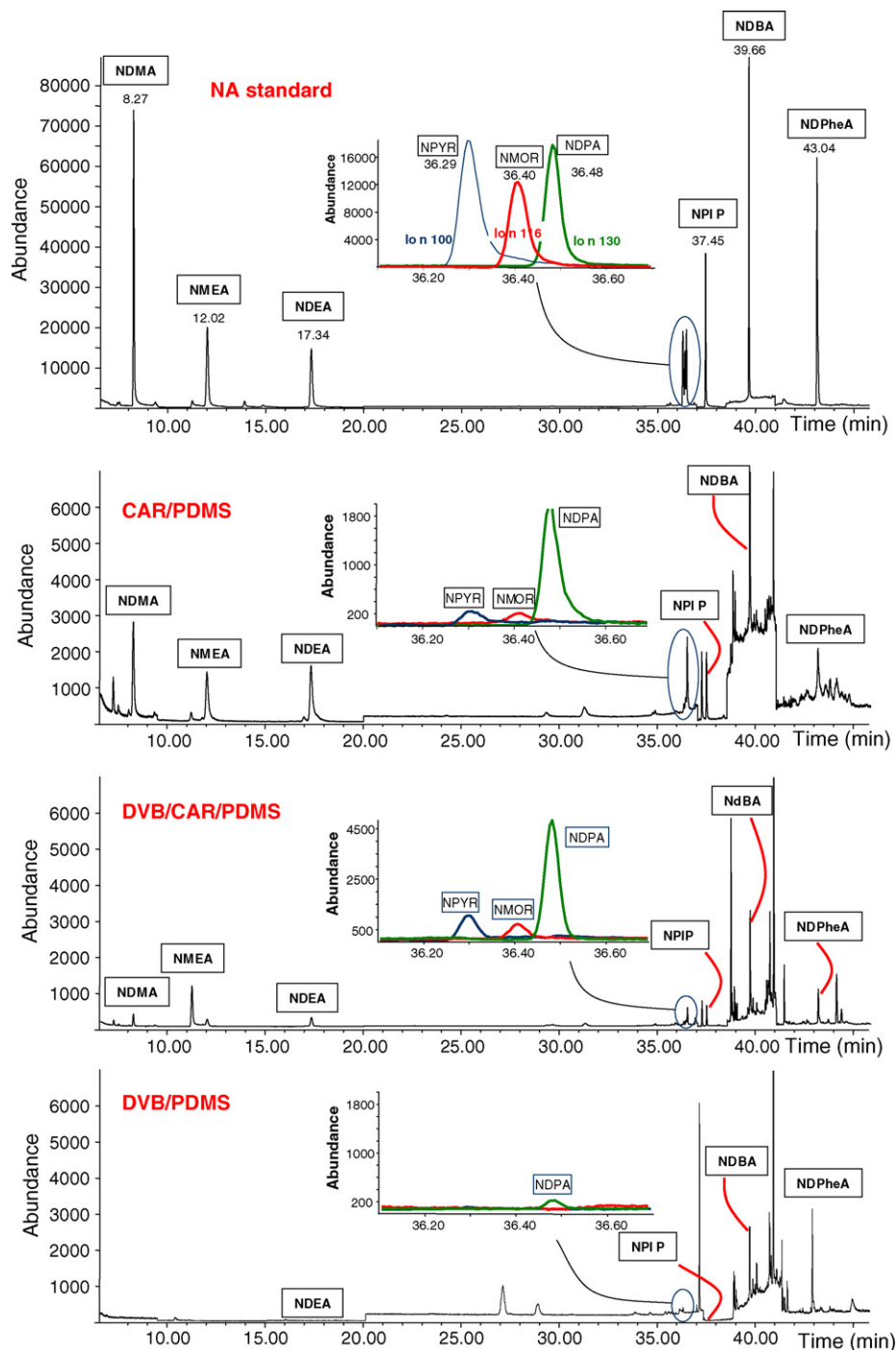


Fig. 2. SIM chromatograms of the NA standard directly injected in the GC (NA standard) and extracted using SPME-DED from gelatines containing $10 \mu\text{g L}^{-1}$ of each NA with CAR/PDMS, DVB/CAR/PDMS and DVB/PDMS SPME fibres. The inner graphics show the selected ions for detection of NA eluted between 36 and 38 min.

Fig. 2 shows the chromatogram of the stock solution of NA directly injected in the GC–MS and the chromatograms obtained using different SPME stationary phases for SPME-DED extraction of NA at $10 \mu\text{g L}^{-1}$ from solid gelatines. Chromatographic areas obtained after extraction of NA at $10 \mu\text{g L}^{-1}$ from solid gelatines using SPME-DED are shown in Fig. 3. Both CAR/PDMS and DVB/CAR/PDMS SPME phases satisfactorily extracted all the NA in the standard working gelatines using SPME-DED, whereas DVB/PDMS SPME coated fibres only

allowed the extraction of five out of nine NA (NDEA, NDPA, NPIP, NDPA and NDPheA). Therefore, SPME-DED coupled to the appropriate SPME fibre appears as a potential extraction technique for NA analysis in solid samples, since allows the satisfactory detection of NA at levels similar to those usually reported in foodstuffs such as bacon, cheese or fish products [18,19]. Moreover, extraction using SPME-DED lasted 60 min, no heating of the sample was required (25°C) and no sample manipulation was necessary. The latter feature means that the

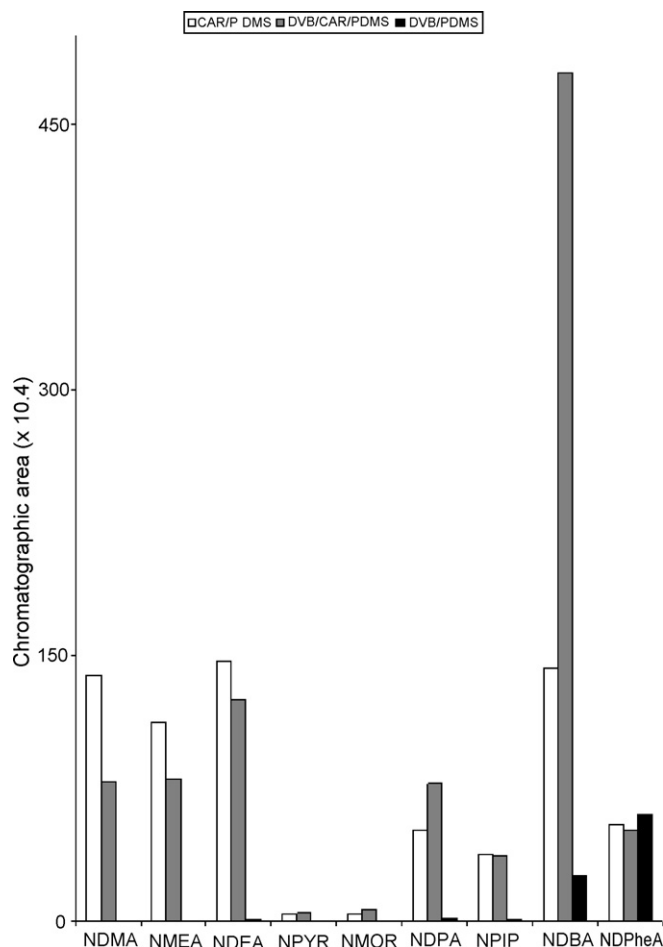


Fig. 3. Effect of different SPME fibre coating on the extraction efficiencies for NA ($10 \mu\text{g L}^{-1}$) from solid gelatines by SPME-DED (extraction time 60 min).

analysis of solid foodstuffs would scarcely (or not at all) damage their quality. In order to confirm the feasibility of SPME-DED for extracting NA, further experiments on real foodstuffs are presently being carried out at our laboratory.

NA contained in the studied EPA standard show molecular weights between 74 (NDMA) and 198 (NDPheA) and different polarities and volatilities, as shown in Table 1. Fig. 3 shows the chromatographic areas obtained for individual NA using SPME-DED with the three studied coatings. The better ability of stationary SPME phases containing carboxen for extracting a wide range of compounds, especially those showing low molecular weight and high volatility, have been previously highlighted by different authors [19,20]. This is not strange since carboxen containing phases were designed to increase the extraction of low molecular weight compounds [21]. Carboxen is a porous and adsorbent carbon based material (Carboxen 1006), showing a surface of $1200 \text{ m}^2 \text{ g}^{-1}$ [22]. This material contains both macropores (more than $500 \text{ \AA}$ diameter) and micro-(<20 $\text{\AA}$) and mesopores (between 20 and $500 \text{ \AA}$ diameter), allowing the extraction of a wide range of molecular weight compounds [22]. However, the higher proportion of micropores than in other porous phases, such as divinylbenzene, makes carboxen particularly efficient for extraction of small molecules [22].

Table 2

Reproducibility (R.S.D.%) for studied NA extracted from solid gelatines by SPME-DED using different types of PSME fibre coatings

	CAR/PDMS	DVB/CAR/PDMS	DVB/PDMS
NDMA	22.95	19.71	n.d.
NMEA	27.18	17.16	n.d.
NDEA	24.51	20.40	16.75
NPYR	66.52	43.23	n.d.
NMOR	59.60	21.42	n.d.
NDPA	21.11	15.87	14.30
NPIP	39.31	24.49	39.27
NDBA	17.23	91.03	27.99
NDPheA	27.59	30.22	38.03

DVB is also a porous material that was included in the stationary phases of SPME together with PDMS to increase the retention of polar analytes [23]. However, this material is not as efficient as carboxen at extracting low molecular weight polar compounds, since its proportion of micropores is lower. DVB/PDMS fibre allowed the extraction of those NA with lower polarities (those with $\log K_{ow} > 0.36$) whereas did not extract more polar analytes. Moreover, the chromatographic area of the NDPheA, which was the heavier studied analyte and the one showing the highest $\log K_{ow}$, was greater using DVB/PDMS fibre than using the other two fibres containing carboxen. A previous study [24] also found a better ability of DVB/PDMS SPME phases for extracting less polar compounds from coffee.

3.2. Reproducibility

Reproducibility of the method expressed as relative standard deviation (R.S.D.) for each studied NA using each type of SPME coating is shown in Table 2. Values ranged from 14.3 to 91.03%. Reproducibility was worse than that reported for other methods, which usually are around 10% or less [13,14,25], although others have obtained values around 15% for some NA [26]. This poor reproducibility could be ascribed partly to the intrinsically higher variability of SPME compare to other methods, especially when the equilibrium is not reached [27]. In this study, extraction was carried out for 60 min at 25°C . This time was not enough for the equilibrium to be achieved, since in some samples extracted for 24 h at 25°C the chromatographic areas for all NA were at least tenfold higher. In non-equilibrium situations, small variations in extraction conditions strongly increase variability [27,28]. In addition, in complex matrixes as the solid gelatine system is, analytes can be trapped in the cells or interact with polar groups from the protein, hindering the achievement of the final equilibrium [29]. In fact, Baines and Morris [30] observed that polymer networks in viscous systems obstructed the diffusion of small molecules from the depth to the surface of the gel. Other studies have also found higher coefficients of variation for solid matrixes than for liquids [19,31], since the latter are more homogeneous and stirring is possible. In our gelatine systems two different equilibria for each analyte should be achieved: the one between the solid matrix and the headspace in the internal chamber of the DED and that between the headspace and the fibre coating (Fig. 1). Moreover, there were a dead volume

between the surface of the gelatine and the parafilm limiting the system. Analytes should therefore equilibrate between the two headspaces and the matrix, leading to a complex situation for the whole equilibria to be achieved.

DVB/PDMS coated fibres showed slightly better reproducibility using SPME-DED for extracting studied NA, CAR/PDMS and CAR/DVB/PDMS fibres showing overall higher RSD values. Several authors have previously reported worse reproducibility for SPME fibers containing carboxen for extraction of different analytes [19,32,33]. It has been hypothesised that this could be ascribed to condensation of compounds into carboxen due to the small diameter of its pores [34].

3.3. Linearity

The linearity of the SPME-DED method for analysing NA from solid gelatines was evaluated by plotting the calibration curves of obtained chromatographic area versus the concentration of the analytes in the gelatines with concentrations ranging from 1 to 50 $\mu\text{g L}^{-1}$. Most NA showed acceptable regression coefficients above 0.95 with all fibres. However, some (four for CAR/PDMS fibres and two for DVB/CAR/PDMS ones) showed R^2 below 0.9. This poorer linearity in the studied range could be partly due to the low reproducibility with fibres containing carboxen [35].

3.4. Limit of detection

The limits of detection (LOD), defined as the concentration of analytes in gelatines, which caused a peak with a signal-to-noise ratio greater than 10, were also determined and are showed in Table 3. LOD ranged from 0.011 to 0.357 $\mu\text{g L}^{-1}$ for CAR/PDMS fibres, from 0.015 to 0.983 $\mu\text{g L}^{-1}$ for DVB/CAR/PDMS and from 0.142 to 9.539 $\mu\text{g L}^{-1}$ for DVB/PDMS. Reported values for most NA in the scientific literature are often above obtained LOD for SPME-DED. For example, levels between 0.08 and 0.93 $\mu\text{g kg}^{-1}$ of NPYR have been reported for whey containing food products [6]. In Gruyere cheese reported values for NDEA ranged from 0.04 to 0.38 $\mu\text{g kg}^{-1}$ [10], while average values of NDMA reported for ham, bacon and fermented fish were 0.79, 0.90 and 7.95 $\mu\text{g kg}^{-1}$, respectively [36]. In many countries a mon-

itoring program was applied to follow the level of volatile *N*-nitrosamines in food. For example, USA set the level of 10.0 $\mu\text{g kg}^{-1}$ of *N*-nitrosopyrrolidine as the limit permitting to introduce products to market [10]. Therefore, using the appropriate SPME fibre, SPME-DED coupled to GC-MS could satisfactorily detect NA at levels even below those set by health authorities in solid foods. Moreover, the LOD of this method is lower than that reported for several NA using other analytical procedures, such as SPE followed by GC-TEA (LOD between 0.3 and 3.076 $\mu\text{g L}^{-1}$) [37]. Using SPME, Sen et al. [14] found higher LOD for NA in hams, even though a TEA detector was used, which is supposed to be more sensitive than MS for NA. The lower LOD found in our study is probably not due to the use of the DED, but to simplicity of the solid matrix compared to real hams, in which salt, fat and other components might interfere with the extraction.

The small volume of the chamber inside the DED will also contribute to the sensitivity of the method. In headspace SPME, the lower the ratio between the volumes of the headspace and of the sample, the higher the sensitivity [27]. In our experiment, the volume of the headspace is that of the chamber inside the DED in which the SPME fibre is exposed, which in our prototype was 0.09 cm^3 . The sample volume is that of the gelatine (30 mL), giving a very small ratio between both volumes of 0.003, which surely contributes to the good sensitivity of the method.

DVB/PDMS coated fibres showed higher LOD for all NA, except for NDPheA, than CAR/PDMS and DVB/CAR/PDMS ones. In fact, for the latter two types, LOD for all NA were below 1 $\mu\text{g L}^{-1}$. These latter two fibres showed similar values of LOD for most NA, except for NDPheA, which was considerably higher for DVB/CAR/PDMS ones. These lower LOD for carboxen containing fibres were not unexpected, given the great capacity of this porous polymer discussed before. In fact, carboxen containing coated SPME fibres have been highlighted as the most sensitive ones for volatile compounds with medium polarity [21].

3.5. Analysis of real meat patties

Meat patties spiked with NA were also analysed by SPME-DED using a PDMS/DVB SPME fibre. This stationary phase was selected due its overall best performance in the study carried

Table 3
Limits of detection (LOD) and regression coefficient (R^2) of the linear equation for studied NA extracted from solid gelatines by SPME-DED with different SPME fibre coatings

	CAR/PDMS		DVB/CAR/PDMS		DVB/PDMS	
	LOD ($\mu\text{g L}^{-1}$)	R^2	LOD ($\mu\text{g L}^{-1}$)	R^2	LOD ($\mu\text{g L}^{-1}$)	R^2
NDMA	0.097	0.9896	0.115	0.9986	n.d.	n.d.
NMEA	0.028	0.9959	0.024	0.9981	9.539	0.9644
NDEA	0.011	0.9968	0.015	0.9972	1.205	0.9767
NPYR	0.206	0.8751	0.335	0.8998	n.d.	n.d.
NMOR	0.357	0.8743	0.118	0.7992	n.d.	n.d.
NDPA	0.075	0.9953	0.016	0.9943	1.594	0.9922
NPIP	0.080	0.9519	0.153	0.9848	3.076	0.9646
NDBA	0.042	0.8803	0.039	0.9936	0.258	0.9816
NDPheA	0.149	0.7998	0.983	0.9659	0.142	0.9679

out in gelatine systems. As for gelatines, all NA were detected at all concentration studied in meat patties, which reinforce the potential use of SPME-DED for preliminary and rapid detection of such toxic compounds in foodstuffs. However, reproducibility values were worse for all studied NA (R.S.D. between 17.11 and 70.32), except for NPYR, NMOR and NPIP, which showed similar values to those obtained for gelatine systems. This in turn led to poorer linearity values, showing even no linear response for NMOR and NDPA (R^2 of 0.17 and 0.15, respectively). This was most likely due to the higher complexity of the matrix. Meat patties were constituted by meat, water and sodium chloride. As a consequence, different types of proteins, lipids, salts, nucleotides and so on were constituents of the matrix. This would involve much more complex relationships between studied analytes and the solid matrix, interfering in the extraction process. Moreover, the heating step needed for obtaining a meat gel could have also contributed to this variability, since some of the NA are not heat stable. Therefore, quantitative results using for NA in meat solid samples using SPME-DED are not fully reliable. However, its use for qualitative assessment of the presence of NA in solid foodstuffs without sampling is promising.

4. Conclusions

It can be concluded that SPME-DED satisfactorily extracts volatile NA from gelatine at lower levels than those set by health authorities. It would allow determining NA from solid matrixes avoiding sampling and without any laboratorial manipulation. Therefore, it could be used as a screening method for a preliminary evaluation of the presence of such toxic compounds in solid foodstuffs without damaging the product. Nevertheless, the presence of other components in the matrix, such as fat or salt, and the effect of different factors affecting the extraction should be studied.

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On-line sample treatment and FT-IR determination of doxylamine succinate in pharmaceuticals

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Abstract

A low solvent consumption method for Fourier transform infrared spectroscopy (FT-IR) determination of doxylamine succinate in pharmaceuticals has been developed. The analyte was continuously and selectively extracted with a 13% (v/v) ethanol:chloroform solvent mixture, recirculating the solvent through the sample and monitoring the process by FT-IR. Doxylamine succinate was determined by on-line standard addition measuring the peak area in the regions 1730–1710 and 1485–1462 cm^{-1} corrected with a two-point baseline established between 2000 and 1800 cm^{-1} . This new method implies low volumes of chloroformic solvent mixture, only 2.6 mL per sample, in front of classical batch FT-IR methods, improving analytical efficiency and reducing waste generation. The on-line extraction and standard addition determination of doxylamine succinate allowed a throughput of 10 h^{-1} .

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Keywords: FT-IR; On-line treatment; Doxylamine succinate; Pharmaceuticals

1. Introduction

Halogenated compounds had been identified as ozone-layer depleting agents, and both their toxic properties for humans and environmental damage derived by their use, recommend a progressive reduction of their consumption restricted by recent laws developed in the frame of the Montreal Protocol [1].

Fourier transform infrared spectrometry (FT-IR) is an analytical technique that offers excellent possibilities for quantitative analysis, based on the selective signals obtained from the characteristic spectra, being widely employed in application laboratories [2]. Transmittance IR measurements in liquid phase require the use of solvents which present a low absorption in this spectral region, halogenated hydrocarbons and carbon disulfide (CS_2) being the main available solvents for these purposes [3].

The introduction of flow injection analysis strategies [4,5] has increased the potential of FT-IR spectrometry as a tool for quantitative analysis and the hyphenation with solid phase extraction [6] and the on-line sample extraction [7] allows us the determination of analytes at the levels of mg L^{-1} and minimizes

the sample manipulation, also reducing the consumption of solvent.

On the other hand, useful methods in quality control must be as fast and low cost as possible, the on-line extraction being a good compromise between productive parameters and a reduced sample handling.

Most active principles in pharmaceuticals are determined by UV–vis spectrometry, which suffers from a lot of interferences, and HPLC, which are time consuming and require large amounts of organic solvents, and in front of these commonly employed methodologies. Excellent results could be obtained by flow analysis and FT-IR. Precedents on that are the determination of octyl succinate [8], ibuprofen [9], dimenhydrinate [10], ketoprofen [11], paracetamol [12–14] and caffeine and acetylsalicylic acid [15]. However, none of these previous studies involved the on-line sample treatment.

Doxylamine succinate (phenyl 2-pyridylmethyl-beta-*N,N*-dimethylaminoethyl ether succinate 1:1) is an antihistaminic drug widely used for treating nausea during pregnancy and it is present in counter-cold and sedative pharmaceuticals [16]. Some chromatographic methods have been proposed for its determination at trace levels [17–19] besides mass spectrometry [20], capillary electrophoresis [21] and UV spectrometry in micellar medium [22].

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The aim of this study has been the development of a mechanized procedure for the determination of doxylamine succinate in pharmaceutical tablets, using FT-IR measurements after on-line sample extraction. The method uses a closed-flow system and involves the consumption of a minimum volume of chloroform per sample, thus contributing to the reduction of chlorinated solvent use and the development of a green analytical chemistry.

2. Experimental

2.1. Apparatus

The FT-IR measurements were carried out using a Nicolet Magna 750 spectrometer (Madison, WI, USA), equipped with a global IR source, a stabilized temperature deuterated triglycine sulphate detector and a Ge/KBr beamsplitter, and using a microflow cell (Graseby-Specac, Orpington, UK) with ZnSe and CaF₂ windows and an optical pathlength of 0.11 mm.

FT-IR spectra and data treatment were recorded and processed with the Omnic v2.1 software from Nicolet.

A UV–vis diode array spectrometer model HP8452A from Hewlett-Packard (Waldbronn, Germany) was used for obtaining UV–vis spectra of samples. UV–vis absorbance spectra were recorded in the range between 190 and 280 nm, using a 10 mm quartz cell from Hellma (Jamaica, NY, USA).

A Medifugue centrifuge (Heraeus Sepatech, Germany) and a J.P. Selecta ultrasound water bath (Barcelona, Spain) were employed for the preparation of samples in the batch procedure. A Hamilton microsyringe of 50 μ L was used for the injection of standards in the on-line standard addition.

2.2. Reagents

Chloroform stabilized with 150 ppm of amylene (Scharlau, Barcelona) and absolute ethanol Multisolvant grade from Scharlau, dried with anhydrous sodium sulphate r.g. (Baker, Deventer, Holland) were employed in order to obtain a 13% (v/v) ethanol:chloroform solvent mixture for sample treatment and FT-IR measurements.

HCl 0.1 M prepared from concentrated analytical grade HCl 36% (v/v) from Scharlab (Barcelona, Spain) and ultrapure deionized water, with a resistivity of 18.2 M Ω obtained from a Millipore MilliQ system (Molsheim, France) were used for the UV reference procedure.

Doxylamine succinate standard with a purity higher than 99% was acquired from Sigma–Aldrich (Steinheim, Germany). DormidinaTM samples produced by Laboratorios Esteve (Barcelona, Spain) were obtained from Spanish market. Tablets containing 25 mg of doxylamine succinate were homogenized appropriately and kept out from light in a desiccator before their analysis.

2.3. On-line extraction manifold

Fig. 1 shows the manifold employed for the on-line extraction and FT-IR determination of doxylamine succinate in pharmaceu-

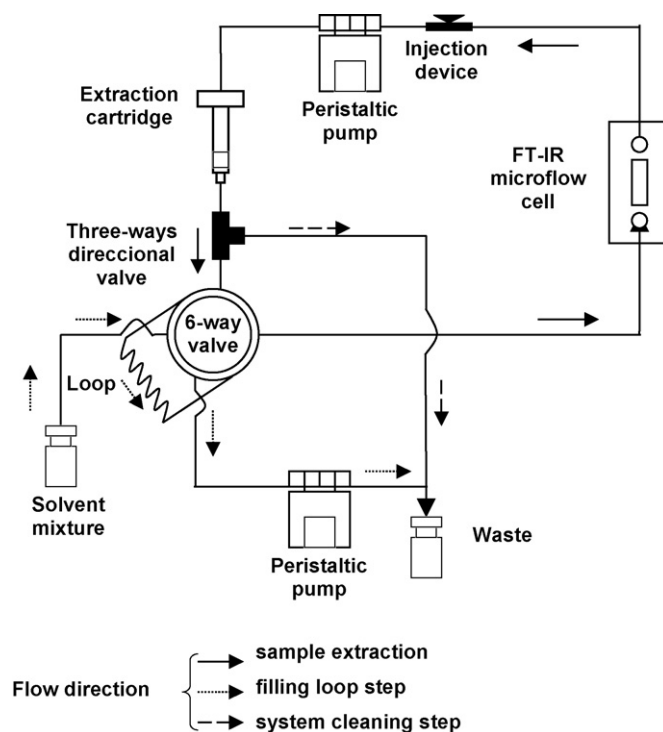


Fig. 1. Manifold employed in the on-line extraction and determination of doxylamine succinate.

ticals. The assembly consisted of a main circuit that includes a Gilson Minipuls 2 (Villiers-le Bel, France) peristaltic pump with a Viton (isoversinic) tube of 1.5 mm i.d. to impel the solvent, an interchangeable syringe cartridge for sample extraction, a glass injection device for standard introduction and a microflow FT-IR cell. The connection between elements was made with PTFE tubes with 0.8 mm i.d. A Rheodyne 5041 six-way valve (Cotati, CA, USA), equipped with a loop of 2.58 mL volume, was used for the introduction of the carrier and the extraction solvent inside the manifold. The solvent loop was filled using a second peristaltic pump equipped with a Viton tube of 3 mm i.d. A three-way directional valve was employed for cleaning the system between the analyses of different samples. For on-line standard addition discrete volumes of a doxylamine succinate stock solution were injected in the system using a 50 μ L microsyringe through a home-made injector device with a silicone membrane (see Fig. 1 for set-up details).

2.4. Batch FT-IR procedure

Fifty milligrams of sample was accurately weighed inside a glass vial and 5 mL of a 13% (v/v) ethanol:chloroform mixture was added. The mixture was shaken for a few seconds and clarified by centrifugation at 3500 rpm for 5 min. The liquid phase was filtered through a 0.45 μ m syringe membrane filter and introduced in the FT-IR measurement cell by aspiration with the peristaltic pump. The FT-IR spectrum was recorded between 4000 and 600 cm^{-1} with a nominal resolution of 8 cm^{-1} and accumulating 10 scans per spectrum versus a background of the cell filled with the same solvent. To calculate the doxylamine

succinate concentration in each sample, the sum of the peak areas between 1710–1730 and 1485–1462 cm^{-1} , both corrected with a two-point baseline established between 2000 and 1800 cm^{-1} , was interpolated in an external calibration of doxylamine succinate prepared by direct dissolution of standards in the 13% (v/v) ethanol:chloroform mixture and measured in the same way as the samples.

2.5. On-line FT-IR procedure

Fifty milligrams of sample was accurately weighed in a 3 mL internal volume cartridge and inserted in the manifold. The 2.58 mL loop was loaded with the 13% (v/v) ethanol:chloroform solution using the six-way valve, being opened the three-way directional valve. Then the solvent was injected into the sample extraction cartridge, closing the directional valve at this moment. Finally, the six-way valve was disposed in the initial position and the directional valve opened, being recirculated the extract obtained from solvent action through the solid sample contained in the cartridge. This extract containing doxylamine succinate, was recirculated with a flow rate of 8.5 mL min^{-1} through the FT-IR spectrometer measurement cell and sample cartridge until the end of the extraction process, when the steady state was reached. After that the extract was conducted with the directional valve to the waste recipient. During the recirculation of the solvent mixture through the extraction cartridge the FT-IR spectra between 4000 and 600 cm^{-1} are continuously recorded as a function of time, with a nominal resolution of 8 cm^{-1} and accumulating four scans per spectrum, until a constant signal is obtained. The recirculation period was established from the chemigram obtained for peak area between 1730 and 1710 cm^{-1} (corrected with an horizontal baseline at 1800 cm^{-1}).

The sample signal was measured from the final spectrum obtained by the co-addition of the spectra for a time interval of 0.038 min after 1.7 min of the beginning of the data acquisition. The sum of peak area between 1730–1710 and 1485–1462 cm^{-1} , both corrected with a two-point baseline established between 2000 and 1800 cm^{-1} , was employed for calculations.

So, on using the aforementioned set-up, it is possible to follow continuously the evolution of the extraction process, being the chemigram directly related with doxylamine succinate concentration. External calibration was obtained by injecting small volumes (25 μL) of a 50 mg mL^{-1} stock doxylamine succinate solution in the manifold through the glass injector silicon membrane and using the corresponding co-added spectra in the same measurement conditions as the sample.

On-line standard addition was carried out by injecting successive 25 μL aliquots of the stock solution to the extracted sample solution after to reach the steady state of the extraction process. The content of doxylamine succinate in the sample was obtained by extrapolation from the standard addition line established from each addition.

2.6. UV-vis reference procedure

0.1500 g of sample was accurately weighed and 65 mL of HCl 0.1 M solution was added. The mixture was shaken for

10 min and transferred to a 100 mL volumetric flask and the volume completed with HCl 0.1 M. The suspension was filtered through a Whatman 42 paper and the first 20 mL of the solution discarded being used the rest. One milliliter of sample clear solution was diluted to a final volume of 10 mL with HCl 0.1 M. The UV-vis spectrum was recorded between 190 and 280 nm in front of a background of HCl 0.1 M. Finally, doxylamine succinate concentration was determined by interpolating the sample absorbance at 262 nm in a calibration line of standard solutions of doxylamine succinate directly prepared in HCl 0.1 M and measured in the same conditions as samples [23].

3. Results and discussion

3.1. FT-IR spectral characteristics of doxylamine succinate

Doxylamine succinate is highly soluble in water and has also a good solubility in ethanol and chloroform. Fig. 2 shows the FT-IR spectra corresponding to a doxylamine succinate standard and an extract of a sample, both prepared in a 13% (v/v) ethanol:chloroform mixture. The background was collected in the same conditions as the sample and standard, and corresponds to the microflow cell filled with the solvent mixture. As can be seen, the main bands present in the spectrum of doxylamine succinate standard were similar to the bands appearing in the extract of the sample, suggesting the use of these bands for the direct determination of doxylamine succinate directly extracted from the samples without any previous treatment. The most intense band appears at 1710 cm^{-1} and corresponds to the succinate carboxylate group absorption. Other important bands are present at 1580, 1470, 1440, 1375 and 1340 cm^{-1} that correspond to vibration modes of monosubstituted benzene, $-\text{CH}_3$ deformation asymmetric, C–OH in-plane bend and $-\text{CH}_3$ symmetric bending, respectively [24].

Different bands and modes of measurement were studied including peak area and peak height with different baseline corrections (see Table 1). Finally, the selected measurement criteria was the use of the sum of the peak area between 1710 and 1730 cm^{-1} and the peak area between 1462 and 1485 cm^{-1} ,

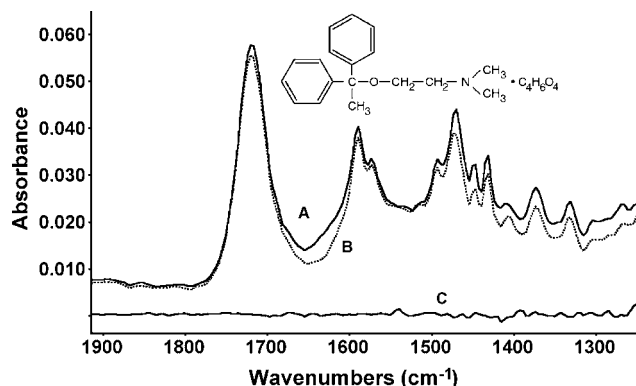


Fig. 2. FT-IR spectra of (A) 4.24 mg mL^{-1} doxylamine succinate standard prepared in 13% (v/v) ethanol:chloroform mixture, (B) sample extract in the same solvent mixture corresponding to 4.51 mg mL^{-1} of doxylamine succinate, and (C) solvent blank. Measurement conditions: 10 accumulated scans per spectrum, 8 cm^{-1} nominal resolution, background of 13% (v/v) ethanol:chloroform.

Table 1

Calibration lines obtained for “in batch” FT-IR determination of doxylamine succinate using different criteria

Measurement criterion	Baseline (cm ⁻¹)	Calibration line, $y = (a \pm s_a)C + (b \pm s_b)$	R ²
Peak height 1720 cm ⁻¹	1800	$y = (0.01080 \pm 0.00003)C + (0.00017 \pm 0.00004)$	0.99991
Peak height 1720 cm ⁻¹	1650	$y = (0.00908 \pm 0.00006)C + (0.00050 \pm 0.00011)$	0.9992
Peak area 1710–1730 cm ⁻¹	1800	$y = (0.2288 \pm 0.0006)C - (0.0035 \pm 0.0010)$	0.99990
Peak area 1455–1488 cm ⁻¹	1800	$y = (0.325 \pm 0.002)C - (0.014 \pm 0.003)$	0.9994
Peak area 1580–1596 cm ⁻¹	1800	$y = (0.1302 \pm 0.0008)C - (0.0047 \pm 0.0013)$	0.9994
Peak area 1710–1730 cm ⁻¹	1750	$y = (0.1621 \pm 0.0012)C - (0.003 \pm 0.002)$	0.9991
Peak area 1710–1730 cm ⁻¹	1850	$y = (0.2209 \pm 0.0005)C - (0.0079 \pm 0.0009)$	0.99991
Peak area 1077–1110 cm ⁻¹	1850	$y = (0.1635 \pm 0.0017)C - (0.012 \pm 0.003)$	0.998
Peak area 1419–1439 cm ⁻¹	1800	$y = (0.1104 \pm 0.0012)C - (0.000 \pm 0.002)$	0.998
Peak area 1710–1730 + 1485–1462 cm ⁻¹	1800–2000	$y = (0.443 \pm 0.003)C - (0.001 \pm 0.003)$	0.9991

both corrected with a baseline established between 1800 and 2000 cm⁻¹ which provided the best sensitivity.

3.2. Batch FT-IR determination of doxylamine succinate

Previously to the development of the on-line method it was necessary to study doxylamine succinate extraction process in order to design correctly the corresponding manifold in which the experimental conditions could be reachable.

Chloroform presents a relative high transparency in the middle IR region and because of that it was selected as a solvent for the extraction of doxylamine succinate from pharmaceuticals. Unfortunately, it was verified that the extraction was not complete. So, we evaluated the effect of incorporating ethanol to chloroform in order to increase the polarity of the extraction solvent. Increasing amounts of ethanol were considered for the extraction of doxylamine succinate being found that for a proportion of ethanol equal to or higher than 13% (v/v) the extraction was complete (see Fig. 3). So, a 13% mixture of ethanol:chloroform was selected for following experiments.

Studies about the effect of shaking time showed that mechanical or ultrasonic shaking is unnecessary for extracting doxylamine succinate completely from pharmaceuticals using the above-mentioned solvent mixture. So, on-line extraction is possible with minimum experimental requirements.

Nominal resolution and number of accumulated scans per spectrum were also evaluated, selecting those conditions which

Table 2

FT-IR spectrometer measurement conditions used in the batch and on-line methods for the determination of doxylamine succinate in pharmaceuticals

Spectrometer parameter	Value
Aperture (%)	100
Gain	8
Mirror velocity (cm s ⁻¹)	0.6329
Number of scans per spectrum	Batch = 10 On-line = 4 (chemigram data spacing = 0.038 min)
Resolution (cm ⁻¹)	8
Spectral window (cm ⁻¹)	4000–600
Apodization	Happ-Genzel
Zero filling factor	0

resulted as a compromise between a good precision and high signal-to-noise ratio with the minimum consumption of time (see Table 2).

In the selected conditions three samples of different batch of a pharmaceutical containing 25 mg doxylamine succinate per unit were analyzed. Results are shown in Table 3 and it can be seen they are statistically comparable with those obtained by using the reference UV–vis spectrometry procedure.

In order to evaluate the accuracy of the FT-IR procedure a recovery study was carried out, having found quantitative results for added amounts from 2.6 to 9.9 mg of doxylamine succinate.

Both, recovery studies and sample analysis, indicated that the extraction of the doxylamine succinate is complete and that FT-IR method provides comparable data to those obtained in the UV–vis region.

Table 3

Analysis of doxylamine succinate in pharmaceuticals and recovery studies by FT-IR in batch mode

Sample	UV–vis method (mg/tablet)	FT-IR batch method (mg/tablet)	Doxylamine succinate added (mg)	Recovery (%)
1	24.0 ± 0.3	24.3 ± 0.7	2.6	97.6 ± 0.2
2	23.6 ± 0.5	23.9 ± 0.7	5.1	100.4 ± 0.9
3	25.2 ± 0.7	24.8 ± 0.5	9.9	100.1 ± 0.2

Note: Results shown correspond to the average value ± standard deviation of three independent analyses.

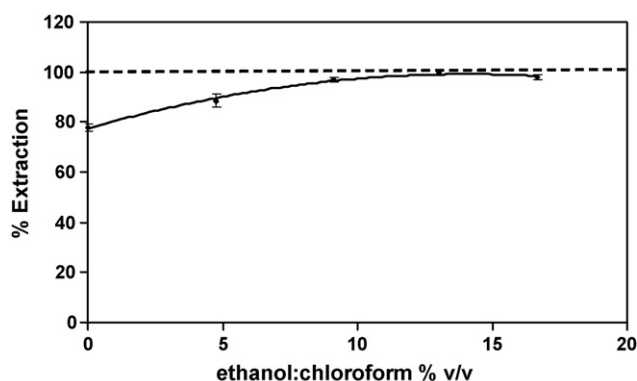


Fig. 3. Effect of the solvent composition in the batch extraction of doxylamine succinate from pharmaceuticals. Values indicated are the average of three independent analyses ± standard deviation.

3.3. On-line extraction and FT-IR determination experimental conditions

A simple manifold was designed in agreement with the experimental conditions fixed in the batch method. However, parameters as solvent volume, recirculating flow and number of scans per spectrum for the continuous measurement are limited because of the specific features of on-line systems.

In the case of solvent volume, the lowest value was tried, but the limitation is due not only to the internal volume of cell and connecting tubes, but for the apparition of air bubbles which were formed when high flow rates were employed and which provide severe changes in the spectra. To avoid bubbles formation, the volume selected was a little bit greater than the minimum theoretical. So, in this manifold the volume used was 2.6 mL.

The number of scans per spectrum was limited because the correlation between spectrum and analyte concentration depends on the time as a consequence of the continuous extraction. A great number of scans accumulated per spectrum improves the signal-to-noise ratio but increases the data spacing of the chemigram since it is not possible to monitor the real situation of the analyte in the measurement cell as a function of time. So, when four scans were accumulated per spectrum a good compromise between precision and time resolution was achieved, this value being selected as adequate for the continuous measurement.

The selection of the extraction flow rate was limited by the time necessary to reach the steady state. A study of the recirculation time of solvent in the manifold evidenced that a flow rate of 8.5 mL min^{-1} was suitable for obtaining a recirculation period of around 0.3 min, allowing to reach the steady state, or constant signal, in a few minutes (see Fig. 4). The continuous mixing of the solution in the extraction cartridge causes the

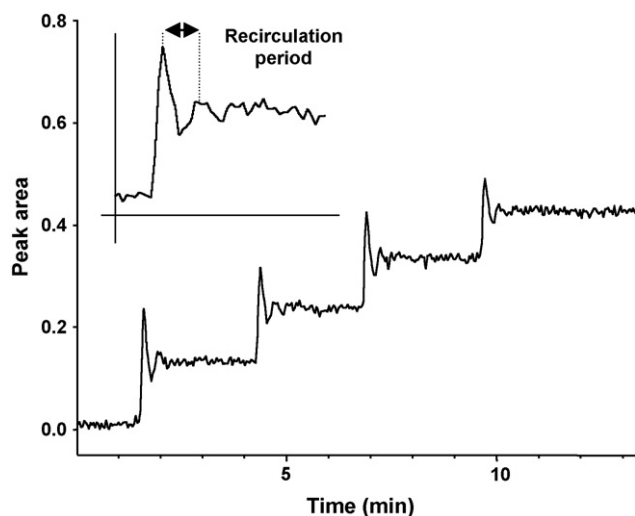


Fig. 4. Chemigram obtained from consecutive injections of a doxylamine succinate standard. *Experimental conditions:* $25 \mu\text{L}$ of a 56.8 mg mL^{-1} standard, 8 cm^{-1} nominal resolution, four scans per spectrum, peak area values between 1730 and 1710 cm^{-1} (baseline 2000 – 1800 cm^{-1}). *Inset:* Detail on recirculation period.

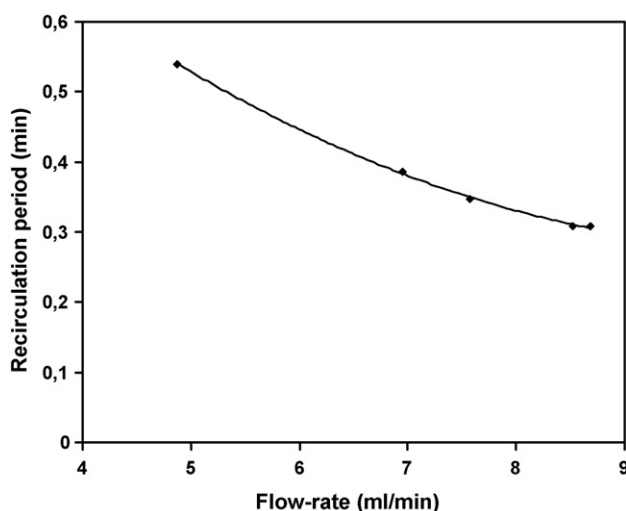


Fig. 5. Effect of the flow rate on the recirculation period. *Experimental conditions:* $50 \mu\text{L}$ of 45 mg mL^{-1} standard, 8 cm^{-1} nominal resolution, four scans per spectrum, peak area values between 1730 and 1710 cm^{-1} (baseline 2000 – 1800 cm^{-1}).

approximation of the signal to a constant value, corresponding to the steady state. From this time all the spectra recorded were similar.

Fig. 4 shows the evolution of the signal corresponding to the extraction of a sample and, after achieving the steady state, the injection of three volumes of a standard of doxylamine succinate. As it can be seen the extraction process is very fast and the shapes of the signals corresponding to the initial extraction and the standard additions are very similar. It indicates that the time required to reach the steady state corresponds, basically, to the homogenization of the solution inside the manifold.

So, it is possible to reduce the time required for the analysis if a co-addition of spectra, through a fixed time interval, is used to register the dynamic process. In this case, spectrum variation due to the non-uniform distribution of the analyte through the system is compensated along the time interval, because of its periodicity (see inset of Fig. 4). With this approach, the frequency analysis is improved drastically.

Fig. 5 shows that there is a strong reduction of the recirculation time, for a carrier flow increase from 5 to 8.5 mL min^{-1} . The use of a carrier flow higher than 8.5 mL min^{-1} does not provide a significant variation of the recirculation time but generates air bubbles inside the system and overpressure problems.

The initial time and the amplitude of the interval considered to obtain the co-added spectrum can involve an important reduction of the waiting time required to do the consecutive injection of standards. In this sense we have carried out a study to evaluate the effect of these two variables (co-addition time interval and initial time) on the final spectrum as compared with the signal that corresponds with the spectra obtained in the steady state.

Taking into consideration that the data point interval in the continuous acquisition is 0.038 min we have considered different multiples of this value for the co-addition interval as 4, 7, 9, 11 and 14, which provide co-addition times from 0.152 to 0.532 min . For a carrier flow of 8.5 mL min^{-1} an interval of

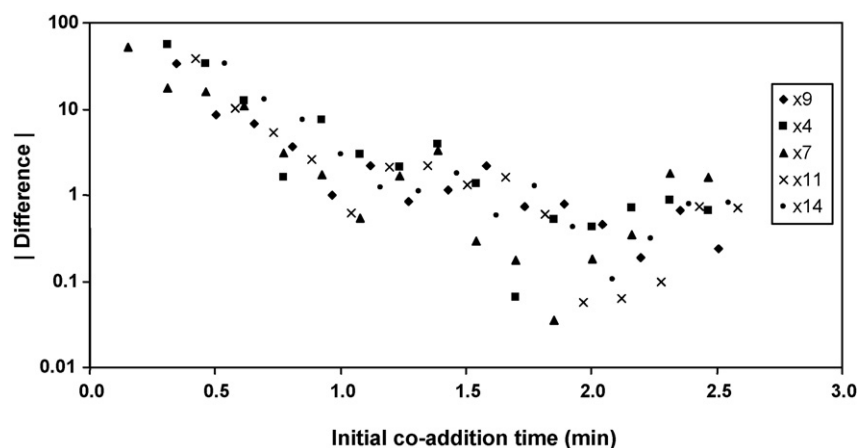


Fig. 6. Effect of the initial and interval time on the difference between absorbance peak measurements of the co-added spectra and data found in the steady state. *Experimental conditions:* 8 cm^{-1} nominal resolution, four scans per spectrum, peak area values between 1730 and 1710 cm^{-1} (baseline $2000\text{--}1800\text{ cm}^{-1}$). *Note:* $\times 4$, $\times 7$, $\times 9$, $\times 11$ and $\times 14$ are the co-addition time intervals expressed as a function of data spacing, and are equivalent to 44, 78, 100, 122 and 156% of the solvent recirculation period for a carrier flow of 8.5 mL min^{-1} .

$\times 9$ times the data spacing corresponds to the time required to complete a recirculation cycle. The co-added spectrum has been obtained for the selected intervals at different initial times from the injection of the standard. The peak absorbance of the co-added spectra were compared with those corresponding to the steady state and the difference calculated as absolute value expressed in percent.

Fig. 6 shows that a co-addition time equal to nine times the data spacing provides results with differences, as compared with those obtained from steady state, lower than 1%. With these conditions, the analysis could be completed in only 1.7 min.

3.4. On-line analysis of commercial samples

Commercial samples were analyzed by the on-line developed method with external calibration using the aforementioned conditions. The calibration line obtained was $S_c = (0.312 \pm 0.004)C$ (mg) + (0.129 ± 0.009) , $R^2 = 0.998$, S_c being the product of the sum of peak areas and the total volume of the solvent in the closed-flow system, and C the mass of doxylamine succinate. Results obtained for the determination of doxylamine succinate by interpolation in this external calibration were not in good agreement with those obtained with batch FT-IR method. The reason of this deviation could be the dragging of excipients by the solvent mixture during the extraction process, causing critical changes in the baseline or in the characteristic doxylamine succinate peaks. So, it was necessary to avoid dragging by inserting a filter after the sample cartridge. However, with this configuration, it was not possible to work with high flow rates due to overpressure of the system that involves changes in the carrier flow. For this reason, standard addition was selected as an alternative strategy for the determination of the analyte in the on-line system.

Standard addition was carried out extracting the sample and injecting $25\text{ }\mu\text{L}$ aliquots of a doxylamine succinate stock solution in the system, operating at 8.5 mL min^{-1} . Each aliquot was injected when the steady state was reached after sample extraction or injection. On using the co-addition of spectra, it was

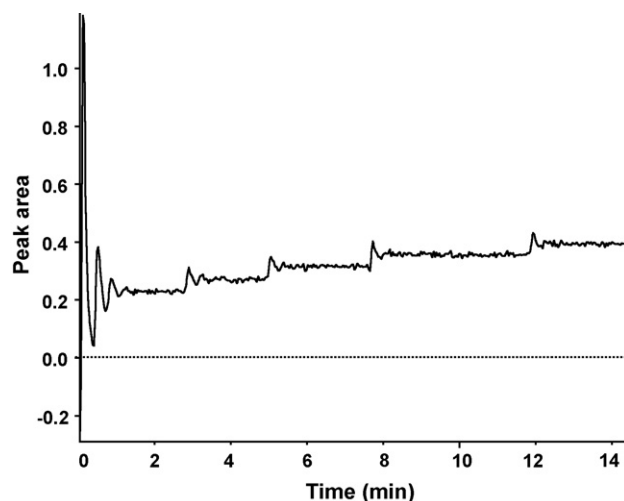


Fig. 7. Chemigram (area between 1710 and 1730 cm^{-1} corrected with a baseline established at 1800 cm^{-1}) obtained for the on-line determination of doxylamine succinate by standard addition. Four $25\text{ }\mu\text{L}$ aliquots of a stock solution (46.8 mg mL^{-1}) were injected in the system consecutively after the extraction of the analyte. *Instrumental conditions:* 8 cm^{-1} nominal resolution, four scans per spectrum, peak area values between 1730 and 1710 cm^{-1} (baseline $2000\text{--}1800\text{ cm}^{-1}$).

unnecessary to wait for the steady state, carrying out measurements in 1.7 min after each consecutive injection (see Fig. 7).

Results obtained by standard addition were in good agreement with theoretical content of samples, thus recommended for its application in commercial sample analysis (Table 4). How-

Table 4
Analysis of a sample containing doxylamine succinate by FT-IR

Method	Doxylamine succinate (mg/tablet)
FT-IR batch	24.8 ± 0.5
FT-IR on-line external calibration	23.6 ± 0.4
FT-IR on-line standard addition	25.19 ± 0.05
UV-vis	25.2 ± 0.7

Results shown as mean value $\pm$ standard deviation of three independent analyses.

ever, if dragging is avoided in future improvements of the on-line method, external calibration probably could be suitable and standard addition would be unnecessary.

4. Conclusions

An on-line method for doxylamine succinate determination in pharmaceuticals has been developed, evidencing the applicability of this strategy in routine analysis, through the design of a simple manifold which reduces sample manipulation and minimizes possible errors. The consumption of chloroform in the on-line method is four times higher than that required for the batch method using standard addition and two times higher than that employed on using external calibration. Analysis of commercial samples revealed that standard addition was required to obtain accurate results using the on-line extraction approach. Co-addition of spectra allowed us to decrease the time for reaching the constant values below 1.7 min per injection, with a final analysis frequency of 10 h^{-1} in the case of analysis by the standard addition method.

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Identification of rhubarbs by using NIR spectrometry and temperature-constrained cascade correlation networks

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Abstract

Temperature-constrained cascade correlation networks (TCCCNs) were used to identify powdered rhubarbs based on their near-infrared spectra. Different network configurations that used multiple network models with single output (Uni-TCCCN) and single networks with multiple outputs (Multi-TCCCN) were compared. Comparative studies were made by using Latin-partitions and leave-one-out cross-validation methods. Results showed that multiple networks with single output predicted generally better than single network with multiple outputs. Better results with TCCCN models were obtained compared with conventional back propagation neural networks (BPNs). The effects of parameters on correct identification and parameter optimizations were discussed in detail. With optimized neural network training parameters, NIR spectra from powdered rhubarb samples were classified by a TCCCN model with 100% accuracy.

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Keywords: Temperature-constrained cascade correlation networks; Rhubarb; Near-infrared spectra; Identification; Artificial neural network; Latin-partitions

1. Introduction

Rhubarb, one of the ancient and best-known Chinese herbal medicines, has been used for thousands of years in China and other Asian countries. Benefits of rhubarb include purgation, liver protection, antibacterial, and purging heat, loosening the bowels, promoting blood circulation, and removing blood stasis [1]. Rhubarb also has functions of antitumor and antimutagenicity [2]. Rhubarb is used as an important component in many Chinese patent and compound medicines. There are more than 40 species grown in China. However, people found that only three species among the rhubarbs have the most effective medicinal activities. In the *Chinese Pharmacopoeia* [3], the three species are assigned as official rhubarb, i.e., *Rheum palmatum*, *Rheum tanguticum*, and *Rheum officinale*. The other species of rhubarbs are referred to as unofficial rhubarbs. Because the compositions of the unofficial rhubarbs are different than the official ones, some medicinal functions of unofficial rhubarb are variable

and may be less effective than certified rhubarbs. The evacuative activity of unofficial rhubarb is lower, while the antidiarrheal activity is higher than that of the official rhubarbs. Even in some cases, the unofficial samples can cause stomachaches. Therefore, rhubarbs should be carefully selected and used by the pharmaceutical industry.

In some cases, roots and rootstalks of unofficial rhubarb are mixed into official rhubarb in commercial products by some manufactures. However, for a long time the methods of rhubarb identification were mainly based on visual inspection, and some physical and chemical properties. These methods are not only time consuming, but also depend on the technician's experience and experimental conditions. Therefore, the various identifications may be obtained under different experimental conditions or from different operators. Therefore, the identification of official rhubarbs from unofficial ones is important for quality control in the production of Chinese medicinal products.

Near-infrared (NIR) spectrometry has many advantages over mid- and far-infrared spectrometric methods in terms of speed, simplicity in sample preparation, cost, efficiency, and flexibility. Since the 1990s, near-infrared spectrometry has been burgeoning and has become a pervasive spectral technique that is widely

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applied in many fields for qualitative and quantitative analysis [4–7]. However, peaks in many near-infrared spectra are overlapped, and in some cases, sloping baselines of spectra are observed. Because of the difficulty of assigning NIR absorbance bands, computer technology coupled with chemometrics allow NIR, to be a powerful tool for many diverse applications [8].

Principal component analysis (PCA) and partial least squares (PLS) are commonly used for NIR spectroscopic data interpretation and development of classification models. Classification and calibration with neural networks have furnished many advantages over conventional statistical methods. Neural networks are mathematical models inspired by biological models. Various artificial neural networks (ANNs) have been used for pattern recognition and calibration [9–15], data interpretation in near-infrared spectroscopy, and many other fields [8,16–20].

Back propagation neural networks (BPNNs) are popular among chemists. The BPNNs are feed-forward network trained by propagation of error back through the networks. All the weights of the networks are adjusted simultaneously, which makes the training process chaotic. The network size and architecture (number of processing units, layers, and interconnections) must be determined before training. The main drawbacks of BPNNs are long times for convergence and convergence to local minima. The cascade correlation network (CCN) was developed to alleviate these problems [21]. The cascade correlation network configures its own architectures as it trains. It starts with an empty network (i.e., no processing units). The CCN then sequentially adds hidden processing units until the relative error between the network outputs and targets decreases below a user-defined threshold. Each new hidden unit is connected to the network input and the output from the previously installed hidden units. Therefore, the outputs from the previously units cascade into each new unit. Several candidate units can be trained in parallel, and the one with the largest covariance between candidate unit and its residual errors can be selected as the next hidden unit to install into the network. Once trained, the hidden units are no longer adjusted thus only one unit is trained at one time.

These traits are somewhat unique for the CCN and training is organized in that the processing unit weights are adjusted a single process unit at a time. The number of hidden units of the CCN will increase until the desired error is obtained or the error converges above the threshold. The most significant advantages obtained from the CCN are faster training rate than back propagation and self-configured networks. However, like other neural networks, the CCN is prone to overfitting the training data.

The temperature-constrained cascade correlation network (TCCCN) was developed based on the CCN. A temperature parameter was introduced to control the network training process by constraining the length of hidden unit weight vectors [22]. The advantage afforded by a temperature constraint is the removal of an extra degree of freedom manifested in the lengths of the weight vectors in the processing units of the neural network. Overfitting is related to the weight vector length which is a set of regression coefficients. The temperature of each processing unit is optimized so that a soft or fuzzy logistic function is obtained and the response surface of the model remains steep.

A logistic function is soft in that the linear portion of the sigmoid function spans the distance between the closest points on either side of the decision boundary and allows the unit to interpolate. Ergo, these network models will have better capability to generalized and better reproducibility with respect to training and initial conditions. The steep response function allows faster training when gradient optimization methods are used for training the hidden processing units.

The TCCCN had been successfully used in many examples, such as, the identification of bacteria by the MALDI-TOF-MS [23], classification of carbamate pesticides on the screening GC-MS data [24], prediction of substructure and toxicity of pesticides [25], the library search of UV spectra of organic environmental pollutants [26], and the quality control of the powder pharmaceutical samples of sulfaguanidine by NIR reflectance spectrometry [8]. In our series of studies of rhubarb samples, conventional BPNNs were employed. In this paper, the TCCCN was evaluated for the classification of rhubarbs from their NIR spectra and compared to the BPNN results. The effects of some the neural network training parameters were studied in detail.

2. Experimental

2.1. Samples and instrumentation

Fifty-two rhubarb samples of different species and from different origins were used for the identification studies in the present work. Among the samples, 25 were official and 27 were unofficial rhubarb samples according to *Chinese Pharmacopoeia*. The samples were dried and ground into 60 mesh powder. The spectra of NIR were obtained directly from 5 g of the powdered samples using diffuse reflectance.

The visible and NIR spectra of rhubarb were measured in the range of 400–2500 nm. The spectrometer was a Foss 6500 NIR scanning monochromator (Foss NIR Systems Inc., MD, USA) equipped with a halide lamp and PbS detector. The monochromator scanned at 2 nm intervals. To obtain acceptable signal-to-noise spectra, 50 scans were averaged.

The theoretical basis of temperature-constrained cascade correlation neural network can be found in ref [8]. The TCCCN software was written in C++ and compiled with Borland C++ 5.0. The program ran on personal computer with Pentium (R) 4 CPU 1.80 GHz processor equipped with 256 MB of RAM, which was operated under Window XP Professional (Version 2002).

2.2. Data processing and computation

In this study, the NIR spectra in wavelength range of 1100–2498 nm were selected to furnish 700 data points per spectrum. NIR spectra of all the samples were first smoothed by the Savitzky-Golay (polynomial function order 3, smooth window is 5) method in MATLAB Toolbox (smooth.m function). The sloping baselines of the spectra were attenuated by transforming the spectra to second derivatives (diff.m function in MATLAB Toolbox). Then, in order to reduce the number of variables to enhance the speed of processing, the spectra were then compressed

by using the wavelet transform (Daubechies wavelets) in MATLAB Toolbox. Each NIR spectrum was normalized so that the minimum was zero and its maximum was unity.

The samples were classified into two sets, the training and test sets. The training set was used to build the model, and the testing set was used to evaluate the model. Binary encoding of the target values was used for the classification. For example, two output units were used in Multi-TCCCN models to distinguish official and unofficial samples, and two single output units were used in Uni-TCCCN models.

An output value that is greater than 0.5 indicates class assignment. This criterion is more robust than the ‘winner-take-all’ criterion and allows for multiple classification of the same object.

2.3. Cross-validation

Two approaches were used for partitioning the near-infrared spectra of rhubarb into training and test sets. One was leave-one-out cross-validation method. The other was Latin-partitioning of the training and prediction sets [27]. The Latin-partitions built five training–prediction set pairs for which each class had a 20% membership in the prediction set and each spectrum of a sample is represented once and only once among the five prediction sets. The classification results obtained from the five prediction sets were pooled to yield summary statistics. The effects of randomization with the Latin-partitions were investigated by using different seed values. For the Latin-partitions, the networks were evaluated with five different random weight initializations for five training–prediction set pairs obtained using the same random seed for each of the Latin-partitions. The Latin-partitions were implemented 5 times to yield 25 training–prediction set pairs. Each set of five prediction set results was pooled for each Latin-partition. The advantage of this method is that every object is used once as in leave-one-out cross-validation method. An additional benefit is that every class is distributed among the training and prediction sets in the same proportion.

In both validation methods used in this work, samples were divided into two sets, a training set and a testing set. The testing set samples were not included in constructing classification models. Therefore, the separate testing set can be used to validate the effectiveness of trained network models.

3. Results and discussion

3.1. Near-infrared spectra of rhubarb

The chemical compositions of rhubarb samples are complex. The main components in rhubarb include Aloe-emodin, Rhein, Emodin, Sennodide A, Sennoside B, Sennoside C, Sennodide E, Sennodide F, and many other minor components. Because the complexity of overlapped overtones and combinational bands in near-infrared region, it is impossible to establish direct relation between chemical composition and NIR spectra of the rhubarb sample.

Fig. 1 gives the two typical NIR spectra of official and unofficial rhubarb samples at room temperature in the range

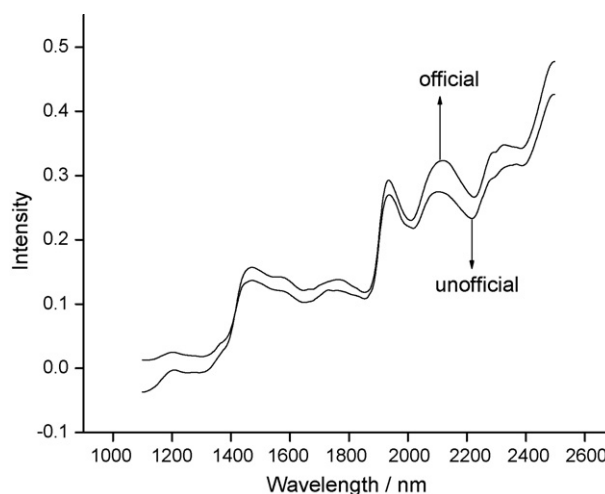


Fig. 1. The conventional NIR spectra of official and unofficial rhubarb in the range of 1100–2498 nm.

of 1100–2498 nm. The differences between the NIR spectra of official and unofficial spectra are not apparent. Correlation coefficients of NIR spectra between official and unofficial rhubarb samples have been calculated to evaluate the similarity of the NIR spectra. Most correlation coefficients between official and unofficial rhubarb samples with conventional NIR spectra were close to 0.999. There are only some minor differences in the NIR between the spectra of official and unofficial rhubarbs. In this study, derivative NIR spectra were used to eliminate the sloping baseline of the spectra and to enhance spectral resolution. The TCCCN model is used to classify rhubarb samples into official and unofficial groupings based on the minor differences in their NIR spectra.

3.2. Effect of wavelet compression levels

To speed up network training speed and avoid overfitting, the NIR spectra were compressed by using wavelet compression. Wavelet compression can facilitate neural network model building and prediction. A comparison was made between TCCCN models from compressed and uncompressed spectra.

The percentages of correctly identified compressed spectra mostly were higher than those of uncompressed spectra for various neural networks. The effect of the degree of wavelet compression on the different neural network classification rate was studied. For Multi-TCCCN with the leave-one-out cross-validation and Uni-TCCCN with Latin-partition, a level 2 wavelet compression (compressed to 175 variables) furnished the best results. Compressed spectra can be characterized by fewer variables, and therefore the number of input units of networks can be reduced. For the Uni-TCCCN leave-one-out cross-validation and Multi-TCCCN Latin-partition, level 3 wavelet compressions (compressed to 88 variables) were optimal. Results showed that the effects of different levels on RMSEP were different for different neural network models, and the RMSEP were lower when the optimum level wavelet

compression with better prediction was adopted. Therefore, in Section 4, the optimal levels of wavelet transform were selected for different neural network models.

Results showed that the differences of various neural network model outputs between compressed spectra and uncompressed spectra sometimes were significant, while the differences of outputs among compressed spectra were not obvious. So, the results from optimal number of variable were better than those from the uncompressed spectra.

3.3. Effect of transfer functions

The types of transfer functions play an important role in network performance. When the sigmoid output units were used, the effects of sigmoid, temperature-constrained sigmoid, and linear functions in hidden units with Uni-TCCCN and Multi-TCCCN were discussed, respectively. The results are given in Fig. 2.

Classification accuracy was higher when temperature-constrained sigmoid hidden units were used for the four different neural networks. The best predictions were obtained from the Uni-TCCCN when evaluated with the Latin-partition method.

When the temperature-constrained sigmoid hidden units were used, the effects of different types of transfer functions with output units on correct identification were also investigated. The neural network with temperature-constrained sigmoid hidden units and output units could not converge. As a result, the models with the linear, sigmoid output units were discussed, respectively. The results are given in Fig. 2. Networks with sigmoid function output gave better predictions, and had faster training rates than networks with linear function for output units. Therefore, temperature-constrained sigmoid function for hidden units and normal sigmoid functions for output units were used in the work.

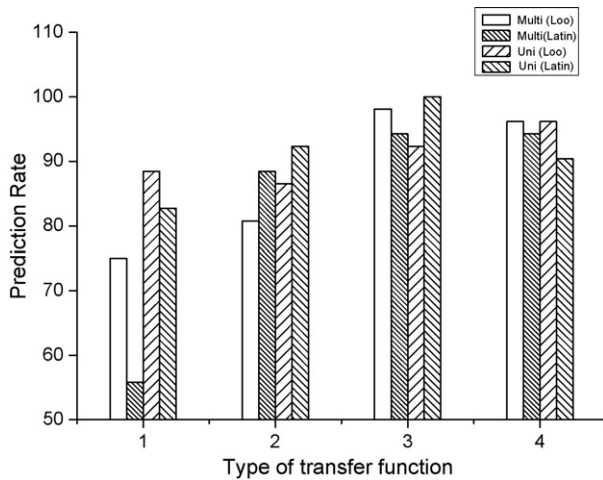


Fig. 2. Effect of transfer functions on correct identification. Notes: 1, 2, and 3, on the abscissa designates the network models with linear, sigmoid, and temperature-constrained sigmoid with hidden units, respectively; 3 and 4, abscissa designates the network models with sigmoid and linear output units, respectively. LOO refers to leave-one-out; Latin refers to Latin-partition.

3.4. Effect of number of candidate units

In TCCCN, the hidden units are added sequentially until the training error decreases below a pre-defined threshold. During the training process, several units can be trained in parallel. The one with the largest covariance between candidate units is selected and incorporated into the network model. To investigate the effect of number of candidate units, network training with various number of candidate units (from two to eight) were investigated.

Results showed that the effects of the numbers of candidate units were various for the Multi-TCCCN and Uni-TCCCN. Best prediction was obtained when three candidate units were used, and when more candidates units were used, the changes of the percentage of correct prediction became not obvious for these two neural networks, while RMSEP became higher. Five and six candidate units were selected for Multi-TCCCN with Latin-partition and Uni-TCCCN with leave-one-out cross-validation networks, respectively. This experiment was repeated six times and the changing trends of average RMSEP with increasing number of candidate units were similar.

3.5. Effect of pre-defined relative errors

In network training, a pre-defined relative error should be given so that the training process can terminate when output error reaches this value. If the calculated relative error reaches the pre-defined relative error, the networks automatically add a hidden unit, and calculate again. The training error is defined as the relative root mean square error of calibration (RRMSEC) and is calculated by

$$\text{RRMSEC} = \sqrt{\frac{\sum_{i=1}^n \sum_{k=1}^p (y_{ik} - \hat{y}_{ik})^2}{\sum_{i=1}^n \sum_{k=1}^p (y_{ik} - \bar{y}_k)^2}} \quad (1)$$

In which n is the number of training spectra, p the number of classed or network outputs, and the target value for the i th object and k th class is y_{ik} , $\hat{y}_{ik}$ the corresponding network estimate of the target value, and $\bar{y}_k$ is the average target value for the k th output.

Prediction performance is evaluated by using root mean square error of prediction (RMSEP), which is defined as

$$\text{RMSEP} = \sqrt{\frac{\sum_{i=1}^n \sum_{k=1}^p (y_{ik} - \hat{y}_{ik})^2}{np}} \quad (2)$$

The relative root mean square error of prediction (RRMSEP) is similar to the RRMSEC in Eq. (1) except only the test data is used.

For the Multi-TCCCN, each output node represents one specific class, and the network is trained so that a specific node produces the largest output among all nodes to show the presence of that specific class. Classification results can be obtained simultaneously by using a Multi-TCCCN. For the Uni-TCCCN network, there is only one output unit in the network, so only one class can be predicted at one time. The whole Uni-TCCCN is composed of several uni-output

networks, therefore, the Uni-TCCCN systems can also be used to multi-class classification. In the discussion of the effects of pre-defined relative errors on neural networks, the temperature-constrained sigmoid function was used for the hidden units, and sigmoid function was used for the output units.

For different neural networks, the effects of pre-defined error were different. The percentage of correct identification with Uni-TCCCN changed very little with the increase of pre-define error, while the Multi-TCCCN percentage first increased, then decrease, and at last kept unchanged. Better predictions were obtained from the Uni-TCCCN than from Multi-TCCCN, and higher percentage of correct identification were obtained from the Uni-TCCCN with Latin-partition than Uni-TCCCN with leave-one-out cross-validation. Therefore, for the four neural networks, good predictions were obtained when a 10% training error threshold was selected.

4. Evaluation of TCCCN models

4.1. Evaluation using Latin-partition approach

For the aforementioned hierarchical uni-output and multi-output networks, the networks were used to discriminate between pairs of classes. For comparison study of different TCCCN models, Latin-partition method was applied. Rhubarb samples were divided into training and testing sets according to the ratio of 80%:20%. For single partition, 20% samples from each class (official and unofficial) were randomly selected as prediction set. Each sample was used as prediction set only once. For each Latin-partition, five training–prediction set pairs were obtained for which each samples occurred once and only once in the five prediction sets. The results of these partitions were pooled and comprise 52 prediction objects. Five Latin-partitions with different randomized samplings to Multi-TCCCN and Uni-TCCCN were accomplished with different seed values. The results of each Latin-partition are given in Table 1. The difference between the prediction errors of the two models Multi and Uni were not statistically significant at a 95% level of confidence.

Noted that Uni-TCCCN or Multi-TCCCN models had better robustness, generalizing abilities and yielded satisfactory

results, and the prediction results did not vary much among the different partitions. In a statistical sense, there were no significant differences that were observed in the classification performance. Better predictions were obtained with Uni-TCCCN models, and lower root mean square errors were obtained than those using Multi-TCCCN. Especially, for the Uni-TCCCN models with optimized parameters, the official and unofficial rhubarb could be identified completely for two of the partitions or 40% of the time.

4.2. Prediction results from TCCCN and BPNNs

BPNNs are popular methods for computer-assisted chemicals structure elucidation and classification. The combination NIRS and BPNNs had been investigated, and that this method was used to identify official and unofficial rhubarbs in our group [28]. For comparison, the results from the BPNNs and TCCCN are given in Fig. 3 and Table 2.

The models of the TCCCN with single outputs had better performance than those with the BPNNs. The samples of no. 2 and 36 were misclassified with the BPNN models, while the TCCCN models identified them correctly. The outputs from the TCCCN were closer to the target outputs than using BPNNs, especially for the official samples. In addition, results in Table 2 show that the average errors of 25 official and 27 unofficial samples from TCCCN model were lower than those from BPNNs model. Therefore, the TCCCN models converged faster, give better robustness and generalizing capability, and also improved performance in identification of rhubarbs compared with the BPNNs. However, a two-way analysis of variance and a matched sample *t*-test did not indicate that the errors between the two methods were statistically significant at 95% level of confidence. However, the classification rate was statistically different at a 95% level of confidence.

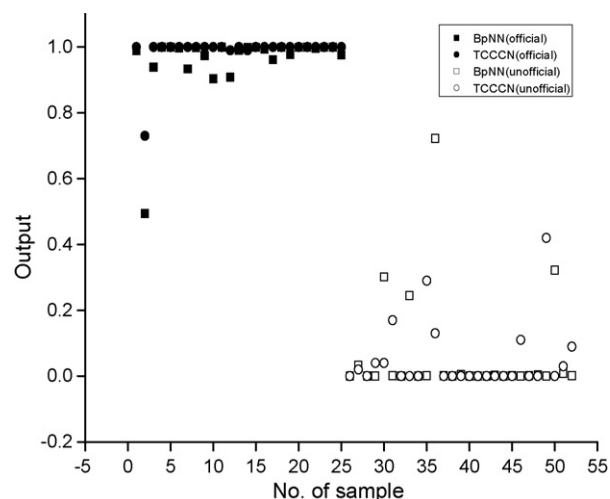


Fig. 3. Prediction results from BPNNs and Uni-TCCCN. Notes: BPNN (official) and TCCCN (official) stand for the prediction results from BPNN and TCCCN with official samples, respectively. BPNN (unofficial) and TCCCN (unofficial) stand for the prediction results from BPNN and TCCCN with unofficial samples, respectively.

Table 1
Results of TCCCN with different Latin-partitions

	Percentage of correct prediction		RMSEP	
	Multi	Uni	Multi	Uni
Partition 1	98.08	100	2.683	0.4
Partition 2	98.08	98.08	2.297	0.948
Partition 3	96.15	98.08	4.188	0.989
Partition 4	96.15	96.15	4.198	1.712
Partition 5	96.15	100	4.028	0.428
Average	97 ± 1%	98 ± 2%	3.5 ± 1	0.9 ± 0.7

Notes: Multi stands for the results from Multi-TCCCN with optimize parameter. Uni stands for the results from Uni-TCCCN with optimized parameter. Symbol '/' stands for no samples wrong identification.

Table 2

Cross-validation prediction results for TCCCN and BPNN models

Official samples no.	Expected output	BPNNs network output	Error (%)	TCCCN network output	Error (%)	Unofficial samples no.	Expected output	BPNNs network output	Error (%)	TCCCN network output	Error (%)
1	1	0.9889	−1.11	1	0	26	0	0	0	0	0
2	1	0.4398	−50.62	0.73	−27	27	0	0.033	3.3	0.02	2
3	1	0.9386	−6.14	1	0	28	0	0.0002	0.02	0	0
4	1	0.9997	−0.03	1	0	29	0	0.0006	0.06	0.04	4
5	1	0.9997	−0.03	1	0	30	0	0.3012	30.12	0.04	4
6	1	0.9971	−0.29	1	0	31	0	0.0015	0.15	0.17	17
7	1	0.9337	−6.63	1	0	32	0	0	0	0	0
8	1	0.9978	−0.22	1	0	33	0	0.245	24.5	0	0
9	1	0.9736	−2.64	1	0	34	0	0.0001	0.01	0	0
10	1	0.9036	−9.64	1	0	35	0	0.0007	0.07	0.29	29
11	1	1	0	1	0	36	0	0.7228	72.28	0.13	13
12	1	0.9078	−9.22	0.99	−1	37	0	0.0016	0.16	0	0
13	1	0.9897	−1.03	1	0	38	0	0.0004	0.04	0	0
14	1	0.9991	−0.09	0.99	−1	39	0	0.0048	0.48	0	0
15	1	0.9991	−0.09	1	0	40	0	0.0001	0.01	0	0
16	1	0.9939	−0.61	1	0	41	0	0	0	0	0
17	1	0.9615	−3.85	1	0	42	0	0.0003	0.03	0	0
18	1	0.9994	−0.06	1	0	43	0	0.0028	0.28	0	0
19	1	0.9777	−2.23	1	0	44	0	0.0003	0.03	0	0
20	1	0.9992	−0.08	1	0	45	0	0.0018	0.18	0	0
21	1	1	0	1	0	46	0	0.0001	0.01	0.11	11
22	1	0.9957	−0.43	1	0	47	0	0.001	0.1	0	0
23	1	0.9988	−0.12	1	0	48	0	0.004	0.4	0	0
24	1	1	0	1	0	49	0	0.0002	0.02	0.42	42
25	1	0.9755	−2.45	1	0	50	0	0.322	32.2	0	0
						51	0	0.008	0.8	0.03	3
						52	0	0.0017	0.17	0.09	9

5. Conclusions

Near-infrared spectroscopy technique and temperature-constrained cascade correlation neural network models have been used to discriminate between official and unofficial rhubarb samples. The powdered rhubarb samples can be identified directly when proper data processing methods are used. Using the TCCCN models with Latin-partition appears to predict better than those with leave-one-out cross-validation, and that the models with mono-output give higher percentage of correct identification of rhubarb. The models with different Latin-partitions are investigated. The results prove that TCCCN has great robust and generalizing ability. Temperature-constrained cascade correlation neural network has been approved to be a good and effective approach for the identification of official and unofficial powdered rhubarb samples.

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Rapid determination of aluminum by UV–vis diffuse reflectance spectroscopy with application of suitable adsorbents

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Abstract

Diffuse reflectance spectroscopy (DRS) can be used as a rapid and sensitive method for the quantitative determination of low amounts of aluminum. In this analytical technique, the analyte in samples are extracted onto a solid sorbent matrix loaded with a colorimetric reagent and then quantified directly on the adsorbent surface. Alternatively, colored aluminum complexes formed in solution can also be immobilized onto adsorbent surface and be measured by DRS technique. Octadecyl silica disk, methyltriocetylammmonium chloride–naphthalene and MCM-41 were examined as adsorbents. Eriochrome cyanine R and quinalizarin were used as coloring reagents. Optimal sorption conditions were found for each system of analyte–reagent–adsorbent. The concentration of analyte is determined using the appropriate form of the Kubelka–Munk function. We obtained for each of the aluminium–reagent–adsorbent system a calibration curve by plotting the absorbance versus the $\log 10^2[\text{Al}^{3+}] \mu\text{g ml}^{-1}$. The linear dynamic range extends over two orders of magnitude within $0.01\text{--}15 \mu\text{g ml}^{-1}$ with little differences in the range and in the correlation coefficients among the adsorbents. We consider that for a rapid determination of aluminum a spot-test-DRS combination with a detection limit of $1.0 \times 10^{-2} \mu\text{g ml}^{-1}$ is the more facile and preferred technique.

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Keywords: Aluminum; Diffuse reflectance spectroscopy; Spot-test; MCM-41; Octadecyl silica; Eriochrome cyanine R

1. Introduction

Instrumental techniques such as atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry, neutron activation analysis and ICP-mass spectrometry are powerful tools for the trace determination of metal ions. However, these methods have the disadvantages of high cost of instruments and matrix interference. Separation of analyte from other elements present and use of a pre-concentration step prior to the determination is a useful alternative approach. Solvent extraction of different metal ions has been widely employed in chemistry for many years. However, the use of classical extraction methods are usually time-consuming labor-intensive and require relatively large volume of high purity solvents. Additional concern is the disposal of the solvent used, which can create a severe environmental problem. Replacing the conventional solvent extraction methods with solid-phase extraction provides a

technique capable of highly selective for removal of metal ions from solution containing matrices, which normally make separation difficult. In this technique usage of organic compounds is reduced considerably. However, final part of the procedure usually contains stripping the membrane disk or columns from the extracted metal ions using water or other solvent prior to the determination. One other way to perform a determination of this type involves the use of diffuse reflectance spectroscopy (DRS) in the final part of the procedure. By employing this technique the elution step can be eliminated from the process. In this technique, direct measurement of the adsorbed species on the solid supports offers the advantage of avoiding the loss usually encountered during elution of the analyte from the adsorbent. Also, this technique simplifies and speeds up the analytical procedure. This technique would also be in the program of the analytical methodologies designed to achieve the green chemistry ambitions [1]. There is an evident demand to the development of greener, yet reliable, analytical procedures to minimize or eliminate the use of toxic reagents.

The development of reflectance spectroscopy was initially considered for several applications such as dye-stuffs, printing

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inks, paints and pigments, textiles, paper, ceramics, building materials, metallurgy and optical materials. But extensive progresses have been recently achieved in diffuse reflectance spectroscopy for studying inorganic materials [2–4]. Although, the reflectance spectroscopy is not widely recognized in analytical chemistry as is the use of transmittance spectroscopy, but with the development of optical devices such as integrating sphere assemblies, the situation has changed and satisfactory results were obtained [4–6].

Application of diffuse reflectance spectroscopy has been reported for many cations [7–19] and some anions [20–22] after adsorption onto a proper solid support. There are different ways for formation and collection of colorimetrically measurable analyte. These include complexation in solution and extraction of the soluble complex [7,8], reagent immobilization with the subsequent extraction and complexation of the analyte [9–14,21,22], analyte extraction followed by exposure to complex reagent solutions [16–19] and also formation of an insoluble complex and collecting it on an extraction membrane [15].

In this paper, three sensitive and efficient spot-test and pre-concentration techniques in combination with diffuse reflectance spectroscopy were developed for measurement of aluminum. The determination of trace amount of aluminum has become increasingly very important because exposure of the metal to human threaten his health due to the diseases such as Alzheimer disease and Parkinson disease [23–25]. In our primer technique either of the reagent eriochrome cyanine R (ECR) or quinalizarin was loaded on octadecyl-bonded silica (ODS) membrane disks and then aluminum solution was passed through the modified disks. We also employed adsorbent columns containing either MCM-41 (a mesoporous material) or methyltriocetylammmonium chloride–naphthalene for extraction of Al–ECR complexes prior to DRS measurements. Finally we examined the efficiency of a spot-test method by adsorbing ECR on a home made MCM-41 disk followed by spotting the disk by drops of aluminum solution. To the best of our knowledge, these modifications have not been employed previously for aluminum measurements.

2. Experimental

2.1. Reagents and adsorbents

All the reagents used were of chemical purity or analytical purity grade. All solutions were prepared with deionised water. The stock aluminum solution (1000 µg/ml) was prepared by dissolving the exact amount of high purity aluminum powder (Aldrich no. 20258, 99.99%) in concentrated HCl and diluting to 500 ml. More diluted solutions were prepared daily from the stock solution. A 0.01 M solution of ECR (Merck no. 103164) was prepared and used throughout. Buffer solution of pH 6.1 was prepared by addition of drops of 1 M hydrochloric acid to 50% (w/v) sodium acetate solution using the pH meter to adjust the desired pH.

The MCM-41 mesoporous adsorbent was synthesized by a room temperature method with some modification in the described procedure in the literature [26]. We used tetraethylorthosilicate (TEOS, Merck, 800658) as a source of silicon

and hexadecyltrimethylammmonium bromide (HDTMABr, BDH, 103912) as a surfactant template for preparation of MCM-41. The details of the procedure have been described elsewhere [27]. The molar composition of the reacting mixture was as follows: SiO₂:1.6 EA:0.215 HDTMABr:325 H₂O. EA stands for ethylamine. The as-synthesized MCM-41 sample is calcined at 550 °C for 5 h to decompose the surfactant and generating the adsorption property of the material. This powder sample is white and used for loading the colored species (ECR or Al–ECR).

The naphthalene adsorbent was prepared by addition of 0.6309 g of methyltriocetylammmonium chloride (Merck, 818079) to 12.5 g naphthalene in 23 ml acetone on a hot-plate stirrer. The mixture was stirred at 40 °C until a clear solution was obtained. The solution was transferred into 750 ml of deionised water while stirring it at room temperature. It was then stirred for 1 h. After being allowed to stand for 30 min, the supernant solution of naphthalene coprecipitated with methyltriocetylammmonium chloride was drained off by decantation and washed with deionised water for several times. This adsorbent was kept in a capped bottle for further use.

Quinalizarin (Fluka, 22570) solution was prepared by dissolving 10.0 mg of reagent in 25 ml acetone. Octadecyl-bounded silica membrane disks were of the type Empore (47 mm × 0.5 mm, 3 M Co).

2.2. Apparatus

A Shimadzu UV-2100 spectrophotometer equipped with an integrating sphere assembly was used to collect all the spectral data from the 12 mm disks or pressed materials. The spectrum is recorded at room temperature against barium sulfate and plotted in terms of absorbance. The crystalline structure of our-made MCM-41 was checked by a Philips PW1840 diffractometer with Cu K_α radiation at room temperature. XRD pattern was recorded using an automatic divergent slit system within $2\theta = 2\text{--}10^\circ$. Chemical analysis of the MCM-41 was done by energy dispersive X-ray analysis (EDX) joined to a Philips XL-30 scanning electron microscope.

The 12 mm disk (~230 mg MCM-41 is lightly pressed to obtain a disk with 12 mm diameter) elaborated in our laboratory is used as adsorbent support for spot-test performance.

2.3. Extraction procedure

2.3.1. ODS disk

Extraction was performed with 47 mm × 0.5 mm (diameter × thickness) Empore membrane disks containing octadecyl-bounded silica used as a matrix for collection of the colored complex. The disk was used in conjunction with a standard 47 mm filtration apparatus. After placing the membrane in the filtration apparatus, it was washed with 10 ml methanol and 10 ml acetonitrile to remove all contaminants arising from the manufacturing process and environment. After drying the disk by passing air through it for several minutes, a solution of 5.364 mg ECR dissolved in 10 ml water or 10 mg quinalizarin dissolved in 4 ml acetone was introduced to the reservoir of the apparatus and was drawn slowly through the disk by applying

a slight vacuum until the reagent penetrated the membrane completely.

The disk modified by ECR or quinalizarin was then washed with 10 ml water followed by passing 2 ml of buffer solution (pH 6.1) through it. Finally the disk was dried at room temperature. The procedure for extraction of Al^{3+} ions on the modified disk was as follows: the modified disk was first wetted with 1 ml water prior to extraction. Then, aluminum solution with known concentration was passed through the disk (flow rate $\approx 7 \text{ ml min}^{-1}$). After the extraction, the disk was dried at room temperature and then part of it was cut for diffuse reflectance measurement.

2.3.2. Methyltriocetylammmonium chloride–naphthalene column

A funnel tipped glass tube (50 mm $\times$ 5 mm i.d.) with a very fine bore was used as a preconcentration column. It was filled with the adsorbent slurry to a height of 2 cm after slightly pressing the adsorbent into the column with a flat glass rod. The column was then conditioned to pH 6.1 with 2–3 ml of buffer. After 2 min, solution of Al–ECR was passed through the column at a flow rate of $\sim 1 \text{ ml min}^{-1}$. Finally the column packing is removed out and part of it was pushed down into a sample holder designed for the solid materials ready for diffuse reflectance measurement.

2.3.3. MCM-41 column

A glass tube (50 mm $\times$ 5 mm i.d.) was packed with the calcined MCM-41 powder employing a vacuum pump to obtain a homogenous packing. The column was first eluted with 5 ml of deionized water. Then 3.5 ml of buffer pH 6.1 was passed through the column. Now, the adsorbent column is ready for elution of Al–ECR solution. Procedure for elution is the same as that described for naphthalene-modified column. At the end of elution the packing is removed out and part of it was used to fill the specific sample holder.

2.4. Spot-test

The calcined MCM-41 was used for preparation of the support disks for spot-test reaction. About 0.5 g of the calcined MCM-41 was added to 25 ml of ECR solution ($5 \times 10^{-4} \text{ M}$) and was stirred for 24 h. After 24 h the color of the matrix is stabilized implying that the dye molecules are adsorbed on MCM-41. The mixture is then centrifuged and solid was washed with deionized water and dried at 40°C . The fine powder of the solid is then pressed with our elaborated disk-preparing system to provide disks with a diameter of 20 mm and a thickness of $\approx 0.4 \text{ mm}$. When drops of aluminum solution come into contact with the surface of the disk a colored product is formed on the surface. The aluminum solution is buffer to pH 6.1 prior to spotting.

2.5. Diffuse reflectance measurements

The Kubelka–Munk equation provides an adequate approach for quantitatively relating the observed signal to the sample concentration in diffuse reflectance measurements [28]. The

Kubelka–Munk function, $F(R)$, is defined as

$$F(R) = \frac{(1 - R)^2}{2R}$$

where R is the percent reflection measured with respect to a standard white. $F(R)$ is related to analyte concentration by

$$F(R) = \frac{\varepsilon C}{s}$$

where ε is the molar absorptivity, C the concentration of the complexed analyte, and s is the scattering coefficient of the sample surface. By assuming the absorptivity and scattering coefficient of our surface are constant at a given wavelength, $F(R)$ can therefore be related directly to analyte concentration. For quantitative measurements, the literature proposed many types of plots relating the signal and concentration [9]. In this work, we have obtained quantitative data by plotting: $\text{Abs} = -\log I_R/I_R^\circ$ versus $\log C$. I_R is the intensity of radiant energy reflected by the sample and I_R° is the intensity reflected by the white blank. Abs is analogous to absorbance with the only difference being that Abs represents the optical density for reflectance measurement and not for absorbance.

3. Result and discussion

3.1. Al–ECR complex formation as a function of pH

It was found that the formation of aluminum–eriochrome cyanine R complex (Al–ECR) was more complete in the pH range 5.8–6.2 (Fig. 1). Effect of pH on absorbance for Al–ECR adsorbed on MCM-41 and MTOAC–naphthalene are shown in Fig. 2a and b. According to those, pH 6.1 was chosen as optimum pH. Adjustment of the pH of the adsorbents was done either by eluting the adsorbent columns (MTOAC–naphthalene or MCM-41) with buffer solutions, passing the buffer through

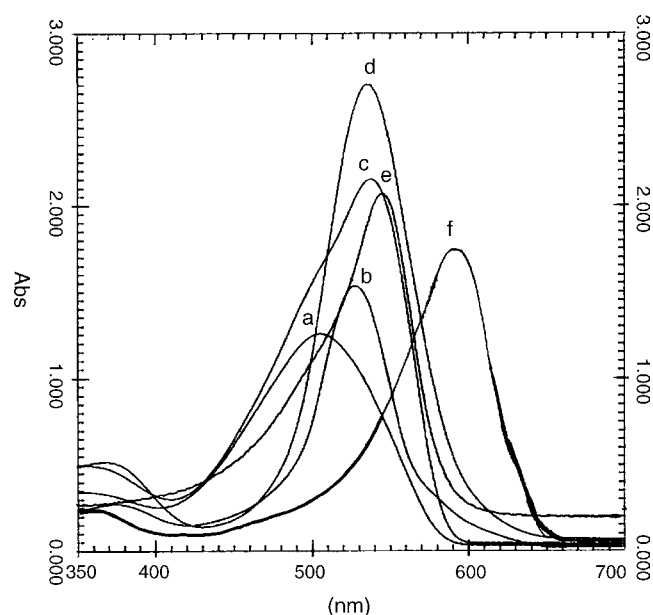


Fig. 1. Absorption spectra of Al–ECR at the indicated pH ($a=2$, $b=4$, $c=5$, $d=6$, $e=7$ and $f=10$).

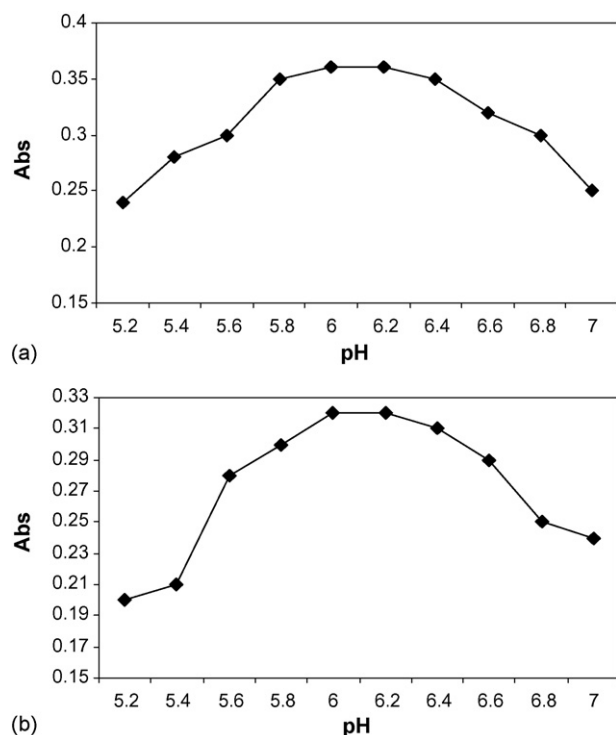


Fig. 2. Effect of pH on the absorbance of Al-ECR adsorbed on (a) MCM-41, $\lambda_{\max} = 530$ nm and (b) MTOAC-Naph, $\lambda_{\max} = 570$ nm; conditions: $[Al^{3+}] = 0.1 \mu\text{g ml}^{-1}$, $C_{\text{ECR}} = 1 \times 10^{-5}$ M.

the adsorbent disk (ODS) or addition of buffer to aluminum solution (spot-test).

3.2. Optical characteristics of aluminum complexes on the surface of adsorbents

The diffuse reflectance spectra of Al/ECR-ODS, Al/Q-ODS, Al-ECR/MTOAC-Naph, Al-ECR/MCM-41 are shown in Fig. 3. The DR spectra of ECR-MCM-41 and Al/ECR-MCM-41 obtained for spot-test are shown in Fig. 4. Table 1 describes that how these designations have been chosen to represent the method used for determination of aluminum.

Diffuse reflectance spectrum of ECR-MCM-41 (Fig. 4a) shows a band at about 420 nm which corresponds to the ECR. This indicates that ECR is adsorbed by MCM-41. The maximum of the DR spectrum obtained for the aluminum spotted on MCM-41 (Al/ECR-MCM-41) is observed at about 530 nm (Fig. 4b). The comparison of the DR spectra of reagent and

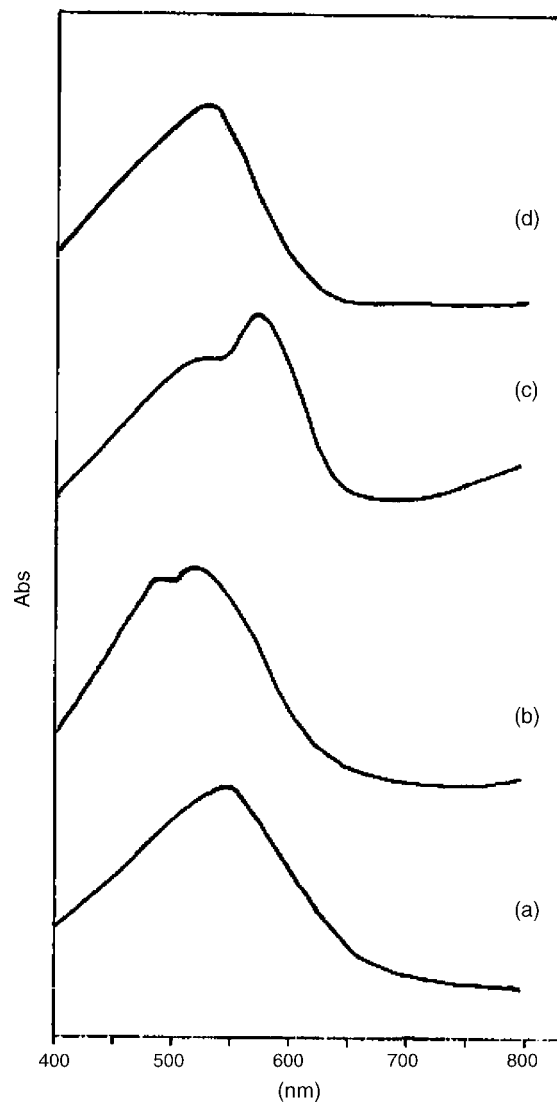


Fig. 3. Diffuse reflectance spectra of (a) Al/ECR-ODS, (b) Al/Q-ODS, (c) Al-ECR/MTOAC-Naph and (d) Al-ECR/MCM-41.

complex clearly shows the progress of reaction occurred on the surface of adsorbent. The reflectance spectra for Al-ECR complexes formed on other solid phases (Fig. 3) shows this characteristics band within 530–570 nm which can be used for calibration and quantitative measurements. The shift in maxima is related to the guest-host effects usually observed in spectra of adsorbed species on the solid supports.

Table 1
Method used prior to a diffuse reflectance spectrophotometric determination of aluminum

Solid adsorbent	Techniques ^a	Reagent	Designation of the reflecting sample
Octadecyl silica disk	SPE	Eriochrome cyanine R	Al/ECR-ODS
Octadecyl silica disk	SPE	Quinalizarin	Al/Q-ODS
Methyltriethylammonium chloride-naphthalene	SPE	Eriochrome cyanine R	Al-ECR/MTOAC-Naph
MCM-41	SPE	Eriochrome cyanine R	Al-ECR/MCM-41
MCM-41	ST	Eriochrome cyanine R	Al/ECR-MCM-41

^a SPE stands for solid phase extraction and ST stands for spot-test.

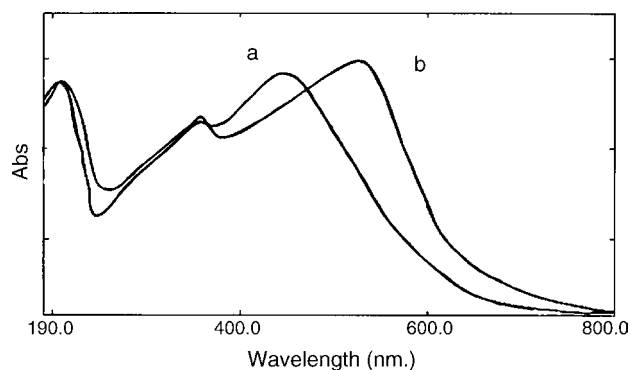


Fig. 4. Diffuse reflectance spectra of (a) ECR-MCM-41 and (b) Al/ECR-MCM-41; condition: pH 6.1, C_{ECR} , 5×10^{-4} M.

3.3. Calibration curves

The calibration curves for Al/Q-ODS and Al/ECR-ODS are shown in Fig. 5. The details of experimental conditions such as pH, λ_{max} , flow rate of sample through the disks are described in caption to the Fig. 5. The calibration graph was linear ($r^2 = 0.997$) in the range 0.08 – $1.6 \mu\text{g ml}^{-1}$ of aluminum for Al/Q-ODS and the correlation coefficient was $r^2 = 0.988$ in the

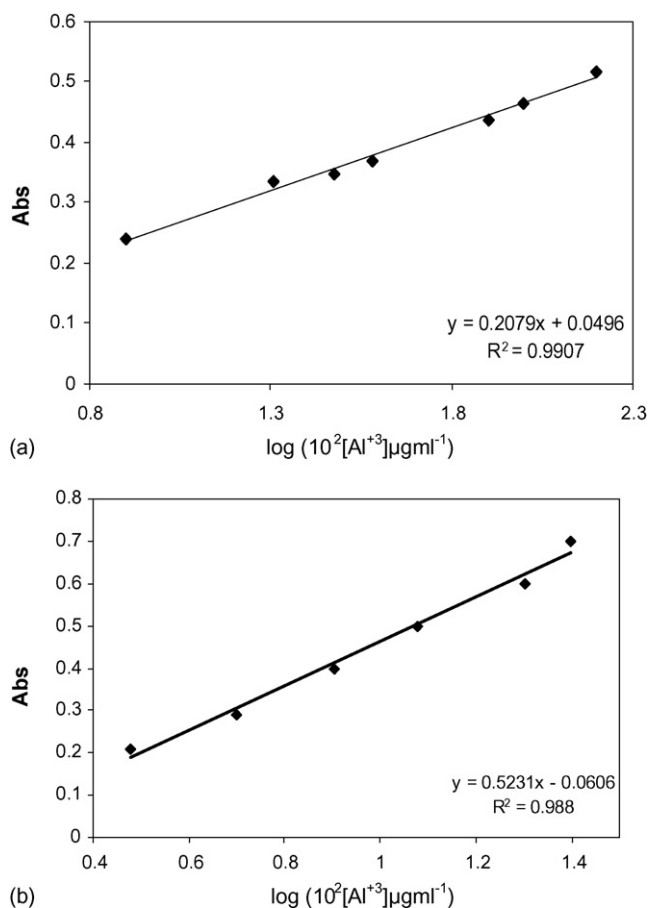


Fig. 5. Calibration curve for aluminum (a) Al/ECR-ODS adsorbent system, pH 6.1, $C_{\text{ECR}} = 1 \times 10^{-3}$ M, $\lambda_{\text{max}} = 540$ nm, flow rate = 6 ml min^{-1} and (b) Al/Q-ODS adsorbent system, pH 6.8, $C_Q = 1 \times 10^{-3}$ M, $\lambda_{\text{max}} = 519$ nm, flow rate = 7 ml min^{-1} .

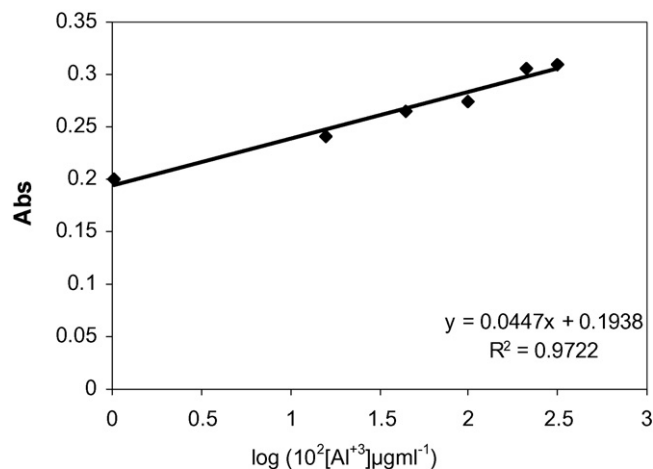


Fig. 6. Calibration curve for aluminum for Al-ECR/MTOAC-Naph adsorbent system, pH 6.1, $C_{\text{ECR}} = 1 \times 10^{-3}$ M, $\lambda_{\text{max}} = 570$ nm, flow rate = 2.5 ml min^{-1} .

range 0.03 – $0.25 \mu\text{g ml}^{-1}$ of aluminum for Al/ECR-ODS. The relative standard deviations (R.D.S.) of six replicate determination of $1 \mu\text{g ml}^{-1}$ of aluminum were 9.6×10^{-5} and 1.9×10^{-5} for Al/Q-ODS and Al/ECR-ODS, respectively. The detection limit was found from the calibration curve by plotting the intersection of the curve with the abscissa. The LOD was $0.004 \mu\text{g ml}^{-1}$ for Al/ECR-ODS and $0.02 \mu\text{g ml}^{-1}$ for Al/Q-ODS.

The calibration curve for Al-ECR/MTOAC-Naph is shown in Fig. 6. The calibration curve was performed from 0.1 up to $3 \mu\text{g ml}^{-1}$. The correlation coefficient r^2 shows a good linearity between Abs and $\log C$. The same procedure was carried out in order to find out the detection limit. The LOD was $0.001 \mu\text{g ml}^{-1}$ for this system.

The calibration curves for Al-ECR/MCM-41 (column pre-concentration method) and for Al/ECR-MCM-41 (spot-test) are shown in Fig. 7a and b, respectively. In order to improve the performance of aluminum determination by spot-test on MCM-41, factors such as concentration of reagent, thickness of adsorbent and time of measurement following the color appearance on the adsorbent disk was modified. Fig. 8 shows how these parameters have been obtained. The pH used was previously chosen as 6.1.

The calibration curves for Al-ECR/MCM-41 and Al/ECR-MCM-41 shows good linearity for both ($r^2 = 0.996$ for Al-ECR/MCM-41 and $r^2 = 0.973$ for Al/ECR-MCM-41). However the linear dynamic range is higher for Al-ECR/MCM-41. The detection limits are rather similar and is about $0.01 \mu\text{g ml}^{-1}$ for both of Al-ECR/MCM-41 and Al/ECR-MCM-41 systems.

3.4. Interference problem

There are three main species: beryllium, vanadium and zirconium where interfere in the determination of aluminum with ECR under chosen condition (pH 6.1) [29]. In the present study our material was free from these ions. Our complementary investigation will be focused on studying the problem of interference by mixing these species separately with our pure aluminum sample and carrying out the DR studies. The type and property of the

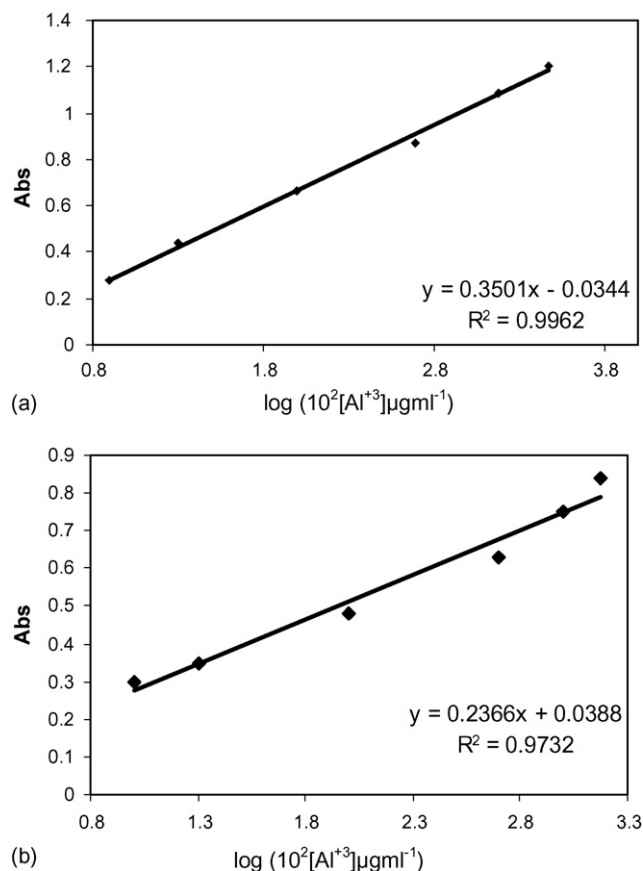


Fig. 7. Calibration curve for aluminum for (a) Al-ECR/MCM-41 adsorbent system, pH 6.1, $C_{\text{ECR}} = 1 \times 10^{-3}$ M, $\lambda_{\text{max}} = 530$ nm, flow rate = 2.5 ml min^{-1} and (b) Al/ECR-MCM-41 spot-test system, pH 6.8, $C_{\text{ECR}} = 5 \times 10^{-4}$ M, $\lambda_{\text{max}} = 530$ nm.

adsorbent in better adsorbing desirable species and not adsorbing the interference ions will be our target.

The results of some common interfere ions for our spot-test measurement of aluminum is summarized in Table 2. Synthetic solution containing $0.2 \mu\text{g ml}^{-1}$ of aluminum and various concentration of ions were prepared and the procedure was followed (at $\lambda_{\text{max}} = 530$ nm). A relative error of $\pm 3\%$ in the Abs reading was considered tolerable.

3.5. Analytical application

The proposed methods were applied to aluminum determination in tap water in Rasht. The results obtained are shown in

Table 2
Effect of interfere ions

Ion added	Tolerance limit ($\mu\text{g ml}^{-1}$)
Ca^{2+}	200
Mg^{2+}	165
Mn^{2+}	130
Ba^{2+}	150
Cu^{2+}	220
Cl^-	125
Co^{2+}	25
Ni^{2+}	40

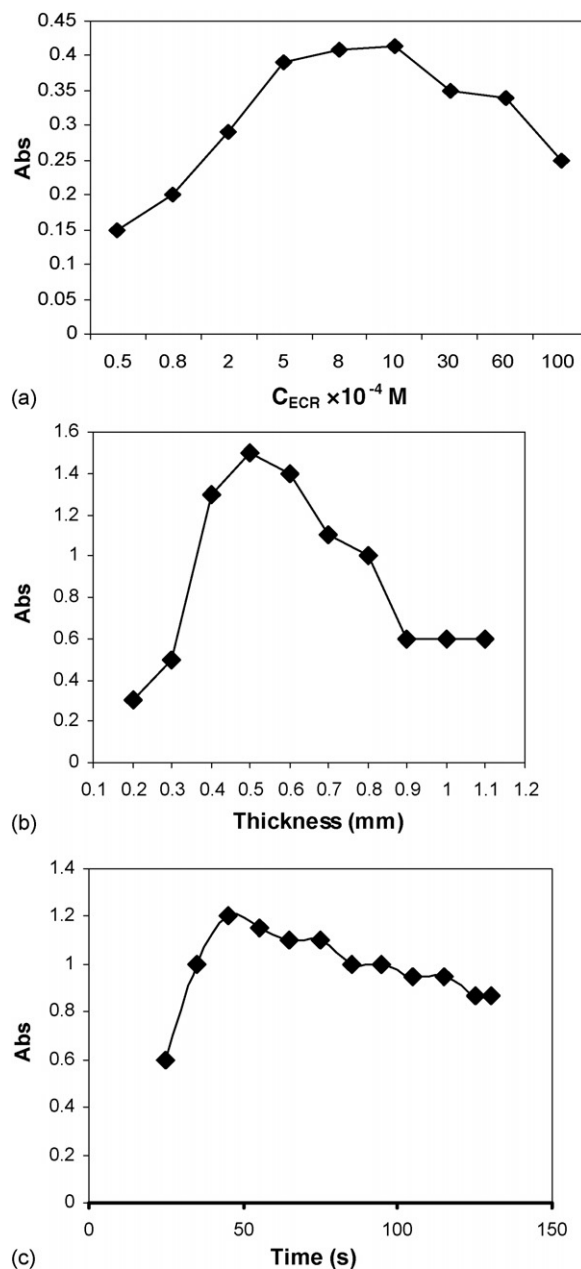


Fig. 8. Effect of some experimental parameters on the efficiency of spot-test for determination of aluminum spotted on MCM-41 adsorbent, (a) ECR concentration, (b) thickness of adsorbent and (c) time of measurement after spotting.

Table 3. The results obtained by DRS methods are higher in comparison with that given by Rasht Water Authority Laboratory ($0.12 \mu\text{g ml}^{-1}$). This is most probably due to interference from Mg^{2+} and Ca^{2+} cations present in high amounts in the city water. In this condition, pre-separation of these cations should be recommended.

In order to evaluate the accuracy and applicability of the rapid Al/ECR-MCM-41 technique for the analysis of real samples, it was also applied to the determination of aluminum in two commercial bottled mineral waters (distributed in Iran) and in “soft-water” used in a textile factory in Rasht (water passed through zeolite and green sands). The results are compared with atomic absorption data (Table 4).

Table 3
Determination of aluminum in tap water by DRS techniques

Reflecting sample	Sample no.	Standard aluminum added ($\mu\text{g ml}^{-1}$)	Found ($\mu\text{g ml}^{-1}$) ^a
Al/ECR–MCM-41 (spot-test)	1	–	0.16 (± 0.01)
	2	0.5	0.71 (± 0.01)
	3	1.0	1.21 (± 0.04)
Al–ECR/MCM-41	1	–	0.17 (± 0.01)
	2	0.5	0.71 (± 0.02)
	3	1.0	1.23 (± 0.01)
Al–ECR/MTOAC–Naph	1	–	0.18 (± 0.01)
	2	0.5	0.73 (± 0.02)
	3	1.0	1.26 (± 0.01)

^a Mean of three determinations.

Table 4
Analysis of aluminum by Al/ECR–MCM-41 reflecting system and atomic absorption (concentrations in $\mu\text{g ml}^{-1}$)

Sample	Proposed technique ^a	Atomic absorption
Damash mineral water	0.039 (± 0.004)	0.042
Damavand mineral water	0.029 (± 0.004)	0.033
Soft water	0.018 (± 0.005)	0.013

^a Mean of three determinations.

4. Conclusion

The results show that the appropriate use of the diffuse reflectance spectroscopy allows determining low concentration of aluminum preadsorbed on a suitable adsorbent disk by pre-concentration or spot-test techniques. Commercial octadecyl silica disk or home-made methyltrioctylammonium chloride–naphthalene and MCM-41 disks provide proper media for adsorbing the colored species. Eriochrome cyanine R or quinalizarin were employed as coloring reagents. To ensure satisfactory results, some conditions such as pH of the adsorbing media, concentration of the reagent, thickness of the home-made disks and time of the reflectance reading were optimized. The adaptation of spot-test method in conjunction with the DRS, provide a very simple and relatively rapid determination of aluminum. The linear range is relatively wide for all of the combination of the employed reagents and the adsorbents within general range of 0.01–15 $\mu\text{g ml}^{-1}$.

In this method, no solvent elution is required because the amount of the colored analyte complex is directly measured on the disk by reflectance spectroscopy. Also, a better environmental impact is achieved due to lower production of toxic wastes. Possibility of construction of a portable reflectometer can be envisaged for the spot-test technique improving the performance and speed of the measurement of aluminum.

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The utilization of modified BCR three-step sequential extraction procedure for the fractionation of Cd, Cr, Cu, Ni, Pb and Zn in soil reference materials of different origins

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Abstract

A modified three-step sequential extraction procedure for the fractionation of heavy metals, proposed by the Commission of the European Communities Bureau of Reference (BCR) has been applied to the Slovak reference materials of soils (soil orthic luvisols, soil rendzina and soil eutric cambisol), which represent pedologically different types of soils in Slovakia. Analyses were carried out by flame or electrothermal atomic absorption spectrometry (FAAS or ETAAS). The fractions extracted were: exchangeable (extraction step 1), reducible-iron/manganese oxides (extraction step 2), oxidizable-organic matter and sulfides (extraction step 3). The sum of the element contents in the three fractions plus aqua-regia extractable content of the residue was compared to the aqua-regia extractable content of the elements in the origin soils. The accuracy obtained by comparing the determined contents of the elements with certified values, using BCR CRM 701, certified for the extractable contents (mass fractions) of Cd, Cr, Cu, Ni, Pb and Zn in sediment following a modified BCR-three step sequential extraction procedure, was found to be satisfactory. © 2006 Elsevier B.V. All rights reserved.

Keywords: Chemical speciation; Heavy metals; Sequential extraction; Soils

1. Introduction

The mobility of metals in the environment depends strongly on their chemical forms or type of the binding of the element. The determination of total concentrations in soils does not give sufficient information about the mobility of metals. In many instances, biological toxicity of the elements is related more closely to the concentrations of particular physico-chemical forms [1]. Speciation studies of trace elements in various phases in the environment are widely used in investigating the effects of a particular element in the ecosystem. Metal cations in soils may be present in several different physico-chemical forms, i.e. as simple or complex ions, as easily exchangeable ions, as organically bound, as occluded by or coprecipitated with metal oxides or carbonates or phosphates and secondary minerals, or as ions in crystal lattices of primary minerals [2].

In order to assess the chemical forms of heavy metals in soils, extraction procedures have been applied, both as single and sequential schemes. The use of sequential extraction furnishes detailed information about origin, mode of occurrence, mobilization and transport of trace metals. Many of the sequential extraction schemes employed are based on the five-stage procedure of Tessier et al. [3]. The literature on metal speciation in solid matrices, taking into account its application in the analysis of soils is reviewed by Das et al. [4]. An improvement in analytical measurements for single and sequential procedures applied to soil and sediment analysis has been reported by Quevauviller et al. [5].

In the framework of BCR, a three-step sequential extraction procedure has been developed, which has been applied to a variety of matrices, including sediments, soils and sewage sludges [6–9]. The study has revealed some sources of uncertainty in application of the BCR three-stage sequential extraction procedure to sediments and has indicated modifications to the protocol [10,11]. The modified extraction scheme is an operationally defined procedure [12] in which the reagent used at each stage is

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Table 1
Modified BCR three-step sequential extraction procedure

Extraction step	Reagent(s)	Nominal target phase(s)
1	CH ₃ COOH (0.11 mol l ⁻¹)	Soil solutions, carbonates, exchangeable metals
2	NH ₂ OH·HCl (0.5 mol l ⁻¹)	Iron/manganese oxyhydroxides
3	H ₂ O ₂ (8.8 mol l ⁻¹) then CH ₃ COONH ₄ (1 mol l ⁻¹), pH 2	Organic matter and sulfides
Residual	Aqua regia	Remaining, non-silicate bound metals

intended to release metals associated with particular soil phases (Table 1). This procedure is widely used in European Union laboratories for improving the reproducibility among laboratories [10].

Sequential extraction procedures are usually applied to assess metal association with the different solid phase components in soils. Standard procedures need certified reference samples. Two materials were certified for the BCR procedure but these are both sediments, CRM 601 and CRM 701. There is an evident need for a wider range of reference materials with values for the BCR procedure, in particular to address the lack of soil reference materials. The three-step sequential extraction procedure followed the modified BCR protocol was applied to Slovak soil reference materials (soil orthic luvisols, soil rendzina and soil eutric cambisol) with certified total values of the elements. Hence, we have established the extractable concentrations of six heavy metals in mentioned three soil reference materials for which the total concentrations have been established. The main purpose of the work was to characterize these soils using the BCR procedure and to provide additional valuable information for these certified materials, which are easily available to the analytical community. The soil extracts were analyzed by flame atomic absorption spectrometry (FAAS) or electrothermal atomic absorption spectrometry (ETAAS). The six elements determined in the extracts were cadmium, chromium, copper, nickel, lead and zinc.

2. Experimental

2.1. Apparatus

A Perkin-Elmer flame atomic absorption spectrometer 1100B (Norwalk, Connecticut, USA), with air–acetylene flame (air flow rate 8.0 l min⁻¹, acetylene flow rate 2.5 l min⁻¹), was used to determine zinc in the extracts and copper, nickel, zinc in the digests and air–acetylene flame (air flow rate 5.0 l min⁻¹, acetylene flow rate 2.5 l min⁻¹ with adding of 1% solution of NH₄Cl

as releasing agent) to determine chromium in the digests. Cadmium, chromium, copper, nickel and lead concentrations in the extracts were determined by graphite furnace atomic absorption spectrometry (THGA: transverse heated graphite atomizer) with Zeeman-effect background correction 5100ZL, using a Perkin-Elmer Zeeman atomic absorption spectrometer 5100PC, fitted with AS 71 furnace autosampler. Recommended modifiers, magnesium nitrate and palladium (0.005 mg Pd + 0.003 mg Mg(NO₃)₂) for copper; ammonium phosphates and magnesium nitrate (0.05 mg NH₄H₂PO₄ + 0.003 mg Mg(NO₃)₂) for cadmium and lead; magnesium nitrate (0.015 mg Mg(NO₃)₂) for chromium were used for the determination using THGA tubes with integrated L'vov platform. The injection volume was 30 µl (20 µl of the extract + 10 µl of the modifier). The operational instrumental parameters and optimum charring and atomization temperatures recommended by the manufacturer for the determination of the analytes, are shown in Table 2.

A KS125 basic IKA-shaker (IKA Labortechnik, Germany) and a K70D centrifuge (MLW, Germany) were used for extraction and to separate extractants from residues in the sequential extraction procedure. The vessels made from PTFE were used throughout the whole procedure. The aqua-regia soil digestion of the residue after sequential extraction procedure and original reference materials of soils was performed using the recommended procedure ISO 11466. All glass-ware and plastic containers were previously soaked overnight in 10% nitric acid and rinsed with deionized water. PRO-PS Labconco (Kansas City, KS, USA) was used for producing of deionized water.

2.2. Reagents

All reagents were obtained from Merck, Darmstadt, Germany. Calibrants were prepared by serial dilution of 1000 µg ml⁻¹ Merck solutions of the appropriate elements in 0.2% nitric acid. BCR-701 (EUR 19775), with the certified extractable contents (mass fraction) of Cd, Cr, Cu, Ni, Pb and

Table 2
Instrumental parameters and optimum charring and atomization temperatures for determination of analytes by ETAAS (°C)

	Cd	Cr	Cu	Pb	Ni	Zn ^a
Wavelength (nm)	228.8	357.9	324.8	283.3	232.0	213.9
Lamp intensity (mA)	6	25	15	10	25	20
Slit-width (nm)	0.7	0.7	0.7	0.7	0.2	0.7
Charring temperature	700	1500	1200	850	1100	
Atomization temperature	1400	2300	1900	1500	2300	
Type of signal	Peak area					

^a FAAS used for determination.

Zn in sediment following the modified BCR-three step sequential extraction procedure, was used to verify the accuracy of the procedure and determination of the elements.

2.3. The samples

The pedologically different types of Slovak soil reference materials with certified total values of Cd, Cr, Cu, Ni, Pb and Zn, according to the international classification FAO, S-VM No. 12-1-07 Soil Eutric Cambisol (Hills of Medzev near the town Kosice), S-MS No. 12-1-08 Soil Orthic Luvisols (Interhill of Saris near the town Presov), S-SP No. 12-1-09 Soil Rendzina (Plateau of Silica near the town Roznava), produced by the Institute of Radioecology and Applied Nuclear Techniques, Kosice, Slovakia, were used for this study [13].

Certified and recommended total studied and major elements in the reference materials of soils: S-VM, S-MS and S-SP are in Table 3.

2.4. Procedures

2.4.1. Sequential extraction

Sequential extraction was performed using the modified three-stage procedure recommended by BCR (Table 1) on 1 g of soil sample in PTFE vessels (100 ml volume) with PTFE covers (originally used for digestion under elevated pressure in autoclave):

- *Step 1.* A 40 ml volume of acetic acid (0.11 mol l^{-1}) was added to 1 g of soil in a PTFE vessel. The vessel was shaken for 16 h (overnight) at room temperature in a mechanical shaker operating at $300 \text{ motions min}^{-1}$ for 20 min, the shaking speed was adapted in order to ensure a continuous suspension of the mixture. The extract was separated from the solid residue by centrifugation for 20 min and decanted into a polyethylene container and stored in a refrigerator at 4°C for analysis. The residue was washed with 20 ml of distilled

water by shaking for 20 min, centrifuged and the washings discarded.

- *Step 2.* To the residue from step 1, 40 ml of a freshly prepared hydroxylamine hydrochloride, 0.5 mol l^{-1} (pH was adjusted by adding of 25 ml of $2 \text{ mol l}^{-1} \text{ HNO}_3$ to 1 l volumetric flask to the dissolved hydroxylamine hydrochloride (34.75 g)) in deionized water. The extraction procedure was then performed as described above.
- *Step 3.* A 10 ml of hydrogen peroxide (8.8 mol l^{-1}) was added carefully in small aliquots to the residue from step 2. The vessel was loosely covered with the watch glass and digested at room temperature for 1 h with occasional manual shaking. Digestion was continued by heating the vessel to 85°C in a water bath for 1 h, and then reduced the volume to less than 3 ml by further heating of the uncovered vessel. A second 10 ml of aliquot of hydrogen peroxide was added and the digestion procedure was repeated. Fifty milliliters of ammonium acetate (1 mol l^{-1} , adjusted to pH 2 with nitric acid) were added to the cool moist residue. The sample was shaken, centrifuged and the extract was separated as described in step 1. The solid residue was retained for aqua regia digestion.

2.4.2. Aqua regia digestion

Pseudototal metal content was determined by digestion with aqua regia, according to the ISO Norm (11466). One gram of the soil was weighed into the reaction vessel, 0.5–1.0 ml of water was added to obtain a slurry, then 7.0 ml of $12.0 \text{ mol l}^{-1} \text{ HCl}$ followed by 2.3 ml of $15.8 \text{ mol l}^{-1} \text{ HNO}_3$, drop by drop, to reduce foaming, was added. The reaction vessel was allowed to stand for 16 h (overnight) at room temperature for slow oxidation of the organic matter of the soil. The temperature of the reaction mixture was slowly raised, until reflux conditions were reached and maintained for 2 h. After cooling the reaction vessel to room temperature the digests were filtered through Whatman No. 541 filter paper into 50 ml volumetric flasks, the insoluble residue onto the filter paper was washed with a $0.5 \text{ mol l}^{-1} \text{ HNO}_3$ and the volumetric flask was filled with $0.5 \text{ mol l}^{-1} \text{ HNO}_3$ up to the

Table 3

Certified and recommended values of the total studied and major elements in the reference materials of soils S-SP, S-MS, S-VM [13]

Element	S-SP content ($\mu\text{g g}^{-1}$)		S-MS content ($\mu\text{g g}^{-1}$)		S-VM content ($\mu\text{g g}^{-1}$)	
	CV	CI	CV	CI	CV	CI
Al ^{*#}	7.48		5.76		8.96 [#]	
Ca [*]	6.34	6.09–6.59	0.49 [#]		0.692 [#]	
Fe [*]	3.73	3.55–3.92	2.70	2.59–2.81	3.73	3.56–3.89
K [*]	2.63	2.41–2.86	1.85	1.74–1.95	3.08	2.89–3.27
Mg ^{*#}	1.19		0.627		0.593	
Mn	734	700–768	910	876–945	897	853–942
Na ^{*#}	0.446		0.807		0.321	
P [#]	1370		1010		1350	
Si ^{*#}	20.4		31.4		24.8	
Zn	119	112–125	63.7	59.5–67.8	88.8	85.6–92.1
Cu	30.9	29.1–32.8	21.2	19.5–22.9	30.0	27.9–32.1
Ni	37.4	34.2–40.7	40.0	37.5–42.5	30.8	28.7–32.9
Pb	41.3	36.9–45.7	18.9	16.7–21.2	19.6	17.5–21.8
Cd	0.285	0.226–0.343	0.198	0.169–0.227	0.214	0.176–0.253
Cr	75.3	72.1–78.4	87.4	77.0–97.7	79.8	74.0–85.5

CV: certified value; CI: confidence interval (95%); * denotes the values in %; # denotes the non-guaranteed values.

Table 4
Detection limits for analytes in the sequential extraction reagents and 20% aqua regia ($\mu\text{g g}^{-1}$)

Analyte	Step 1	Step 2	Step 3	Aqua regia
Cd	0.003	0.003	0.005	0.003
Cr	0.020	0.077	0.019	0.012
Cu	0.030	0.030	0.068	0.120
Ni	0.042	0.050	0.067	0.038
Pb	0.001	0.001	0.001	0.003
Zn	0.12 ^a	0.12 ^a	0.19 ^a	0.29 ^a

^a FAAS (all other values are ETAAS).

Table 5
Summary of the results obtained by sequential extraction and pseudototal digestion for cadmium, chromium, copper, nickel, lead and zinc ($\mu\text{g g}^{-1}$)

Element	Soil	Step 1	Step 2	Step 3	Residual	$\sum 1 + 2 + 3 + \text{residual}$
Cd*	S-MS	80.9 $\pm$ 3.2	76.9 $\pm$ 3.4	27.9 $\pm$ 2.3	6.09 $\pm$ 0.33	192
	S-SP	88.0 $\pm$ 2.8	174 $\pm$ 4.0	25.6 $\pm$ 2.1	6.85 $\pm$ 0.45	294
	S-VM	114 $\pm$ 11	81.2 $\pm$ 3.75	18.7 $\pm$ 1.4	3.81 $\pm$ 0.28	218
Cr	S-MS	0.106 $\pm$ 0.009	1.73 $\pm$ 0.32	3.93 $\pm$ 0.45	33.3 $\pm$ 1.8	39.1
	S-SP	0.062 $\pm$ 0.006	1.35 $\pm$ 0.25	4.76 $\pm$ 0.38	35.0 $\pm$ 2.0	41.2
	S-VM	0.098 $\pm$ 0.008	1.26 $\pm$ 0.20	2.88 $\pm$ 0.29	23.5 $\pm$ 1.5	27.7
Cu	S-MS	0.468 $\pm$ 0.042	2.50 $\pm$ 0.12	0.526 $\pm$ 0.036	15.0 $\pm$ 0.9	18.5
	S-SP	0.186 $\pm$ 0.022	2.02 $\pm$ 0.09	1.22 $\pm$ 0.042	26.4 $\pm$ 1.3	29.8
	S-VM	0.725 $\pm$ 0.036	5.64 $\pm$ 0.20	1.41 $\pm$ 0.045	19.9 $\pm$ 1.1	27.7
Ni	S-MS	2.69 $\pm$ 0.08	5.14 $\pm$ 0.15	3.09 $\pm$ 0.14	20.4 $\pm$ 1.2	31.3
	S-SP	0.830 $\pm$ 0.038	4.85 $\pm$ 0.12	2.73 $\pm$ 0.12	19.3 $\pm$ 1.1	27.7
	S-VM	0.876 $\pm$ 0.026	2.07 $\pm$ 0.08	1.76 $\pm$ 0.09	17.3 $\pm$ 0.9	22.0
Pb	S-MS	0.121 $\pm$ 0.013	12.0 $\pm$ 0.9	0.637 $\pm$ 0.025	2.09 $\pm$ 0.08	14.8
	S-SP	0.093 $\pm$ 0.007	21.5 $\pm$ 1.2	1.43 $\pm$ 0.08	14.8 $\pm$ 1.5	37.8
	S-VM	0.155 $\pm$ 0.015	15.0 $\pm$ 1.1	0.809 $\pm$ 0.021	1.72 $\pm$ 0.22	17.7
Zn	S-MS	2.02 $\pm$ 0.05	6.85 $\pm$ 0.09	3.98 $\pm$ 0.05	41.5 $\pm$ 1.3	54.4
	S-SP	1.98 $\pm$ 0.04	17.4 $\pm$ 0.55	5.72 $\pm$ 0.07	84.9 $\pm$ 2.0	110
	S-VM	3.81 $\pm$ 0.07	6.37 $\pm$ 0.07	7.23 $\pm$ 0.08	65.5 $\pm$ 2.5	82.9

* in ng g^{-1} .

Table 6
Comparative results of analyses of the direct aqua regia digestion and the BCR sequential extraction procedure of the soil samples ($n = 4$), based on the mean element contents ($\mu\text{g g}^{-1}$)

Element	Soil	Sum of the extraction steps	Direct procedure of aqua regia	Recovery (%) (extraction/digestion)
Cd*	S-MS	192	200	96
	S-SP	294	290	101
	S-VM	218	210	104
Cr	S-MS	39.1	41.4	94
	S-SP	41.2	43.4	95
	S-VM	27.7	29.3	95
Cu	S-MS	18.5	18.5	100
	S-SP	29.8	28.3	105
	S-VM	27.7	27.4	101
Ni	S-MS	31.3	34.6	90
	S-SP	27.7	28.9	96
	S-VM	22.0	24.0	92
Pb	S-MS	14.8	14.6	101
	S-SP	37.8	39.0	97
	S-VM	17.7	16.5	101
Zn	S-MS	54.4	55.5	98
	S-SP	110	109	101
	S-VM	82.9	84.2	98

* in ng g^{-1} .

mark. Residual material remaining after step 3 of the sequential extraction was digested in the same manner after quantitative transferring of the residual from PTFE vessel to the glass flask.

Reagent blanks were negligible and no detectable contamination was found when aliquots of the sequential extraction reagents were processed and analyzed with the samples. Detection limits (three times the standard deviation of the blank, divided by the slope of the calibration graph) are given in Table 4.

Table 7

Certified and determined contents of extractable contents of Cd, Cr, Cu, Ni, Pb and Zn in certified reference material of fresh sediment CRM 701

Element	Element contents ($\mu\text{g g}^{-1}$)					
	Step 1		Step 2		Step 3	
	Certified value	Determined value	Certified value	Determined value	Certified value	Determined value
Cd	7.34 ± 0.35	7.39 ± 0.23	3.77 ± 0.280	3.81 ± 0.31	0.27 ± 0.060	0.21 ± 0.08
Cr	2.26 ± 0.16	2.07 ± 0.18	45.7 ± 2.0	52.0 ± 3.0	143 ± 7	104 ± 8
Cu	49.3 ± 1.7	50.3 ± 1.8	124 ± 3	124 ± 5	55.2 ± 4	53.5 ± 3
Ni	15.4 ± 0.9	15.4 ± 1.2	26.6 ± 1.3	27.1 ± 1.6	15.3 ± 0.9	15.8 ± 1.2
Pb	3.18 ± 0.21	3.26 ± 0.34	126 ± 3	122 ± 6	9.3 ± 2.0	8.29 ± 1.3
Zn	205 ± 6.0	207 ± 8.0	114 ± 5	115 ± 7	45.7 ± 4	45.6 ± 2.6

3. Results and discussion

The contents of Cd, Cr, Cu, Ni and Pb in the extracts were determined by electrothermal atomic absorption spectrometry, Zn in the extracts and Zn, Cr, Cu, Ni in the aqua regia digest of original soil sample were determined by flame atomic spectrometry. The results are presented in Table 5.

An internal check was performed on the results of the sequential extraction by comparing the total amount of metals removed in the procedure with the results of the pseudototal digestion. The recovery of the sequential extraction was calculated as follows:

$$\text{recovery} = [(\text{step 1} + \text{step 2} + \text{step 3} + \text{residual})/\text{pseudototal}] \times 100$$

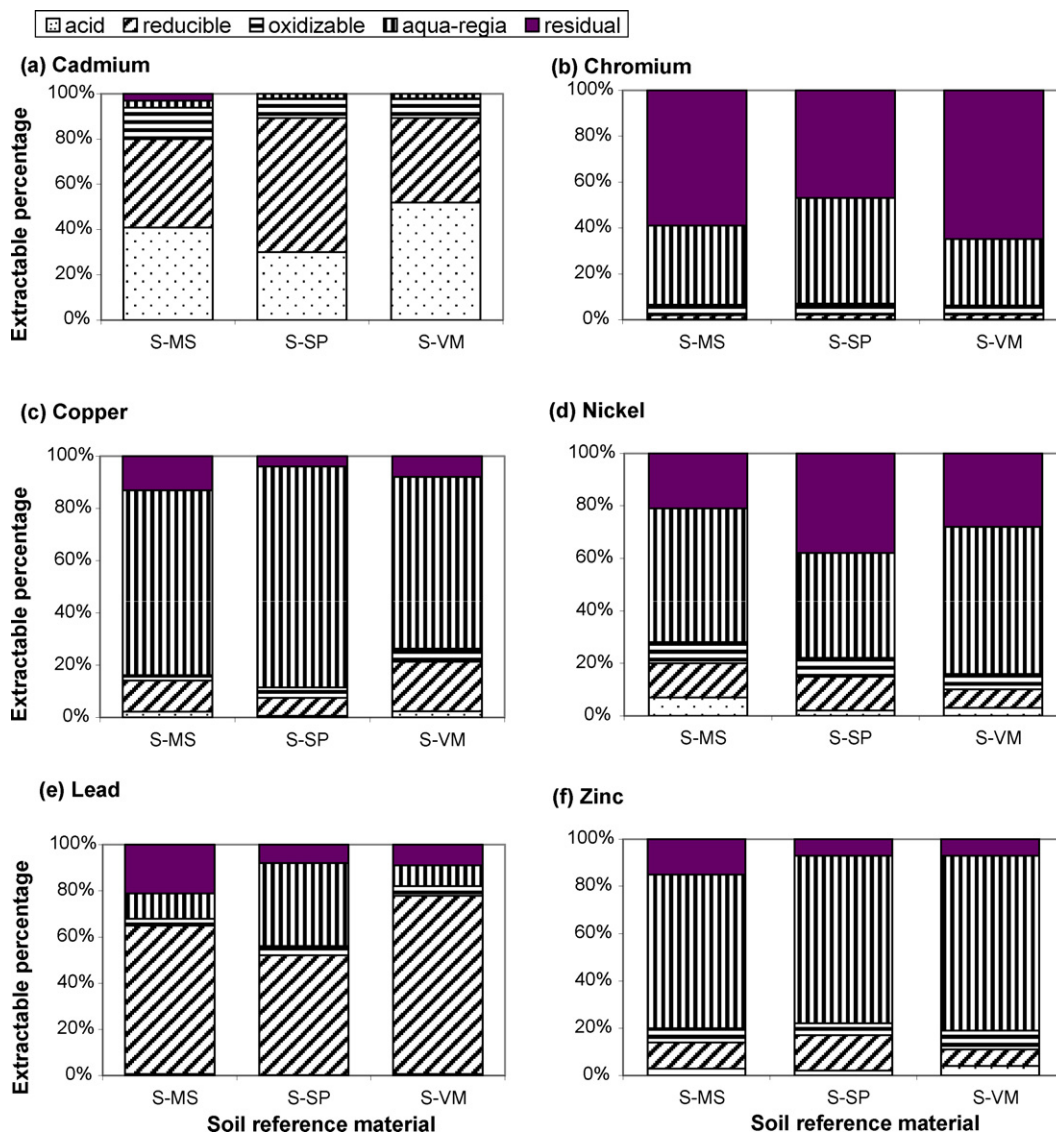


Fig. 1. Metal distributions obtained for the soils S-SP, S-VM, S-MS using the modified BCR sequential extraction procedure.

Comparison of the sum of the metal concentrations in the sequential extraction steps with this pseudototal metal concentrations showed a good agreement for all elements except of chromium and nickel. The results are given in Table 6.

The accuracy of the sequential extraction procedure and the determination of Cu, Pb, Cd, Cr, Ni and Zn in the extracts was verified by using certified reference material CRM 701 (BCR, Brussels). The sequential extraction procedure of the certified reference material of the sediment, CRM 701, was performed as part of the batch and analyzed at the same time as the samples. The determined contents of the elements were compared with the certified extractable contents of Cd, Cr, Cu, Ni, Pb and Zn. The results (mean values $\pm$ standard deviation (S.D.) for $n = 4$) of the determined contents using FAAS, and ETAAS in comparison to certified values are in Table 7. From the results very good agreement can be seen.

Fig. 1 shows the extracted percentages of Cd, Cr, Cu, Ni, Pb and Zn in all steps of the sequential extraction procedure of the soils S-SP, S-VM, S-MS. The figure presents the distribution of the elements among fractions: (1) exchangeable and associated with carbonates, (2) associated with easily and moderately reducible iron and manganese oxyhydroxides, (3) associated with organic matter and sulfides, (4) aqua-regia extractable residual fraction, non-silicate bound metals, and (5) the metal residual fraction—metals bound in mineral lattice. The metal contents in the fractions are evaluated in percentages comparing to the certified total value, which represents 100%.

From the figure is obvious, that most cadmium is divided into the first (31–53%) and the second fraction (38–61%). The proportion of cadmium in the third fraction is relatively small (9–14%). Almost any cadmium does not occur in the residual fraction.

On the contrary, chromium was found exclusively in the residual fraction (47–65%) bound in mineral lattice, the aqua-regia extractable fraction represents non-silicate bound chromium (29–46%), from which release of chromium is unlikely under environmental conditions. Copper and nickel were found mainly in association with aqua-regia extractable fraction (66–85%, 40–56%, respectively), but both reducible (7–19%, 7–13%, respectively) and oxidizable (2–5%, 6–8%, respectively) forms of copper and nickel occur. The fractionation, that enables the distinguishing of copper and nickel bound to the humic compounds and the sulfides [14] has showed, that more copper is bound to the humic compounds than nickel, on the contrary larger amount of nickel is bound as sulfide. It is particularly interesting to note that only a small fraction of lead is presented as acid exchangeable species, a predominance of reducible lead (52–77%) has been found in sequential extraction, and a significant proportion (9–36%) is in aqua-regia extractable fraction. The rest of lead (8–21%) is bound in mineral lattice. Zinc is presented predominantly in aqua-regia extractable fraction (65–74%), in reducible fraction occurs 7–15% and also 7–15% of total content of Zn is associated with residual fraction.

4. Conclusions

The extraction procedure validated by the Measurement and Testing Programme – MAT (formerly BCR) – of the European Commission, was applied to different types of soil reference materials. The used sequential extraction procedure permits the evaluation of the various chemical forms present in the soils. The applicability of the BCR method is easy, the method was found to be both, repeatable and sufficiently reproducible for environmental monitoring purposes and is suitable for hygienic and soil laboratories.

Our results obtained by the modified BCR sequential extraction procedure can be utilized as indicative values for the study of fractionation of cadmium, chromium, copper, nickel, lead and zinc in similar types of soils as the Slovak reference materials of soils, soil orthic luvisols S-MS, soil rendzina S-SP, and soil eutric cambisol S-VM.

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