Talanta 116 (2013) 827–834

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a multiresidue method for the determination of endocrine disrupters in fish fillet using gas chromatography–triple quadrupole tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 29 April 2013
Received in revised form
21 July 2013
Accepted 22 July 2013
Available online 7 August 2013

Keywords:
GC–MS/MS
QuEChERS
Endocrine disrupters
Fish fillet

ABSTRACT

Endocrine Disrupter Compounds (EDCs) are responsible for alterations in the endocrine system functions. Aquatic organisms are able to accumulate EDCs residues, being the major source of contamination for top predators and human consumers. This study aimed to develop and validate a method for the determination of 40 EDCs in fish fillet using modified QuEChERS and Gas Chromatography coupled with Mass Spectrometry in tandem (GC–MS/MS). A factorial design was used to optimize the extraction procedure. Method validation presented recoveries from 70.1% to 120.0% with RSD < 20% and method limit of detection ranged from 0.3 to 7.5 $\mu g\,kg^{-1}$, showing good accuracy and precision. This method was successfully applied to the analysis of fish fillet from different species and residues of bisphenol A, chlorpyrifos and bifenthrin were detected. The proposed method proved to be effective for the determination of EDCs in fish fillet at very low concentration levels.

1. Introduction

The group of substances known as endocrine disrupter compounds (EDCs) has been investigated extensively for their effect on the environment [1,2] and a significant amount of research has been dedicated to the phenomenon of endocrine disruption (ED) in wildlife [3–5]. EDCs can interfere with the endocrine system by mimicking the action of naturally produced hormones, by preventing the action of endogenous hormones, by altering the synthesis and function of hormone receptors, or modifying the synthesis, transport, metabolism and excretion of hormones [4,5]. Different types of compounds are classified as endocrine disruption, such as pesticides, alkylphenols, polychlorinated biphenyls (PCBs), bisphenol A, endogenous and synthetic hormones, as many other substances [6–8].

EDCs may reach the natural environment in runoff from non-point sources such as agricultural areas, from manure or biosolid applications, or from point sources such as discharges from municipal sewage treatment plants to surface waters [3]. Aquatic organisms such as fish and shellfish are a suitable indicator for the environmental pollution monitoring [7,9]. Data on the presence and distribution of endocrine disrupters in fish, especially in edible species, are therefore important not only from ecological but also from human health perspective. Fish are able to accumulate EDCs residue concentrations several times higher than the surrounding water via diffusion across the gills and skin. In aquaculture farms, fish feed, contaminated by EDCs is a potential source of direct introduction into fish. Consequently, fish are a major source of contamination for both top marine predators and human consumers [9,10].

QuEChERS which stands for quick, easy, cheap, effective, rugged and safe is a simple and fast method for the extraction of pesticide residues in fruits and vegetables and it was firstly introduced by Anastassiades et al. [11]. This method is characterized by the use of acetonitrile to extract matrix containing water and salts are used to obtain phase separation [11,12]. Salts like sodium chlorine (NaCl) can help in the salting out effect or the use of sodium acetate anhydrous (NaAc) to enable the formation of the acetate buffer when using acetonitrile containing acetic acid. The salting out effect consists the addition of excess salt facilitating, in this way, the analyte to become less soluble in the aqueous phase [13]. In the same time of QuEChERS development a new clean-up procedure called dispersive solid phase extraction (d-SPE) was proposed which consists of adding the extract into a polypropylene tube containing sorbents and salts [11]. Shorter sample preparation, the elimination of evaporation steps and changing the use of traditional SPE cartridges by d-SPE are some advantages of the QuEChERS method. The QuEChERS method has already been applied to the

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0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.07.047

determination of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in fish, acrylamide in food, veterinary drugs in animal tissue and hormone esters in muscle tissues, as many other applications [8,12].

Other techniques can also be used for endocrine disrupters extraction in food as the pressurized liquid extraction (PLE), which operates at pressures and temperatures above the boiling points of conventional organic solvents. The higher temperatures are responsible for faster desorption of the analyte from the matrix and the analyte solubility in the solvent is also improved [14]. An important disadvantage of PLE for fatty matrices, such as fish, is due to the presence of high quantities of lipids co-extracted, requiring extensive clean-up procedures [15]. Microwave-assisted extraction (MAE) can also be used to enhance the efficiency of solvent extractions in solid and semi-solid samples. This technique agitates and heats the sample during extraction, thus it is applicable to thermally stable analytes [14]. Nevertheless, the extract usually contains interfering species that require clean-up prior to chromatographic analysis [16]. Supercritical fluid extraction (SFE) allows a more selective extraction, and provides faster reaction kinetics than most liquids, due to the use of carbon dioxide as the supercritical fluid. Robustness is one of the main problem in SFE when compared to other extraction techniques. Also the use of SFE for fatty samples can require an extensive sample preparation including more clean-up procedures [14]. These techniques require a higher investment in instrumentation than the QuEChERS method.

The use of gas chromatography with tandem mass spectrometry (GC–MS/MS), in particular operating in selected reaction monitoring (SRM) mode, is very important for the analysis of compounds in low concentrations in complex matrices and/or with many interferences, such as fish [7]. The Directive (2002/657/EC) [17] describes that instruments GC–MS/MS and liquid chromatography with tandem mass spectrometry (LC–MS/MS) must monitor two transitions, from the same precursor ion; or two different precursors and one product ion from each precursor. It is due to reduction in the probability of spectral interferences allowing the identification by monitoring two transitions (one for quantification and another for confirmation).

A number of 244 samples of Nile tilapia fillet (*Oreochromis niloticus*), common carpe (*Cyprinus carpio*) and African sharptooth catfish (*Clarias gariepinus*) from three different rivers in Ethiopia were analyzed employing QuEChERS and GC–MS. DDT and its metabolites were found in the highest levels in the most fat containing fish species which also contained considerable amount of endosulfan sulfate (until 65.1 $\mu g\,kg^{-1}$). Chlorpyrifos, HCB, o,p′-DDE and PCBs were also detected, but lower than the LOQs [8]. Shao et al. [18] verified the presence of nonylphenol and bisphenol A in fish fillet from markets from Beijing (China) with concentration levels of 0.33–55.98 $\mu g\,kg^{-1}$. These compounds were detected using PLE followed by solid-phase clean-up, and LC–MS/MS. The authors attributed the presence of such compounds due to contamination of the aquatic environment in which they live. The contamination of fish of various species has also been observed by Liu et al. [19] in China. Combining MAE and GC–MS the authors observed the presence of 4-tert-octylphenol, 4-cumylphenol, 4-nonylphenol and bisphenol A in fish fillet with maximum concentrations of 4.6, 4.4, 18.9 and 83.5 $\mu g\,kg^{-1}$, respectively. The concentration levels were dependent from the locations where the fish samples were collected, but in most of them presence of more bisphenol A, at relatively high levels, was observed. It proves that there is a contamination of the aquatic environment and this should be a constant concern nowadays in order to reduce the environmental impacts caused by EDCs.

As the examples shown above from the literature ilustrate, there is a lack of multiresidue methods for the determination of a wide variety of endocrine disrupters from different chemical classes in fish fillet using a single and simple extraction method combined with a chromatographic detection system. Therefore, the aim of this work was to develop and validate a multiresidue method for the determination of 40 endocrine disrupters from different classes in fish fillet through the use of the modified QuEChERS method and triple quadrupole GC–MS/MS. The present method was developed and validated using fish fillet from catfish (*Rhamdia quelen*) species and then also evaluated for the fish species tilapia (*Oreochromis niloticus*) and striped catfish (*Pangasius hypophthalmus*).

2. Experimental

2.1. Chemicals and apparatus

Analytical standards listed in Table 1 and the internal standards (IS) quintozene and triphenylphosphate were acquired from Dr. Ehrenstorfer (Germany) with purity between 94.0% and 99.5%. As surrogate standard (SS) isotopically modified, trifluralin-d14 (99.1%) purchased from CND Isotopes (Canada) was used. Acetonitrile (MeCN) HPLC grade, florisil 60–100 mesh and anhydrous sodium acetate (NaAc) p.a. were from Mallinckrodt (USA), glacial acetic acid 100%, anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride p.a. (NaCl) were from J.T. Baker (USA), calcium chloride p.a. ($CaCl_2$) from Spectrum (USA) and sorbents primary secondary amine (PSA) and octadecylsilane (C_{18}), with 40 μm of particle size, were purchased from Agilent (USA). Nylon filters of 13 mm and 0.2 μm of porosity were from Vertical Chromatography (Thailand). Ultrapurified water was obtained with a Milli-Q Direct UV3® system (Millipore, USA).

Vortex mixer model QL-901 (Microtécnica, Brazil), precision analytical balances AUW-220D and UX-420H (Shimadzu, Japan), refrigerated centrifuge NT 825 (Novatécnica, Brazil), centrifuge (Centribio, Brazil) and food processor Varimix (Targo, Spain) were used.

Measurements were carried out on a gas chromatography CP 3800 (Varian, USA) coupled to a triple quadrupole mass spectrometer MS 1200. The system was equipped with an autosampler CP 8400; injector 1079 with Programmable Temperature Vaporizing (PTV) and a data acquisition software MS Workstation 6.4.

2.2. GC–MS/MS conditions

The GC–MS/MS system was operated with a capillary column VF-5-MS (5% phenyl 95% dimethylpolysiloxane) with 30 m × 0.25 mm of internal diameter and 0.25 μm of film thickness. The column oven temperature program was 50 °C for 1 min, raised at 10 °C min^{-1} to 65 °C, then at 25 °C min^{-1} to 180 °C and then at 5 °C min^{-1} to 280 °C, resulting in a runtime of 35 min. The injector program was 100 °C held for 0.1 min and then at 200 °C min^{-1} to 280 °C. The quadrupole mass spectrometer was operated in selected reaction monitoring (SRM) mode using two transitions, one for quantification and another for confirmation as shown in Table 1. Transfer line temperature was set at 250 °C, ion source was electron ionization (EI) at 70 eV and temperature at 210 °C. Helium was used as carrier gas at 1 mL min^{-1} and argon as collision gas (2 mTorr). Injection volume was 2 μL in the splitless mode with a carbofrit inserted in the liner. Full scan analysis in *m/z* range from 50 Da to 500 Da was used for identification of possible interferences in the extract which could affect the analysis and result in frequent maintenance of the instrument.

Table 1
Mass spectrometry parameters for the GC–MS/MS determination of selected endocrine disrupters, retention time (t_R), the method limits of detection (LODm) and of quantification (LOQm), water solubility and the partition coefficient octanol/water (K_{ow}).

Compound	t_R (min)	1st Transition quantification		CE[a] (eV)	2nd Transition confirmation		CE[a] (eV)	LODm[b] ($\mu g\ kg^{-1}$)	LOQm[c] ($\mu g\ kg^{-1}$)	Water solubility ($mg\ L^{-1}$ at 20 °C)	log K_{ow}
Triclorfon	6.5	185	93	15	185	109	20	0.3	1.0	120000	0.43
4-Terc-octylphenol	8.9	135	77	31	135	107	31	1.5	5.0	19.0	4.12
Trifluralin	9.3	306	264	10	306	206	15	0.3	1.0	0.221	4.8
Trifluralin-d14 (SS)	9.3	315	267	8	315	209	10	0.3	1.0	–	–
Alpha-HCH	10.0	219	183	10	219	147	20	0.3	1.0	10.0	3.8
Hexachlorobenzene	10.1	284	214	35	284	249	30	0.3	1.0	0.0047	3.93
Dimethoate	10.2	125	79	10	125	125	10	7.5	25	25.0	0.56
Simazine	10.3	201	138	15	201	173	15	1.5	5.0	6.2	2.1
Atrazine	10.4	215	200	10	215	173	10	1.5	5.0	33.0	2.75
4-n-Octyphenol	10.5	107	77	30	206	107	30	1.5	5.0	3.1	5.5
Beta-HCH	10.5	219	183	10	219	147	20	1.5	5.0	5.0	3.78
Quintozene (IS)	10.6	295	237	10	295	265	10	–	–	0.44	4.46
Lindane	10.6	219	183	10	219	147	20	1.5	5.0	8.52	3.80
Diazinon	10.7	304	179	10	304	162	10	0.3	1.0	60.0	3.30
Delta-HCH	11.3	219	183	10	219	147	20	0.3	1.0	10.0	4.14
4-n-Nonylphenol	11.7	220	107	20	107	77	30	0.3	1.0	7.0	5.76
Chlorpyrifos methyl	11.9	286	208	10	286	241	25	0.3	1.0	2.6	4.24
Vinclozolin	12.0	212	145	20	212	172	15	0.3	1.0	3.4[d]	3.10
Parathion methyl	12.1	263	109	25	263	136	10	0.3	1.0	11.0	3.83
Alachlor	12.1	188	160	10	188	130	40	0.3	1.0	170.31	3.09
Heptachlor	12.3	274	239	20	274	237	20	0.3	1.0	0.056	5.44
Malathion	12.9	173	99	15	173	127	10	0.3	1.0	145.0[d]	2.6
Chlorpyrifos	13.1	314	258	15	314	286	15	0.3	1.0	1.4[d]	4.70
Aldrin	13.3	263	193	30	263	191	30	1.5	5.0	0.027[d]	6.5
Parathion ethyl	13.3	291	81	25	291	109	20	1.5	5.0	11.0	3.83
Dicofol	13.6	139	111	10	139	75	25	0.3	1.0	0.8[d]	4.30
Heptachlor-epoxide exo	14.4	353	263	15	353	282	15	1.5	5.0	0.35	5.40
Hepachloro-epoxide endo	14.5	272	237	18	272	141	30	1.5	5.0	0.35	5.40
2,4-DDE	15.2	246	176	25	318	246	25	0.3	1.0	0.065	6.5
Alpha-endosulfan	15.5	241	170	15	241	172	15	3.0	10	0.53	4.74
Bisphenol A	16.1	213	119	15	213	91	15	0.3	1.0	120.0	3.3
4,4-DDE	16.1	246	176	25	318	246	25	0.3	1.0	0.12	5.76
Dieldrin	16.3	277	206	15	277	241	10	1.5	5.0	0.14[d]	4.32
2,4-DDD	16.4	235	165	20	235	199	20	0.3	1.0	0.1	6.91
Endrin	17.0	263	193	30	263	191	30	1.5	5.0	0.24	4.56
Beta-endosulfan	17.4	241	170	15	241	172	15	3.0	10	0.28	4.79
DDT I	17.6	235	165	20	235	199	20	0.3	1.0	0.006	5.9
Endosulfan sulfate	18.6	272	237	15	272	235	10	0.3	1.0	0.22	3.66
DDT II	18.7	235	165	20	235	199	20	0.3	1.0	0.006	5.9
Triphenylphosphate (IS)	19.3	325	169	18	325	226	18	–	–	0.2	4.6
Bifenthrin	20.3	181	165	20	181	166	10	0.3	1.0	0.001	6.6
Mirex	22.2	272	237	10	272	143	40	0.3	1.0	0.085	5.28
Fenarimol	22.5	251	139	20	251	111	35	0.3	1.0	13.7[d]	3.69
Permethrin cis/trans	23.8	165	91	10	165	127	5	3.0	10	0.2	6.1

[a] CE: Collision energy.
[b] LODm: Method Limit of Detection.
[c] LOQm: Method Limit of Quantification.
[d] At 25 °C.

2.3. Optimization of sample preparation and validation conditions

The use of factorial design is of interest since it is possible to minimize the period for optimization of the procedure, permitting to evaluate simultaneously several variables [20]. The best conditions of extraction were obtained using the factorial design with star configuration, also called Central Composite Design (CCD), according to Neto et al. [21] with two factors (NaCl and water quantity). Factorial design was evaluated using recovery values. Therefore, the sample preparation described below is the one that presented the greatest number of compounds with recovery between 70% and 120%.

Firstly, aliquots of 500 g of each fish fillet was processed and homogenized in a food processor and the sample preparation was carried in a polypropylene tube of 50 mL. 10.0 g of fish fillet was weighted and 100 μL of the surrogate standard (10 $mg\ L^{-1}$) was added which was extracted with 10 mL of acetonitrile acidified with 1% (v/v) of acetic acid. Manual shaking was performed for 1 min followed by addition of 2.0 g of NaCl with new manual shaking for 1 min. After that, 0.3 g of anhydrous $MgSO_4$ and 1.7 g of anhydrous sodium acetate were added followed for manual shaking for 1 min. The tube was centrifuged for 8 min at 3400 rpm in order to obtain good separation of the organic phase. After that an aliquot of 3 mL of the supernatant (organic phase) was transferred to a polypropylene tube of 15 mL, containing 450 mg of $MgSO_4$, 75 mg of PSA and 375 mg of C_{18} sorbents for the clean-up step by d-SPE. After centrifuging for 8 min at 3400 rpm, 1 mL of the extract was transferred to a vial and 10 μL of the internal standards mixture (10 $mg\ L^{-1}$) was added. The final extract was filtered and analyzed by GC–MS/MS. In Fig. 1 the steps of the proposed sample preparation procedure are represented.

In order to minimize the source of errors during samples preparation, a surrogate standard (trifluralin-d14) was added in all samples at a concentration of 100 $\mu g\ kg^{-1}$ before the extraction to detect possible errors during this step. To monitor the GC–MS/MS performance, to each extract standard solution was injected and the internal standards quintozene and triphenylphosphate were added. Furthermore, the traceability of standards and solutions prepared

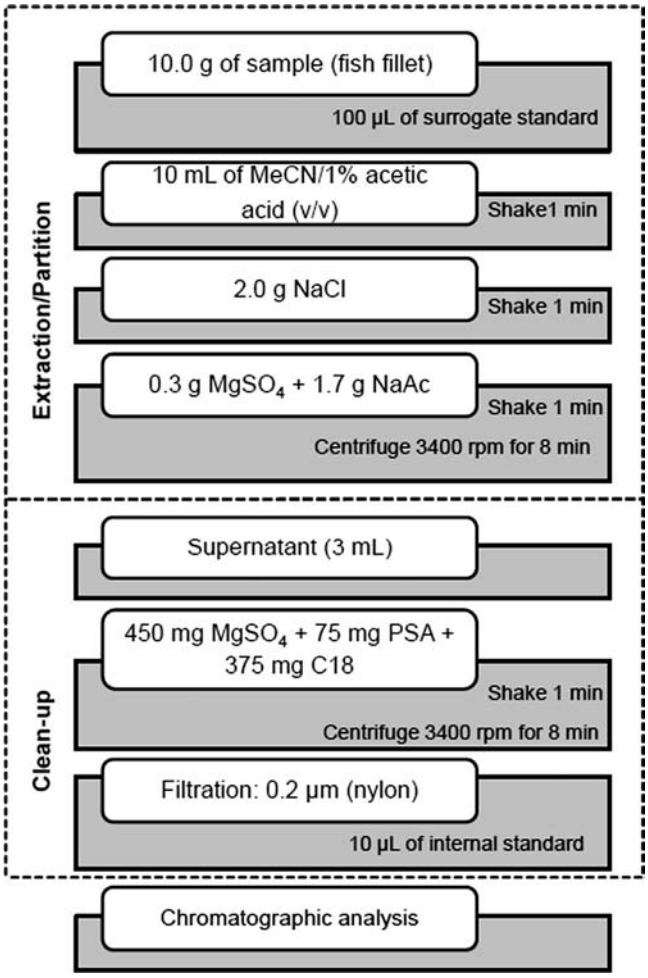

Fig. 1. Representation of the modified QuEChERS method established in this study.

in the laboratory collaborate to improve the quality control of the results. In this way it is possible to guarantee the quality of the analysis realized and the performance of the developed method.

A combination of different sorbents was tested to assure the removal of interferences without loss of efficiency in the extraction. Tests were performed using $CaCl_2$, PSA, C_{18}, Florisil and freezing (-20 °C) always in the presence of $MgSO_4$.

Development and validation of the method was performed using fish fillet blank samples, obtained from a controlled fish production, which were used as sample control. Fish fillets from different species were studied due to their importance in Brazil and all around the world. Catfish is a very important species in fish farm in Brazil because it is a fish easy to handle, fast growing and adapts well to diets and environmental variations, and with good acceptance by consumers and good commercial value [22]. Tilapia is a species that adapts very well to the climatic conditions more frequently in Brazil and is also the second species of interest in fish farming [23,24]. Striped catfish is one of the major fish species in the Mekong River fishery, one of the largest and most important inland fisheries in the world. Vietnam is by far the world's largest producer of this fish and exports to over 80 countries [25].

Validation parameters for residues determination in food samples were studied. Accuracy, evaluated though recovery essays at three different concentrations levels (10, 25 and 50 $\mu g\,kg^{-1}$), and precision, under repeatability and intermediate precision conditions, were evaluated. Six replicates of each concentration level were injected once in the chromatographic system. Selectivity, analytical curve (1.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 150.0 and 200.0 $\mu g\,L^{-1}$), linearity (coefficient of determination, r^2), matrix effect (comparing the slope of curves prepared in acetonitrile and matrix matched) and limits of detection (concentration corresponding to a signal/noise ratio of 3) and quantification (signal/noise ratio of 10) also were determined.

3. Results and discussion

3.1. Chromatographic determination by GC–MS/MS

With the GC conditions optimized, a single run in GC–MS/MS permitted the multiresidue analysis of 40 EDCs in 30 min, and it offered good sensitivity and selectivity. Fig. 3D displays a GC–MS/MS TIC chromatogram obtained with the conditions presented in Table 1, from a matrix matched analytical solution containing the EDCs at 50 $\mu g\,L^{-1}$. All EDCs showed determination coefficient (r^2) > 0.996 and linear range from LOQ to 200 $\mu g\,L^{-1}$.

3.2. Optimization of the extraction step

Accurate analyses of fish fillet samples contaminated by endocrine disrupters require an efficient method of extraction and clean-up, especially due to the high fat content that makes the sample preparation step difficult. As described above the optimization of the extraction procedure was performed using factorial design evaluating different quantities of NaCl and water. In a preliminary evaluation higher values were chosen: 10 and 20 mL of water and 3.0 and 6.0 g of NaCl. In a second assessment these quantities were reduced to achieve the greatest number of compounds recovered between 70% and 120%, since it was observed, initially, that lower quantities resulted in a greater number of compounds with appropriate recovery. Therefore, values of 1 and 5 mL of water and 1.0 and 3.0 g of NaCl were employed in the factorial design to find the best extraction procedure for EDCs in fish fillet. Through a factorial design it was possible to optimize the best conditions of extraction with a small number of experiments. The responses obtained through this study can be observed using multivariate statistic techniques like response surface methodology (RSM). RSM is a collection of mathematical and statistical techniques, which describe the behavior of a data set with the objective of making statistical previsions. It can be well applied when a response of interest is influenced by several variables [20]. The objective is to simultaneously optimize the levels of these variables to attain the best system performance. Fig. 2 shows the response surface methodology generated for water and NaCl quantities.

It was verified that the addition of water was not essential for extraction efficiency and thus water was not used. On the other hand, the use of an intermediate quantity of NaCl (2 g) was found to be important. The salting-out effect, resulting from the addition of NaCl, usually leads to increased recoveries of polar compounds [11]. The addition of the proper amounts and combination of salts can be used to control the percentage of water in the organic phase and vice versa for organic solvent in the water phase, thus enabling a certain degree of adjustment in the polarity of the phases. The quantity of salt to be used must be optimized since a high amount of salt can result in an excessive extraction of polar co-extractives, decreasing the recovery of the analytes. Interestingly, the amount of NaCl used during the partitioning also had a great influence on the peak shapes and areas of several pesticides. This effect is related to the amount and nature of the co-extracted matrix components [11].

3.3. Clean-up step optimization

The optimization of the clean-up step was performed using different sorbents. Fig. 3A–C presents chromatograms obtained in

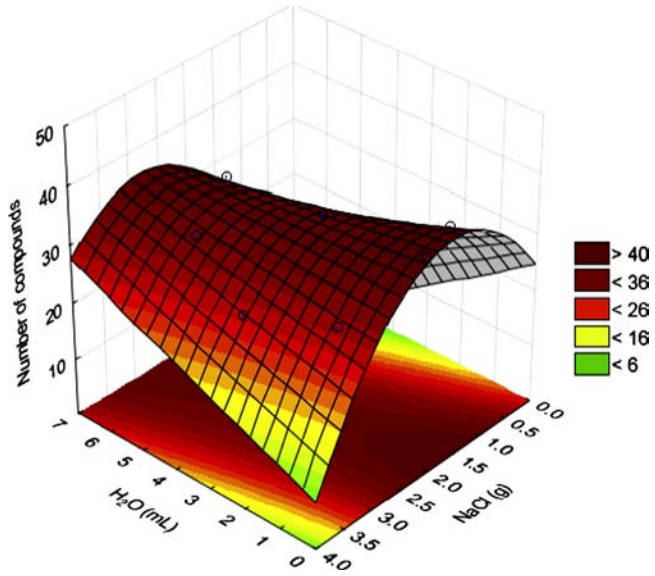

Fig. 2. Response surface methodology considering the total of compounds with recovery from 70% to 120% and RSD below 20% obtained with the factorial designed of different water and NaCl quantities.

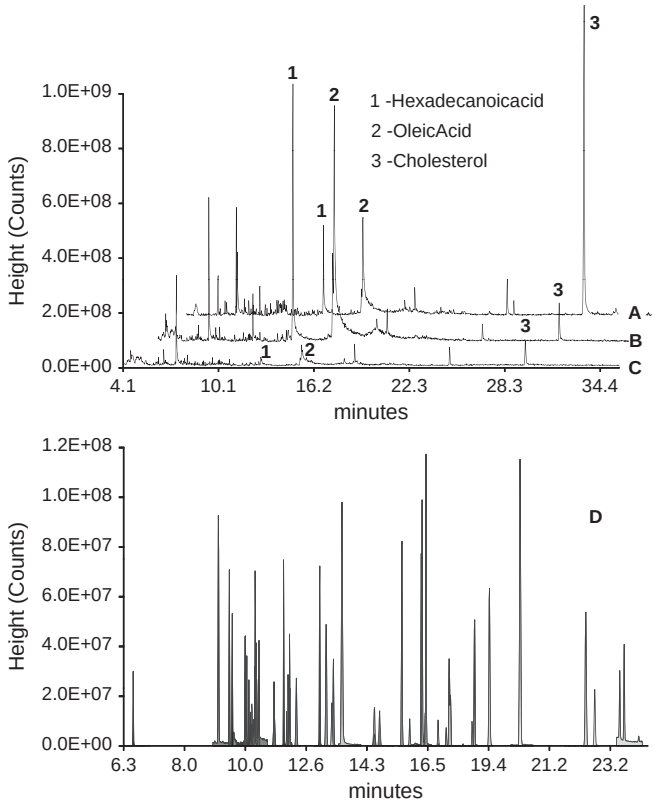

Fig. 3. Chromatograms in the full scan mode for the clean-up tests: (A) with PSA, (B) with C_{18}, (C) with C_{18} and PSA, (D) GC–MS/MS TIC chromatogram from a matrix matched standard at 50 μg L^{-1}.

full scan mode from blank samples after extraction and different clean-up procedures. A very intense peak was observed at 29.7 min, which by comparison with the mass spectral library corresponds to cholesterol. In fish, cholesterol is found in quantities between 31 and 270 mg/100 g of fish fillet [26]. This highlight shows the need of proper removal of this compound as the continuous injection in the system may demand higher maintenance of the instrument. Combinations of sorbents without C_{18} are responsible for this chromatogram. Thus, it is possible to affirm that the use of C_{18} in the clean-up step is necessary. The choice of C_{18} as sorbent for the dispersive solid phase extraction was made in order to obtain good method performance, maintaining recovery of the analytes adequate for trace analysis. Sorbent C_{18} removes apolar substances, like lipids [12]. Lehotay et al. [27] also used C_{18} for cholesterol removal in egg extracts during clean-up step.

Other interferences also could be identified and PSA demonstrated good capacity to remove hexadecanoic and oleic acids, due to its ability to selectively remove several organic acids, polar pigments, carbohydrates, sugars and fatty acids with hydrogen bonding properties. This occurs because PSA forms hydrogen bonds with compounds containing hydroxy or carboxy groups [11]. The combination of freezing, $CaCl_2$ and Florisil does not improve the clean-up, resulting just in one more step in the procedure. In this way the combination of PSA and C_{18} sorbents plus the anhydrous $MgSO_4$ proved to be an efficient way to reduce interferences without loss in recovery of the EDCs and with lower maintenance of the chromatographic system.

3.4. Method validation

Validation parameters were evaluated and the selectivity was confirmed since no interferences were observed in the blank extract compared with a spiked sample of fish fillet. Analytical curves were constructed and good linearity was observed with r^2 higher than 0.996 for all the studied EDCs.

Any international legislation or harmonization values about maximum residues limits (MRL) for these evaluated endocrine disrupter compounds were not found, but the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) established MRL values for some of the evaluated compounds [28]. Aldrin, α, β and δ-HCH, mirex, endrin and heptachlor have MRL of 50 μg kg^{-1} for fish. Their uses are forbidden but they still can be found widely distributed over large regions, including those where they have never been used [9]. This extensive contamination of environmental media and living organisms includes many foodstuffs and has resulted in the sustained exposure of many species, including humans, for periods of time that span generations, resulting in both acute and chronic toxic effects [9].

Method detection and quantification limits were from 0.3 to 7.5 μg kg^{-1} and from 1.0 to 25 μg kg^{-1}, respectively (Table 1). These limits obtained for the proposed method were satisfactory since they are lower than the established by the national legislation.

The results of accuracy, evaluated through recovery tests, and of precision are shown in Table 2. Recovery values in the three concentration levels ranged between 70.1% and 120.0%, except for trichlorfon (34.3–43.2%), hexachlorobenzene (50.1–54.5%), dicofol (56.4–62.5%) and mirex (45.0–51.2%). Good precision was observed for all the substances with relative standard deviation in repeatability terms (RSD_r) between 2.1% and 20.0%.

The organophosphate dimethoate could not be quantified in the spiked concentration level of 10 μg kg^{-1}, since its method LOQ is 25 μg kg^{-1} being evaluated only in the concentration levels 25 and 50 μg kg^{-1}.

The use of trifluralin-d14 as surrogate standard (SS) allows to evaluate the extraction procedure and judge whether the results were satisfactory in all spiked levels. Using the same concentration of SS it was possible to compare the results obtained in all concentration levels. The average recovery of the SS remained practically constant, so it is possible to conclude that no significant alterations during the extraction procedure were observed. The same can be said by the use of internal standards which were used to evaluate the response of the instrument during all the analyses. It is important to emphasize the use of surrogate standard as well

Table 2
Average recovery and precision (n= 6) from repeatability study and matrix effect.

Compounds	10 μg kg^{-1}		25 μg kg^{-1}		50 μg kg^{-1}		Matrix effect (%)
	Rec[a] (%)	RSD[b] (%)	Rec[a] (%)	RSD[b] (%)	Rec[a] (%)	RSD[b] (%)	
Trichlorfon	43.2	18.2	40.8	8.7	34.3	10.1	−5.6
4-Terc-octylphenol	97.0	10.1	98.4	12.0	96.8	4.4	79.8
Trifluralin-d14 (SS)	100.1	11.0	100.9	8.8	97.0	3.3	31.7
Trifluralin	98.8	13.4	98.2	10.7	92.3	3.4	24.5
Alpha-HCH	91.6	13.1	97.4	12.3	95.0	5.0	40.3
Hexachlorobenzene	54.5	15.1	50.5	14.7	50.1	6.7	27.4
Dimethoate	–	–	106.5	4.0	110.3	7.0	474.3
Simazine	101.4	10.2	92.4	15.8	92.5	6.6	47.3
Atrazine	109.0	12.0	100.0	9.3	91.1	6.9	29.1
4-n-Octylphenol	105.1	9.7	105.5	11.3	98.8	5.6	13.1
Beta-HCH	80.0	13.0	82.4	10.6	80.0	3.2	175.7
Lindane	99.5	12.9	101.0	12.0	94.3	8.6	34.7
Diazinon	97.9	13.6	97.1	10.2	93.2	6.0	34.4
Delta-HCH	107.8	12.3	103.0	11.2	92.5	8.8	219.4
4-n-Nonylphenol	78.7	13.8	80.1	11.1	72.4	7.8	185.0
Chlorpyrifos methyl	105.4	10.4	95.9	4.2	92.5	6.6	143.3
Vinclozolin	120.0	13.4	113.2	9.5	105.5	8.3	40.6
Alachlor	113.2	14.7	105.9	10.2	95.2	5.4	32.3
Parathion methyl	114.7	18.1	98.3	10.6	90.0	5.9	231.8
Heptachlor	88.9	14.9	82.0	12.4	77.3	6.8	28.8
Malathion	113.5	10.7	112.3	11.3	107.3	4.5	281.5
Chlorpyrifos	119.8	4.8	110.9	6.6	92.9	9.1	85.8
Aldrin	76.7	20.0	70.1	12.1	70.7	8.0	29.3
Parathion ethyl	117.4	12.7	107.7	12.5	101.9	6.3	214.1
Dicofol	62.5	13.2	60.7	13.3	56.4	4.1	71.2
Heptachlor epoxide exo	97.7	13.0	91.3	11.6	86.4	7.2	41.3
Heptachlor epoxide endo	95.8	13.0	92.0	10.6	85.6	7.3	26.1
2,4-DDE	80.8	13.2	78.1	10.1	73.6	6.5	31.9
Alpha-endosulfan	107.1	19.0	88.3	11.7	82.2	9.2	35.6
Bisphenol A	118.2	13.9	104.5	12.7	90.5	2.1	3476.0
4,4-DDE	74.2	11.0	70.4	8.9	70.4	5.3	21.5
Dieldrin	89.9	13.2	85.9	12.6	91.0	7.8	14.8
2,4-DDD	90.8	13.5	87.7	12.5	81.9	5.5	32.0
Endrin	94.7	14.5	86.9	13.3	81.5	4.5	43.3
Beta-endosulfan	102.7	16.3	85.7	10.9	83.9	6.1	83.8
Endosulfan sulfate	120.0	12.3	108.4	11.4	115.9	16.4	145.9
DDT	96.1	14.9	80.5	14.0	76.6	8.4	156.7
Bifenthrin	98.9	7.8	82.6	9.5	75.9	3.8	35.9
Mirex	51.2	14.1	45.0	14.8	40.6	4.8	16.0
Fenarimol	106.0	10.1	97.9	11.9	90.0	4.8	58.9
Permethrin cis/trans	81.2	19.1	74.3	11.9	73.6	12.0	22.5

[a] Rec: Recovery.
[b] RSD: Relative Standard Deviation.

as internal standards in all samples to assure the quality of the analysis during the stages of sample preparation and of analysis by GC–MS/MS. This control quality realized is extremely necessary since there is no Certified Reference Material for this number of compounds studied in fish fillet.

Mirex, dicofol and hexachlorobenzene are considered very stable pesticides and are persistent in soil and sediment with a partition coefficient octanol/water (K_{ow}) of 5.28, 4.30 and 3.93, respectively; and its low solubility in water can explain the low recovery due to their accumulation in the adipose tissue [29]. The low recovery of trichlorfon cannot be attributed to this condition of accumulation in fish adipocytes, as seen its K_{ow} of 0.43 is too low and does not confer this characteristic [30].

Intermediate precision was performed with the concentration level of 25 μg kg^{-1}. Recovery has remained with values between 70.1% and 119.5% with RSD in the range of 3.9–20.0%. These results are in accordance to international regulations for the analysis of pesticides at low concentration level by chromatographic analysis [31].

In the study of matrix effect (ME) in fish fillet the results were calculated as follows: ME%=[(slope of matrix-matched calibration − slope of analyte in solvent calibration)/slope of analyte in solvent calibration] × 100 [32]. Several approaches have been proposed to reduce matrix effects and the most obvious strategy is the reduction of the amount of matrix components entering the gas chromatographic system, due to the application of extensive sample extract clean-up steps. The use of different injection techniques, such as programmed temperature vaporizer (PTV) and carbofrit inserted in the glass liner, can reduce matrix effect but not eliminate it [33]. All these strategies were employed trying to minimize the matrix effect from fish fillet, nevertheless a considerable effect (Table 2) was observed for the EDCs studied. Co-extractives, as lipids (triglycerides and phospholipids) and other high molecular weight components can remain solubilized in the extracts, even after sample extract clean-up [34]. To compensate this effect, matrix matched analytical solutions were used to obtain the analytical curves to avoid quantification problems.

Positive matrix effect was observed for almost all the compounds, besides trichlorfon that showed negative effect. Specially bisphenol A, malathion and dimethoate showed a percentage of ME rather greater than values reported in literature and must be evaluated in order to confirm this effect, since values of matrix effect above 50% should be considered as sources of a very important quantitative error [35]. Pinho et al. [34] also noted very high matrix effect when they used mass spectrometry coupled to gas chromatography for sulfur pesticides in string bean. Fig. 4 shows a comparison between the analytical curves obtained in solvent and in matrix matched standards, as well as, the chromatogram of bisphenol A at 50 μg L^{-1} where the difference between the signals in solvent and in the blank matrix can be observed.

Robustness was studied with different fish fillet species (catfish, tilapia and striped catfish). Blank samples spiked at an intermediate concentration level (25 μg kg^{-1}) were used to compare the results among the three species. Tilapia showed similar behavior to catfish due to unsatisfactory results for trichlorfon, hexachlorobenzene and mirex, with the exception of dicofol that presented adequate recovery (78.1%). The compounds delta-HCH, 4-n-nonylphenol, endosulfan sulfate and aldrin showed recoveries below 70%. In this way, it is possible to conclude that 33 EDCs can be analyzed using the proposed method in tilapia fillet. Striped catfish robustness tests presented recoveries below 70% for trichlorfon, hexachlorobenzene, mirex, dicofol, delta-HCH, endosulfan sulfate and aldrin, being possible to apply the proposed method for 33 EDCs. This difference among the results can be attributed to the percentage of fat in each fish species. This percentage is quite different mainly by the influence of the diet due to the presence of fatty acids. The increase of this sort of fat (omega 3) could help to value the product [36]. In catfishes this quantity can vary from 2.5% to 5.7%, but in tilapia, that is low fat, this amount is about 1% [8]. These results demonstrated the importance in evaluate the behavior of the extracts obtained with the different species of fish fillet because these ones may not always be extrapolated.

3.5. Real samples

In order to evaluate the proposed method, five real samples of fish fillet were analyzed by the proposed method: two of striped catfish, two of catfish and one sample of tilapia. These samples were bought from supermarkets in Santa Maria, Rio Grande do Sul State, Brazil. Samples of catfish presented residues of bisphenol A (6.2 and 14.5 μg kg^{-1}), chlorpyrifos (34.7 μg kg^{-1}) and bifenthrin (2.1 μg kg^{-1}). In tilapia, only residue of bisphenol A (2.7 μg kg^{-1}) was found and no residues of EDCs were found in the two samples of striped catfish analyzed. Fig. 5 shows the chromatograms of the positive samples compared to a control sample.

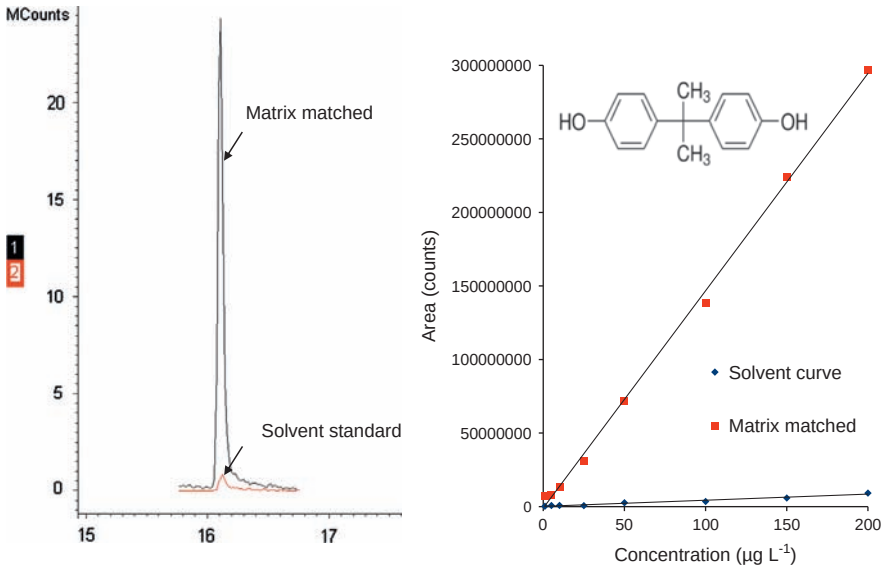

Fig. 4. Comparison of the analytical curves obtained in solvent and in the blank of the matrix for bisphenol A, as well as its chromatograms at 50 µg L^{-1} comparing the difference between the signals in solvent and in the blank matrix.

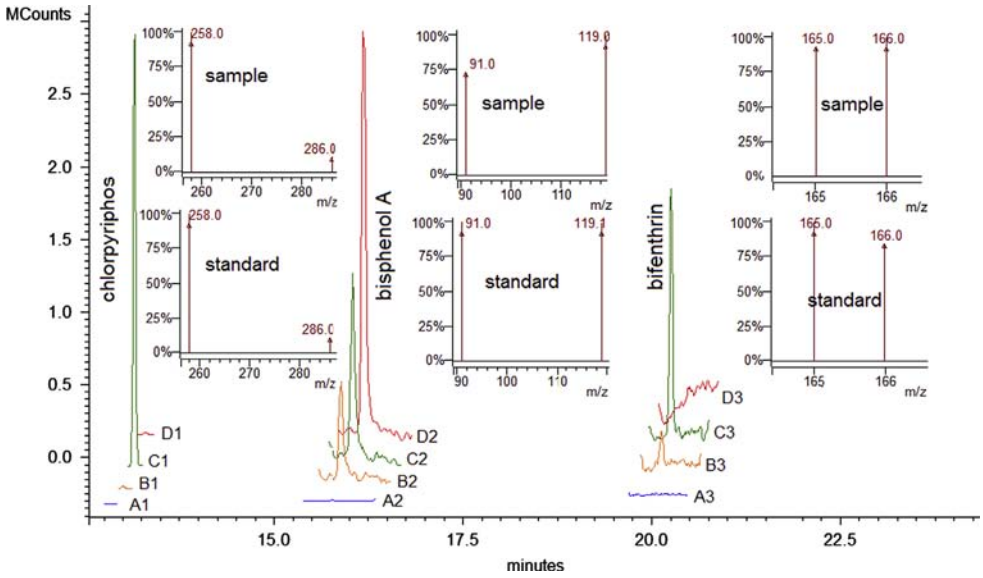

Fig. 5. Chromatograms of the positive samples compared to a control sample.

A few studies have investigated the occurrence of EDCs in fish. Bisphenol A an octanol–water partition coefficient (K_{ow}) of 3.3, which means that this substance can be mainly retained on organic matter. However, its transport in the aquatic environment is its major route of distribution [4]. In studies developed by Shao et al. [18] and Liu et al. [2] the presence of bisphenol A was observed in fish fillet at concentrations of 56 and 83.5 µg kg^{-1}, respectively. The presence of chlorpyrifos can be justified as a possible contamination of this organophosphate pesticide in products used in the fish feed, as reported by Sun and Chen [37]. The insecticide bifenthrin is highly toxic to fish with 96-h LD50 values of 0.10 and 0.18 µg L^{-1} for rainbow trout (*Onchorynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus*), respectively [38].

As well, the positive results for some EDCs show the need of monitoring residues of these compounds in fish and other aquatic species. Because of the large environmental and human impacts these substances generate, greater awareness surrounding their use should be encouraged.

4. Conclusions

The main advantages of the proposed method lie in the fact that it is simple and quick to perform, demands a small amount of solvent and permits the analysis of multiclass pesticides at trace levels in fish fillet samples with good accuracy and precision.

The QuEChERS approach is so flexible and rugged that most organic compounds give excellent results when the conditions for extraction and clean-up are selected properly. Tandem GC–MS/MS was selected as the detection technique and the simultaneous measurement of two transitions for each analyte confirms a positive result without the need to re-inject the sample. Thanks to the simplicity and quickness of the modified QuEChERS method, coupled with the selectivity and sensitivity of triple quadrupole MS the proposed method is capable of analyzing a large number of samples daily. The suitability of the developed method was demonstrated by the complete validation of the sample preparation and instrumental analysis.

The optimized modified QuEChERS method using GC–MS/MS in SRM mode for the determination of EDCs in catfish fillet proved to be effective for 36 of 40 endocrine disrupters evaluated. Recovery values ranged from 70.1% to 120.0% with RSD below 20% demonstrating good accuracy and precision. Linearity values were adequate with values of r^2 higher than 0.996, as well as limits of quantification from 1.0 to 25.0 $\mu g\ kg^{-1}$, which are lower than the described in the Brazilian legislation for residues in fish. Robustness studied with different fish fillet species (catfish, tilapia and striped catfish) demonstrated that small differences in the recovery values may occur and can be attributed to the difference in fat content of each fish species.

The application of the method in real samples showed excellent performance and no interferences from co-extractives were observed. The results proved that the method is adequate for utilization in routine analysis for the determination of EDC residues in fish fillet. In order to complement the study, bioassays could be realized in the future to investigate correlations between the active principle present in water and the quantity of this substance accumulated in the fish fillet. It is also very important to evaluate possible toxic effects of these compounds in different fish species [39,40].

Acknowledgments

Authors gratefully acknowledge the financial support and fellowship grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coodernação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

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