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Fast HPLC-DAD quantification procedure for selected sulfonamids, metronidazole and chloramphenicol in wastewaters using second-order calibration based on MCR-ALS

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ABSTRACT

The present work focuses on the application of multivariate curve resolution-alternating least squares (MCR-ALS) for analysis of five important antibiotics (sulfamethoxazole, metronidazole, chloramphenicol, sulfadizine and sulfamerazine) in highly complex wastewater samples, using solid phase extraction (SPE)-high performance liquid chromatography with diode-array detection (HPLC-DAD) on a short column, regarding the fast elution methodology. The samples were pre-concentrated on Bond Elut-ENV cartridges and separated on an ODS column (7 cm) in less than 4 min using an isocratic mode of elution with methanol–water (55:45, v/v) at pH=3.2. Due to the matrix interferences and the resulting sensitivity changes, a strategy implementing standard addition calibration in combination with MCR/ALS algorithm was applied. In this paper, the signal corresponding to background contribution was considered as a systematic part of the model during MCR/ALS data processing, so the background correction step was not necessary. The qualitative and quantitative results showed that the application of MCR/ALS algorithm in the traditional chromatographic method was appropriately able to resolve highly drifted background constituents as well as heavily overlapped peaks among the analytes and also between the analytes and the matrix interferences. Recoveries were ranged from 69.6% to 120.3% with relative standard deviations of less than or equal to 11.0% and showed the acceptable performance of the method. Additionally, statistical *t*-test as well as computed elliptical joint confidence region (EJCR) confirmed the accuracy of the proposed method and indicated the absence of both constant and proportional errors in the predicted concentrations. The results well explained that the second-order advantage for analytes was achieved in samples containing one or more uncalibrated components, which strongly related to wastewater samples.

1. Introduction

Antibiotics, as an important class of pharmaceuticals, are widely prescribed to prevent and to treat bacterial infections in human and animals. They are also used to promote growth and to enhance productivity in the industrial agriculture and aquaculture. Due to the particular concerns over the occurrence of antibiotics in water resources, several research studies have been conducted for their detection and determination in various environmental media [1]. Antibiotics may be released to the environment, either intact or as metabolites, during one or more of the production, consumption, excretion and disposal procedures. They also may show a wide variety of persistency levels against natural biochemical and/or chemical degradation processes. Inappropriate or insufficient removal procedures, utilized in the wastewater treatment plants (WWTPs), can transform these units into the remarkable sources of pollution by antibiotics, especially in the case of sewage as well as pharmaceutical and agricultural industries. In addition to the adverse effects of antibiotic compounds on the recipient ecosystems, sometimes long term presence of these compounds in water resources results in the appearance of antimicrobial-resistant bacterial communities through the continuous exposure [2–5]. There is also an increasing interest in the biodegradability and the fate of these compounds in the environment. The majority of the antibiotics are regarded as pseudo-persistent pollutants, which may be able to persist enough in the environment to harmfully alter biodiversity of ecosystems and also possibly affect human health [6]. Several classes of antimicrobial compounds have been detected in different water samples, at concentrations of microgram and/or nanogram per liter [7–18]. Practically, accurate and precise quantification of trace amounts of antibiotics in complex matrices, such as wastewater effluents, has been a challenging goal, mainly because

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the matrix components can easily attenuate selectivity and sensitivity of the entire analytical procedure.

Sulfonamides (SAs), as an important class of antimicrobials, are widely used for both human and animals. These compounds are known to have high potential to resist degradation and also, are hydrophilic enough to be transported in the aquatic environment [19]. During the recent years, several analytical methodologies concerning different sample preparation strategies and instrumental techniques have been developed for the analysis of trace amounts of sulfonamides and the other selected antibiotics, in surface water resources and wastewaters [20]. In spite of this fact that the modern method development researches have shown a trend toward proposing faster sample preparation procedures and reducing the required sample volume, SPE procedure is still being implemented in a large number of reported methodologies for preconcentration and extraction of antibiotics from water samples. One of the most challenging problems in the analysis of highly complex matrices by SPE on the conventional sorbents is co-extraction of matrix interferences, of which some are typically present at much higher concentration than the analytes of interest. These unwanted matrix components can interfere with the detection of target analytes and also suppress the extraction efficiency and recovery of the SPE procedure, leading to loss of sensitivity and selectivity of the entire analytical method. It is well known, that the matrix constituents present in the real samples, may affect the interaction of analytes with SPE sorbent, may form complexes with the target analytes or may interact with the sorbent and reduce the number of free sites or the active surface available for the retention of analytes [21]. In fact, lack of selectivity occurs when the blank matrix has a drifting background, interfering constituents or overlapping components, which may also add up to the signal of the analyte and lead to a constant systematic error [22]. Presence of organic and inorganic components in the samples can also affect the slope of calibration curve and produce relative systematic error [23]. One of the most important points in handling such samples is that the matrix constituents in different wastewater samples may vary from sample to sample, in nature and the relative concentration. In such cases, lots of time, effort and resources must be devoted to remove and/or compromise the variant effects of different samples and to obtain validated quantitative or qualitative results.

Regarding the mentioned challenges, the use of second-order calibration methods in combination with hyphenated chromatographic system has been proved to have a high potential for resolution and quantification of analytes in such complex samples through exploiting second-order advantage [24]. Among different second-order calibration algorithms, multivariate curve resolution-alternating least squares (MCR-ALS) [25,26] and parallel factor analysis2 (PARAFAC2) [27] allow deviations to the trilinearity of three-dimensional data. MCR-ALS is an excellent tool for modeling LC-DAD data, especially when retention time shifts exist between chromatograms. In fact, whenever significant matrix effect makes conventional data processing become problematic and inaccurate, combination of second-order calibrations, such as MCR-ALS with standard addition methodology, can be utilized as a powerful strategy for efficient resolution of overlapping peaks and multitarget determination, without the need for the exact knowledge of the interfering components characteristics [28].

The present study in particular, was developed for determination of selected sulfonamids (sulfamethoxazole, sulfadizine and sulfamerazine) as well as metronidazole and chloramphenicol in effluent wastewaters collected from two sewage treatment plants, using fast HPLC-DAD analysis. At first, the matrix effect was confirmed and then the second-order methodology based on MCR-ALS algorithm and without any pre-processing step combined with standard addition was used to determine the target antibiotics in the wastewater samples.

2. Experimental section

2.1. Chemicals and solvents

Antimicrobial standards of sulfamethoxazole (SMX), metronidazole (MET), chloramphenicol (CAP), sulfadizine (SDZ) and sulfamerazine (SMR) were purchased from Sigma-Aldrich (USA). HPLC-grade methanol (MeOH), acetonitrile (ACN) and ethyl acetate (EA) were from Merck (Germany). Hydrochloric acid (32%), acetic acid (98%) and phosphoric acid, which were used in sample preparation and pH adjustment, were of analytical reagent quality from Merck. Ultrapure water was prepared using a Milli-Q water purification system from Millipore (USA). All solvents for HPLC application were filtered before use through the 0.2 μm membrane filter paper (Varian, USA). Bond Elut-ENV SPE cartridges (6 mL), containing 500 mg of polystyrene-divinylbenzene (PS-DVB), were also from Varian.

2.2. Instrumentation and software

The HPLC system (Agilent Technologies Inc., USA) included an Agilent 1200 Series system equipped with a Rheodyne 7725 manual injector with a 20-μL injection loop, a degasser system, a quaternary pump, a column oven compartment, a Hewlett-Packard 1200 series photo diode-array detector (DAD) and Chemstation software package (version B.03.01) to control the instrument, data acquisition and data analysis. Chromatographic separation was carried out on a C18 column; 70 mm × 4.6 mm and 5 μm of particle size. A Visiprep™ solid phase extraction manifold (Supelco, USA), connected to a vacuum mini pump (Aldrich, France), was used for extraction and clean up steps.

HPLC-DAD data which was gathered by Chemstation software, exported as Microsoft Excel® file format for further processing. Routines for MCR-ALS were available at (http://www.ub.edu/mcr/welcome.htm) and all algorithms were written in MATLAB (version 7.2.0.232 R2006a, The Mathworks, Natick, MA).

2.3. HPLC-DAD procedure

Analytical performance characteristics of the SPE-HPLC-DAD method were evaluated by means of a conventional univariate strategy, after complete chromatographic separation of the analytes. A gradient elution program (method A) was implemented for this purpose, using water (pH = 3.2 with acetic acid) and methanol as the mobile phase ingredients at 1.0 mL min^{-1} of flow rate. Elution started at 80:20 composition of water–methanol and remained for 3.5 min. Then this ratio changed to 30:70 in 0.5 min, remained for 2 min and returned to the initial conditions in 1 min. The mobile phase was allowed to reach equilibrium in the column for at least 3 min, between each two successive chromatographic runs.

On the other hand, the final fast chromatographic elution program (method B) consisted of a 55:45 mixture of water (pH = 3.2 with acetic acid) and methanol, running in an isocratic mode of elution. The column oven temperature was set at 30 °C. The flow rate of the mobile phase and injection volume were 1.0 mL min^{-1} and 20 μL, respectively. The total run time was less than 4 min. Samples were filtered through a 0.45 μm Nylon membrane filter before injection. Photometric detection using DAD detector was recorded between 250 and 400 nm with the spectral resolution of 2 nm and integration period of 0.4 s per spectrum.

2.4. Sample collection and preparation

Wastewater effluent samples were collected from two sewage treatment plants (STPs), located in the north (STP1) and the west

(STP2) of Tehran (Iran). The effluents were collected in amber glass bottles (2.5 L) that had been pre-cleaned successively with detergents, water, distilled water and MeOH, and finally dried. The samples were stored in darkness at 4 °C and were processed within 72 h. Before analysis, the samples were vacuum-filtered through a 1 μm micro fiber glass filter and then a 0.45 μm membrane filter.

2.5. Preparation of standard and validation sets solutions

All solutions were prepared in standard volumetric flasks. Antibiotic stock solutions were prepared by dissolving each compound in methanol at concentration of 1 mg mL^{-1} and maintained in amber vials in the freezer (−18 °C). Fresh stock solutions were made every month. Working standard solutions were prepared by successive dilution of the stock solutions with methanol/milli-Q water mixture (1:1, v/v) just before use and stored in the dark at 4 °C.

Two validation sets, containing four aliquots of each STP effluent (including an unspiked sample) were spiked at different concentration levels of five antibiotics, for recovery studies and calibration. The unspiked sample and one of the spiked samples (s3) were prepared and analyzed three times to check the repeatability of the analysis (see Table 1). Then, 0.0, 1.0–3.0, 3.5–5.5, 6.0–9.0 and 9.5–15.0 μg L^{-1} of each analyte were added to five aliquot of each previously fortified samples of the two STP effluents for calibration, using the standard addition strategy. All samples were shaken vigorously to ensure homogenization and were subjected to the following SPE procedure.

2.6. Solid phase extraction procedure

At first, the cartridges were conditioned with 6 mL of methanol, 6 mL of methanol:ethyl acetate (1:1, v/v) and then 6 mL of Milli-Q water (pH = 3) before use. Then, a predetermined volume (450 mL) of the water samples (HPLC water or wastewater samples) was passed through the cartridge, at a flow rate of 5 mL min^{-1} after adjusting the pH at 3.0 by addition of HCl. Cartridges were then cleaned up by passing 5 mL of HPLC water and then were dried under vacuum for 5 min. Desorption step was carried out by eluting the sorbent with 2 × 2 mL of methanol. The resulting extracted solutions were evaporated to dryness under a gentle stream of nitrogen and re-dissolved in 0.5 mL of mobile phase, and finally 20 μL portions of these solutions were injected into HPLC (running in the isocratic mode of elution). The above mentioned procedure would yield a theoretical enrichment factor of 900.

2.7. Matrix effect

The matrix effect, caused by sample components, was also studied by comparing the slope of pseudo-univariate calibration curves in HPLC water and wastewater, after enrichment by SPE. For this purpose, 450 mL portions of HPLC water were spiked at five concentration levels, between 0.1 and 50 μg L^{-1} and extracted according to the described procedure. The same SPE extraction steps were carried out on the wastewater samples, spiked at three concentration levels of 5, 22 and 44 μg L^{-1}. Every concentration point was extracted and analyzed in triplicate, for both of the sample types.

Table 1
Nominal concentrations of the five antibiotics in validation wastewater samples.

Analyte	Spiked concentration in validation samples (μg L^{-1})					
	STP1			STP2		
	s1	s2	s3	s4	s5	s6
SDZ	0.85	4.60	2.30	0.85	2.30	4.60
MET	3.50	6.00	4.70	3.30	5.30	8.30
SMR	1.20	4.90	2.90	1.20	6.50	8.30
SMX	4.00	1.90	5.50	3.50	4.70	5.20
CAP	1.60	6.20	4.30	1.00	4.50	8.90

3. Results and discussion

3.1. General considerations

In the present study, we have developed a fast chromatographic methodology for elution of five selected antibiotics. So, the optimization of elution program was performed on a short C18 column to achieve the minimum retention time value for CAP (the last analyte eluted from the column) and at the same time, maintaining the minimum resolution values for the pairs of SDZ-SMR, SMR-MET and finally MET-SMX. Establishing the final elution program was done also in such a way that an accurate quantification could be obtained by the MCR-ALS algorithm, considering the fact that UV spectra of SDZ, SMR and SMX were similar in pattern. Finally, an isocratic composition of 55:45 of water (pH = 3.2 with acetic acid) and methanol was found to produce a run time equal to 3.5 min. So, compared with method A, it can be considered that though using the current method, the overall run time for chromatographic analysis of calibration matrices will be reduced by 60%. Fig. 1 shows the chromatographic landscape obtained for a standard mixture of five antibiotics, recorded with a diode array detector in the region of 250–400 nm, with the concentration value of 5 mg L^{-1} for all analytes except MET which its value was 10 mg L^{-1}. As can be seen, the first four analytes were eluted in less than 2 min and the retention time of the last analyte was 3.2 min. The total analysis time was very short and a number of overlappings were produced between the first four analytes and so the originated data had to be processed using multivariate algorithms.

The breakthrough of the analytes from the SPE cartridge was also studied in order to establish the maximum volume of water samples that can be passed through the sorbent without significant loss of analytes [29]. Among the most frequently implemented approaches for evaluating the breakthrough volume, variation in the volume of the extracted sample with fixed concentration shows better applicability for environmental samples. So, 50–750 mL aliquots of pure water sample were spiked with the antibiotics at 5 μg L^{-1} (each volume analyzed in triplicate) and were passed through the cartridge, under the conditions mentioned in Section 2.6. Study of the variation of analyte signals under the chromatographic method A

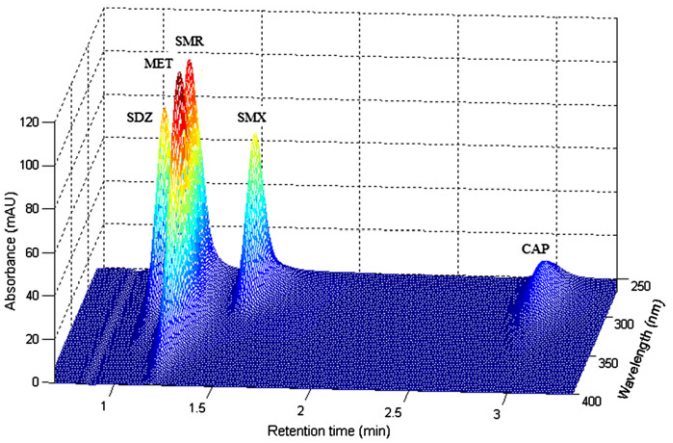

Fig. 1. Landscape obtained for a mixture of antibiotics at concentration values of 10 mg L^{-1} for MET and 5 mg L^{-1} for other analytes recorded between 250 and 400 nm.

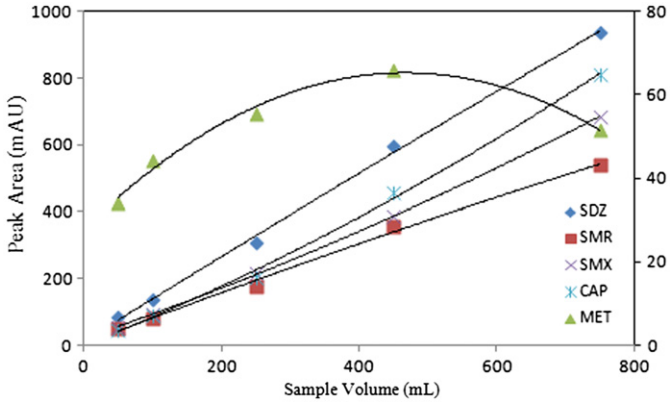

Fig. 2. Breakthrough curves for five analytes obtained through extraction of HPLC water spiked at 5 μg L^{-1}.

(average peak areas at 320 nm for MET and 270 nm for the rest of the analytes, respectively), along with increasing the sample volume passed through SPE cartridge, revealed that only for MET and for the sample volume of more than 450 mL, curvature was observed. As Fig. 2 demonstrates, for other analytes, the proposed extraction method produced a linear relationship between signal (peak area) and sample volume up to 750 mL. Thus, 450 mL was selected as an optimum extraction volume for the rest of analyses.

3.2. Univariate validation parameters

The method was validated through univariate methodology, based on peak areas at 320 nm for MET and 270 nm for the rest of the analytes. Calibration curve was developed for all antibiotics by spiking the HPLC water at seven concentration levels in triplicate, within the range of 0.1–50 μg L^{-1}. Linear curves were obtained with R^2 values between 0.9973 and 0.9995. Also, the lack- of- fit test was used (Statgraphics Centurion XVI, V 16.1.11) to check the adequacy of the linear models for the calibration curves. Since the p-values for lack-of-fits in the ANOVA table were greater than 0.05, the linear models appeared to be adequate for the observed data at the 95.0% confidence level.

The reproducibility was also tested with six replicates and the relative standard deviations (RSDs) were less than 7.8%. Limits of detection (LOD) and limits of quantification (LOQ) were calculated according to the methodology described in Ref. [30]. The estimated LODs of the SPE-HPLC-DAD method varied from 19 to 40 ng L^{-1}. The univariate analytical figures of merit are summarized in Table 2.

3.3. Quantification of antibiotics in wastewater using multivariate data

As stated in Section 3.1, the main goal of this study was to verify whether coupling of a multiset resolution technique with fast elution HPLC-DAD data, could make possible to quantify five antibiotics in the highly complex matrix of wastewater, without losing accuracy and precision. An example of such a matrix has been shown in Fig. 3 for an extracted wastewater sample, spiked with different amounts of five target analytes (STP1, sample s3 in Table 1) and recorded at multiple wavelengths. The elution order of the studied analytes was SDZ, SMR, MET, SMX and CAP. Photometric detection was performed between 250 and 400 nm range with spectral resolution of 2 nm and the period of integration of 0.4 s per spectrum. So, every chromatogram exported as a data matrix, containing 350 rows (according to 0.99 and 3.32 min) and 76 columns (number of wavelengths).

Table 2
Univariate figures of merit for quantification of the selected antibiotics in effluent wastewaters using SPE-LC-DAD signals selected at λ=270 nm, except for MET (λ=320 nm).

Analytes	Linear range (μg L^{-1})	r^2	Repeatability (%)	LODs (μg L^{-1})	LOQs (μg L^{-1})
SDZ	0.1–50	0.9992	9.6	0.02	0.1
MET	0.1–50	0.9979	7.8	0.04	0.1
SMR	0.1–50	0.9986	5.4	0.03	0.1
SMX	0.1–50	0.9973	4.0	0.02	0.1
CAP	0.1–50	0.9995	4.2	0.03	0.1

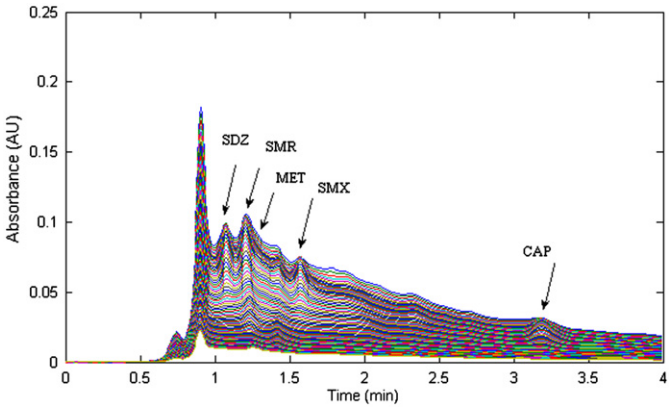

Fig. 3. SPE-LC-DAD chromatograms, each at a single wavelength (250–400 nm), of a wastewater sample (taken from STP1) spiked with five antibiotics (s3, Table 1). The analytes of interest are indicated.

Fig. 3 clearly shows the co-extraction of matrix components, such as humic and fulvic acids, through the sample preparation step. These components are UV absorptive, and so they are responsible for the appearance of the broad band at the beginning of the chromatograms or a hump in the middle, depending on the mobile phase conditions. The appearance of the baseline drift and co-elution problem, between analytes with each other and also with extracted matrix components, led to the fact that neither identification nor quantification could be performed using classical univariate calibration. In fact, erroneous determination of the starting point and the end point of a peak, in case of baseline drift, has a drastic effect on the uncertainty of the predicted concentrations. So, handling the baseline drifts or background contributions, before applying second-order calibration algorithms, has been a critical preprocessing step in many chromatographic analyses [31–34]. Background estimation can be done through fitting of different polynomial functions or splines, which has been adapted to multidimensional data. When the baseline offset is corrected as an individual step, care should always be taken about the suitability of the applied fitting function, the way of background correction (locally or globally), the appearance of probable artifacts and the other parameters that may influence the final resolution results. An alternative approach is to set the background as a systematic part of the model. As reported by Amigo et al. [35], this approach works well when the factor modeling methods have been applied on the local regions, because of the fact that the number of components, in the region around the eluted analyte, is fairly low and the baseline only changes in intensity through run to run. So, in the present work, we tried to consider the background signal as a chemical component, through partitioning the total chromatographic data into four regions. In Fig. 4, partitioning of a typical wastewater chromatogram into the regions has been indicated. Also, chromatograms of a standard mixture containing 3.3 mg L^{-1} of MET and 1.7 mg L^{-1} of other

antibiotics at wavelengths 270 and 320 nm, have been superimposed on this figure to show the constituents of every region. As can be appreciated from this figure, analytes SMX and CAP were completely separated from the others by the applied mobile phase condition. The necessary information about this figure has also been shown in Table 3. As can be seen, four regions R1, R2, R3 and R4 with the following compositions can be specified; R1 (21×76) contains only the analyte SDZ, R2 (21×76) contains co-eluted peaks of SMR and MET, R3 (31×76) contains the analyte SMX and R4 (26×76) contains the analyte CAP.

3.4. MCR-ALS modeling and the standard addition strategy

The chromatograms of wastewater samples clearly showed the overlapping of unwanted matrix components with the analytes of interest. In fact, in the classical univariate methods, contaminants of the sample matrix can mask the analytes peaks or cause the underestimation of peak areas by raising the chromatograms baseline. Another considerable point was the sensitivity changes for all analytes, due to matrix effect. This effect was statistically confirmed (p-values < 0.05) by comparing the slope and intercept of pseudo-univariate calibration curves in HPLC-water and spiked wastewater samples, after enrichment by SPE. An example of this phenomenon has been shown in Fig. 5, corresponding to superimposition of two calibration plots, obtained for SDZ within its linear range. Thus, second-order standard addition methodology combined with MCR-ALS [25,26], was implemented in this work for determination of all analytes, so that matrix effect and co-elution problems could be effectively handled. Knowing the fact that MCR-ALS is a suitable method for processing of HPLC data sets with a non-trilinear structure, setting the background as a systematic part of the model would make it possible to implement the algorithm to all the data sets, without any pre-processing step. As mentioned earlier, before employing MCR-ALS, HPLC-DAD data sets were divided into sub-matrices at specific retention time intervals. There were some reasons for this partitioning, such as simplifying the data processing, including the background as a model component, similarity of UV spectra of some analytes like SDZ, SMR and SMX. Then, each sub-matrix of validation set was augmented with four standard addition counterparts along the time direction. In this analysis, initial estimate of the spectral profiles was performed using either *a priori* known pure profiles or the ones obtained from SIMPLISMA (simple interactive self-modeling mixture) methodology. The latter is a pure variable based method which extracts all pure component spectra from a series of mixtures with varying compositions [36].

Finally, the bilinear decomposition of the augmented matrix **D** was performed, considering the predefined number of components and a spectral matrix of initial estimates, according to the following expression:

$$\mathbf{D} = \mathbf{C}\,\mathbf{S}^{\mathrm{T}} + \mathbf{E} \quad (1)$$

where, the number of rows in matrix **D** equals the total number of recorded elution times in different chromatographic runs for test and standard addition samples ($(I+1) \times K$, I=number of training samples and K=number of elution times in each sample) and the number of columns is equal to the considered number of wavelengths (J). The results of MCR/ALS modeling are in the form of an unfolded matrix **C**, with the columns containing the elution profiles of the N compounds, a matrix **S** with the columns containing their corresponding spectral profiles and **E** which is a matrix of residuals, not fitted by the model. During the ALS fitting, iterative least-squares minimization of $\|\mathbf{E}\|$ was performed through constraints of non-negativity in spectral profiles and non-negativity and unimodality in chromatographic profiles. Highly acceptable fitting values were obtained through mentioned methodology. Also, MCR modeling performed on the same data with a known spectral profile of target analytes and an estimate of spectral profiles of additional interfering components. No significant differences were observed between the results in both cases for all regions.

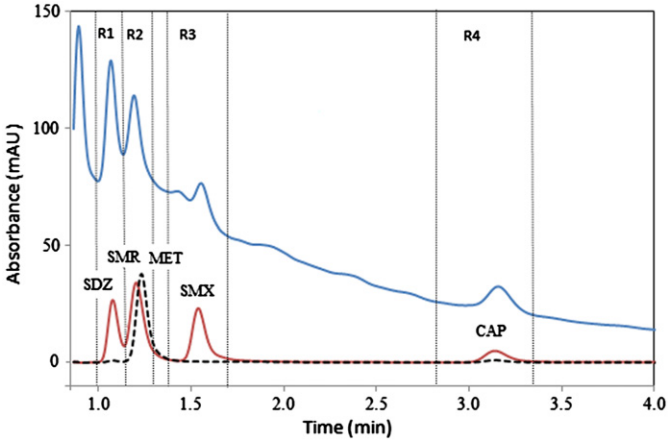

Fig. 4. Simplification of data analysis by dividing the entire wastewater chromatogram (blue solid line) into four regions. The chromatograms of a standard mixture containing 3.3 mg L^{-1} of MET and 1.7 mg L^{-1} of the other analytes at wavelengths of 270 nm (red solid line) and 320 nm (black dashed line) are indicated, too. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

Table 3
Regions in which the total chromatographic data were divided for building MCR-ALS models.

Analytes	Region	Scan No.	Time region ($_{min}$)	Retention time ($_{min}$)	MCR-ALS factors (STP1/STP2)
SDZ	1	150–170	0.99–1.12	1.07	2/3
SMR	2	170–190	1.12–1.25	1.20	4/4
MET	2	170–190	1.12–1.25	1.22	4/4
SMX	3	220–250	1.39–1.65	1.58	2/3
CAP	4	425–500	2.79–3.32	3.20	2/2

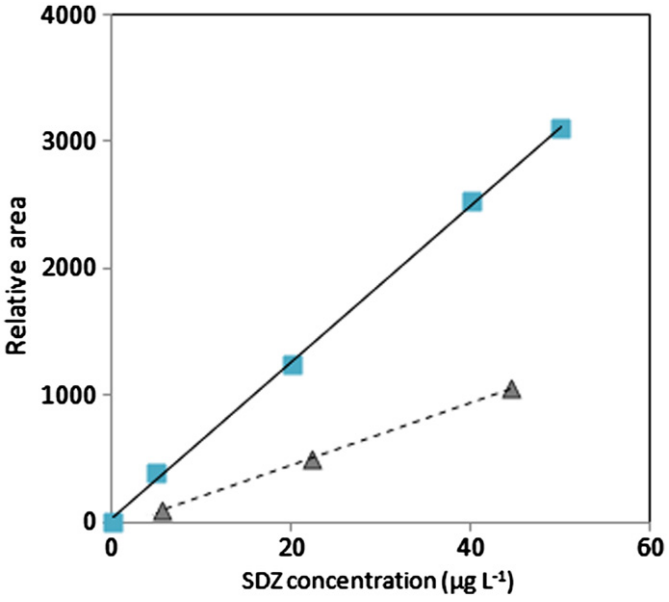

Fig. 5. Comparison of slopes of pseudounivariate calibration plots in HPLC-water (square) and spiked wastewater sample (triangle) after enrichment by SPE for showing the matrix effect.

Three wastewater samples from two sewage treatment plants (STP1 and STP2) were spiked with five antibiotics at different concentration levels (Table 1) and were used for validation studies. Then, four standard addition samples corresponding to increasing concentration values of each analyte in real effluent samples were constructed. The blank wastewater samples were also submitted to the extraction and data analysis processes to investigate the presence of the target compounds in these samples, through the recovered elution profiles, UV spectra and the concentration values. In this way, after partitioning of non-spiked data matrices into four regions, each region in the test matrix was augmented to the corresponding four standard addition matrices and then submitted to MCR-ALS modeling.

Fig. 6 shows the recovered elution profiles for region R2 in test sample s1 (STP1, Table 1) together with four related standard addition matrices retrieved by MCR/ALS modeling. The number of factors in each region was checked through singular value decomposition of non-spiked and spiked chromatographic landscapes, to confirm the presence of interferences. This number depends on the size of the selected subset in chromatographic direction and also, the characteristics of wastewater sample being analyzed. As it is clear, two components in this region have been recovered in addition to analytes SMR and MET. These components corresponded to one interfering compound and the background signal. The correctness of assigning the curve (c) as background signal was further confirmed through performing background correction on the chromatogram (the figure was not shown for brevity). The methodology proposed by Eilers et al. [37] was chosen for background elimination in two dimensional signals, based on asymmetric least squares splines regression approach. As can be appreciated from Fig. 6, there were no significant profile shape changes for background signals across the samples, which in turn confirmed the appropriateness of the model. In addition, the intense co-elution of the analytes with each other and also with the matrix components has been properly resolved. All these evidences confirmed the necessity of a second-order strategy to be implemented for calibration. Similar acceptable results were obtained for the other regions for both STP effluent samples. In all cases the number of factors was 2, 3 or 4, but never 1, which was required and presupposed for univariate calibration. The results of MCR-ALS decomposition in retention time direction indicated an acceptable increasing profile of analytes in the presence of nearly constant profiles of interferences and background. In this manner, the quantification of the isolated analytes was done using estimated relative peak areas for the target analytes and so a pseudo-univariate standard addition curve was built accordingly.

Fig. 7 shows another result of MCR-ALS as estimated chromatographic profiles for region R1 in test sample s6 (STP2, Table 1). As can be seen in this figure, three time profiles corresponding to SDZ, one interfering compound and baseline component are perfectly extracted. Thus, in the same way that was explained for s1, the isolated signal of SDZ together with the pure time profiles of the standard additions was used for its quantification.

The quality of spectral prediction is another parameter which can be used to show the capability of the model. Fig. 8 shows a comparison between the predicted spectral profiles for all analytes through MCR/ALS modeling of STP1 samples with the actual spectra registered from direct injection of pure standards into LC-DAD. As it can be seen, two sets of spectra match together almost completely, thus confirm the high quality of deconvolution process through a good reconstruction of individual profiles. Similar results were obtained by analysis of STP2 samples.

The above mentioned process was repeated for all regions of spiked and non-spiked STP1 and STP2 effluent samples. The important point was that the background contribution appeared in all regions, while it was not the case for interferences. Actually, for regions R2 and R4, no difference was observed in the number of components comparing STP1 and STP2 samples, but this was not the case for regions R1 and R3. In fact these regions contained the analytes SDZ and SMX, one unexpected component co-eluted with these analytes and a baseline component for STP2 sample (Table 3). On the contrary, there was not any interference found in these regions for STP1 sample. This was an interesting result showing the capability of the MCR algorithm to model an analyte in the treated sewage test samples containing different number of matrix constituents.

The quantitative results for all the five analytes in the six validation samples, containing the predicted concentrations, recovery values and relative standard deviations for triplicate analyses are displayed in Table 4. As can be seen, there is a good agreement between predicted and the nominal concentration values considering the complexity of analytical problem and no need to correct the baseline signal. Recovery was calculated using the predicted concentration values through extrapolation of standard addition curves and the nominal concentration spiked in the wastewater samples. For the targeted antimicrobial analytes, recovery calculation was based on the subtracted amount of analyte in the original sewage sample from the spiked sample. Among the studied antimicrobial compounds, only CAP was detected in STP1 and MET and CAP were detected and quantified in STP2. A t-test ($P=0.05$) was carried out to compare the mean recoveries of seven analytes with the ideal value of 100%. As it is shown in Table 4, all t-values were less than tabulated critical

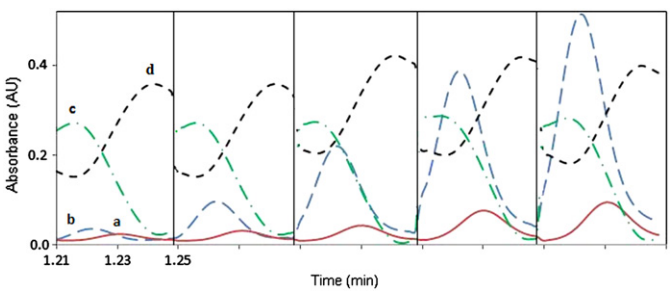

Fig. 6. Successive estimated elution time profiles retrieved by MCR-ALS analysis for region 2 of sample s1 (STP1 effluent) and four corresponding standard addition matrices which includes (a) MET (red solid line), (b) SMR (blue long dashed line), (c) background offset (green dash dotted line) and (d) the interfering compound (black short dashed line). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

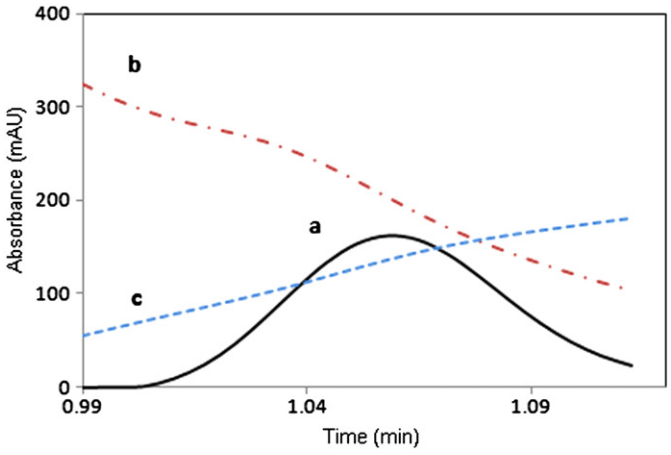

Fig. 7. Estimated chromatographic profiles by MCR/ALS modeling for region 1 of sample s6 (STP2 effluent) which includes a) SDZ (black solid line), b) background offset (red dash dotted line) and c) the interfering compound (blue short dashed line). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

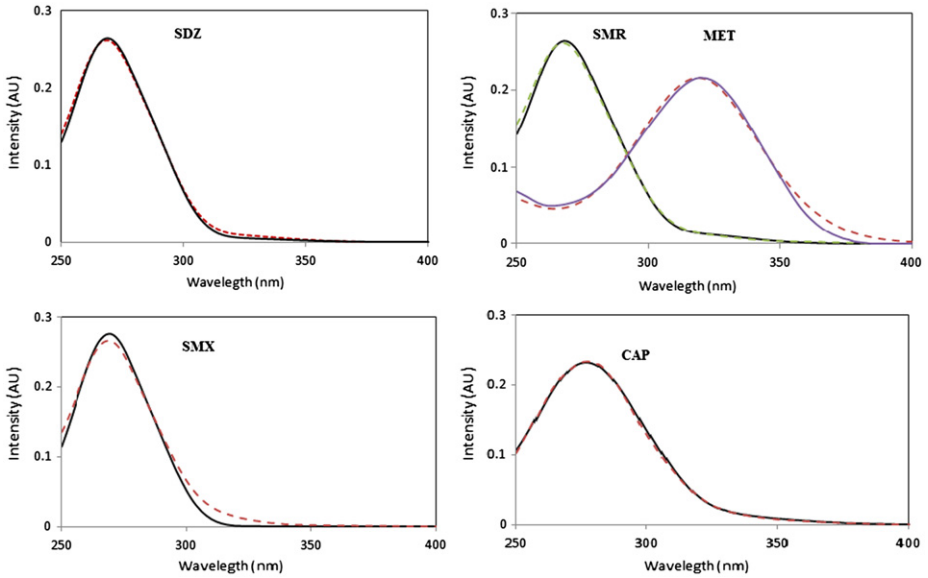

Fig. 8. Spectral profiles recovered by MCR-ALS modeling for five antibiotics. Comparison between the normalized pure analyte spectra (blue solid line for MET and black solid line for the rest of analytes) and the spectra reconstructed by the curve resolution model (green dashed line for SMR and red dashed line for the rest of the analytes). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

Table 4
Predicted concentrations using MCR-ALS on two wastewater treatment plant effluents spiked with different amounts of selected antibiotics.

Analyte	Predicted concentrations ($\mu g\ L^{-1}$)[a]								
	STP1				STP2				t_{calc} (t-test)[c]
	Un-spiked	s1	s2	s3	Un-spiked	s4	s5	s6	
SDZ	n.d.	0.93 (109.4)	4.96 (107.8)	2.26[8.8][b] (98.3)	n.d.	0.97 (114.1)	2.11 (91.7)	4.64 (100.8)	$1.1 < t_{crit.}$
MET	n.d.	2.71 (77.4)	5.02 (83.6)	3.55[5.4] (75.5)	2.8	4.08 (123.6)	5.64 (106.4)	8.24 (99.2)	$0.74 < t_{crit.}$
SMR	n.d.	1.24 (103.3)	5.83 (118.7)	2.02[9.5] (69.6)	n.d.	1.04 (86.6)	7.21 (110.9)	7.45 (89.7)	$0.48 < t_{crit.}$
SMX	n.d.	4.16 (104.0)	8.30 (120.3)	5.80[11.0] (105.0)	n.d.	3.54 (101.1)	4.41 (93.8)	5.85 (112.5)	$1.62 < t_{crit.}$
CAP	0.78	1.92 (120.0)	7.32 (118.1)	5.00[10.0] (116.3)	0.43	0.89 (89.7)	4.47 (99.3)	9.21 (103.4)	$1.56 < t_{crit.}$

n.d., Not detected.

[a] Recoveries in parenthesis.

[b] RSD (%) for three replicates of s3 in square brackets.

[c] $t_{calc}=(\bar{x}-\mu_0)/(s/\sqrt{n})$, $\bar{x}$ is the average recovery, μ_0 is 100%, n is the number of measurements and in confidence level of 95%, $t_{crit.}=2.57$.

values, so the results were satisfactory and proved that the algorithm could provide accurate results for validation samples. In addition, if elliptical joint confidence region (EJCR) [38] be calculated for the slope and intercept of the plot of predicted vs. nominal concentrations, it can be concluded that the computed ellipse for the global data set (at 95% confidence level) includes the theoretical expected point (1, 0). This fact confirms, further, the absence of both constant and proportional error.

Finally, the calculated relative standard deviations (RSD%) of predicted concentration values for three replicates of sample s3, were less than or equal to 11.0%, which can be considered acceptable regarding the complexity of the analytical problems and low concentration values of the analyzed samples and finally this fact that no preprocessing has been carried out.

4. Conclusion

In the present study, we have shown the possibility of combination of SPE-HPLC-DAD with second-order calibration using MCR/ALS, through a standard addition strategy, for the calibration of chromatographic signals and to quantify five antibiotics in wastewater effluent samples. Since the coelution problems of the analytes with each other and the matrix constituents were resolved mainly using "mathematical separation" instead of conventional optimization of physico-chemical parameters, the chromatographic run time was dramatically reduced while compared to the univariate methodologies. As the background correction step can be time consuming and may influence the final resolution results, in case of incorrectness of its contribution estimation, we tried to treat the background signal as a systematic part of the model. Besides, according to inherent capability of MCR/ALS for handling chromatographic data with retention time shift, no preprocessing step was necessary in this work. Finally, the algorithm was used efficiently for direct analysis of divided raw data matrices, through unfolded three-dimensional arrays obtained from HPLC-DAD. Taking into consideration all the problematic concerns, such as the trace concentration level of the analytes, elimination of preprocessing steps, complexity of the sample contamination and resulting matrix effect, still acceptable recovery

and standard deviation values were obtained and the second-order advantage was fully exploited. This low cost, rapid and simple methodology can be used effectively for determination of antibiotics in different aquatic environments.

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