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A vortex-assisted MSPD method for the extraction of pesticide residues from fish liver and crab hepatopancreas with determination by GC–MS

Sergiane Souza Caldas [a], Cátia Marian Bolzan [a], Eliana Jaime de Menezes [b], Ana Laura Venquiaruti Escarrone [b], Camila de Martinez Gaspar Martins [b], Adalto Bianchini [b], Ednei Gilberto Primel [a,*]

[a] Laboratório de Análises de Compostos Orgânicos e Metais, Escola de Química e Alimentos, Universidade Federal do Rio Grande, Av Itália, km 8, s/n, Rio Grande, Rio Grande do Sul State, 96201-900, Brazil

[b] Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Av Itália, km 8, s/n, Rio Grande, Rio Grande do Sul State, 96201-900, Brazil

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ABSTRACT

A method based on matrix solid-phase dispersion (MSPD) and gas chromatography-mass spectrometry to determine pesticides in fish liver and crab hepatopancreas was optimized. Ethyl acetate and acetonitrile were evaluated as elution solvents and their volumes were also checked. The best results were obtained with 1.0 g reused C18 as sorbent, using 5 mL acetonitrile as the elution solvent. Analytical recoveries ranged between 57 and 107% with RSD lower than 26% in fish liver and between 56 and 122% with RSD lower than 21% in crab hepatopancreas. The LOQ values for these compounds ranged from 0.05 to 0.5 mg kg^{-1} for crab hepatopancreas and from 0.125 to 1.25 mg kg^{-1} for fish liver. MPSD was shown to be easy and fast to use, with a clear advantage regarding costs because it does not need any expensive instrument. The proposed method was successfully applied to determine dimethoate, atrazine, clomazone, fenitrothion, malathion, fipronil and tebuconazole in fish liver and crab hepatopancreas samples.

1. Introduction

Pesticides have been widely used all over the world because they have enabled the development of agricultural and farming production by controlling a wide range of pests and diseases. However, it is well known that the application of these substances can lead to damage for human health as well as for the environment [1,2].

Depending on their chemical class, pesticides may pose different toxicities to human health. The organophosphate class, which has been found to have endocrine disrupting properties, is suspected of altering reproductive function not only by reducing brain acetylcholinesterase activity but also by affecting the gonads [3]. Moreover, another characteristic of organophosphates is the tendency to bioaccumulate in lipophilic tissues, such as liver, due to the fact that they are metabolized in this organ [1].

Most pesticides are applied directly to the soil or sprayed over crop fields and, hence, released directly into the environment. Due to their physicochemical properties, such as solubility in water, soil-sorption constant (Koc) and half-life in soil (DT50), pesticides that have a low sorption coefficient, long half-life and high water solubility, tend to be leached [4,5] and reach water sources. It may result in the contamination of the aquatic ecosystem. Aquatic organisms, such as fish and shellfish, are able to accumulate pesticide residue in much higher concentrations than the surrounding water [6]. Thus, pesticides that have this tendency, besides lipophilic characteristics, can be found in biological tissues, such as fish liver and crab hepatopancreas. Studies have shown that the crab hepatopancreas, a large and fatty gland, is a major storage site of lipophilic environmental pollutants [7]. Besides, liver has shown to be the one of the tissues that has the highest concentrations of pesticides, such as the organochlorine ones [8]. Because of these characteristics, liver and hepatopancreas are considered suitable tissues for the determination of pesticides; they may generate additional information regarding the bioavailability of organic contaminants in the environment. It is worth mentioning that information on the pesticide levels in biological tissues associated with biomarker analyses has been used for biomonitoring programs, not only to verify water contamination by these compounds, but also to evaluate the biological impact of these aquatic pollutants.

Methods for pesticide residue determination in aquatic species are generally time-consuming and expensive, besides demanding good laboratory practices and advanced equipment. Therefore, it is highly desirable to have more practical and less expensive analytical methods in order to identify and quantify these residues in biological tissues at trace levels [1]. These methodologies must be efficient at the extraction process to ensure extraction, separation

* Corresponding author. Tel.: +55 5332336956; fax: +55 53 32336961.
E-mail addresses: dqmednei@furg.br, eprimelfurg@gmail.com (E. Gilberto Primel).

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and subsequent quantification of the residues found in samples. Efficient sample preparation depends on the matrix, as well as on the properties and concentrations of the analytes to be determined [9].

Currently, different sample preparation techniques have been used for the isolation of pesticides in fish and crab tissues, such as microwave-assisted extraction (MAE) [10,11], pressurized liquid extraction (PLE) [12] and matrix solid-phase extraction (MSPD) [13,14].

MSPD enables the simultaneous extraction and clean-up of analytes from solid samples with solid, semisolid and highly viscous features [15,16]; it can also be used as an alternative method to classical extraction [1,13,17]. This technique shows high flexibility and selectivity due to the variety of combinations of sorbents and elution solvents [18,19]. Because of its simplicity and high throughput, MSPD has been widely used for extracting pesticide residues from highly viscous samples [13,19,20].

Separation and quantification of volatile organic compounds that are thermally stable have been mainly carried out by gas chromatography, mainly mass spectrometry (GC–MS), since it allows the simultaneous determination of several compounds belonging to different classes [21].

The aim of this study was to optimize an analytical method which employs MSPD and determination by GC–MS for the extraction of atrazine, clomazone, dimethoate, fenitrothion, fipronil, malathion and tebuconazole from fish liver and crab hepatopancreas samples. Some parameters that affect the efficiency of the extraction were evaluated. The research aimed at reusing packing from C18 cartridges generated by the SPE technique. The method is environmentally friendly and minimizes costs regarding the sorbent, without affecting its precision and its accuracy. In addition, the use of a vortex instead of a vacuum manifold for the elution step enabled the analyst to be less exposed to the solvent and sample handling.

2. Experimental

2.1. Chemicals

Atrazine, clomazone, dimethoate, fenitrothion, fipronil, malathion and tebuconazole analytical standards (purity $>99\%$) were purchased from Sigma Aldrich (São Paulo, Brazil). Methanol, acetonitrile and ethyl acetate of chromatographic grade were supplied by Mallinckrodt (Phillipsburg, NJ, USA). The reused packing from C18 cartridges were recycled from Chromabond C18, whose particle size was 45 μm and pore size was 60 Å, supplied by Macherey-Nagel (Duran, Germany).

Standard stock solutions of 1000 mg L^{-1} and 100 mg L^{-1} of the target compounds were prepared in acetonitrile and stored at −18 °C. These solutions were used for preparing the pesticide working solutions which were used for sample spiking and for preparing the calibration curves. Working standard solutions were prepared monthly while the dilutions were prepared daily.

2.2. Apparatus and chromatographic conditions

Chromatographic analyses were carried out by a Shimadzu apparatus, model GC–MS-QP2010 Plus, equipped with an autosampler (AOC-20i) and a mass spectrometric detector with a quadrupole mass filter. Mass spectrometry was performed by electron impact. The GC separation was carried out by an Rtx®–5 MS capillary column (30 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA). Analytical instrument control, data acquisition and treatment were performed by the software *GC–MS solution version 2.7*® (Shimadzu, Columbia, MD, USA). Helium (99.999% purity) was the carrier gas. Injection of 2 μL at high pressure (150 kPa) and split ratio 1:10 were used.

For the optimization of the MS parameters, all compounds were monitored in full scan mode in the range *m/z* 50–500. The range of *m/z* should be reduced to the smallest appropriate scope so as to increase the sensitivity of full scan. Full scan spectra were obtained to select the appropriate precursor ions, which were selected in an attempt to choose the ion with higher *m/z* ratio (increase in selectivity) and abundance (increase in sensitivity) [22]. The other GC conditions, such as injection temperature, separation temperature program, interface and detector temperature, were optimized by using the pesticide standard solution in a 2.5 mg L^{-1} concentration as reference. The spectrometer was operated in electron-impact (EI) mode and the ionization energy was 70 eV.

2.3. Liver and hepatopancreas samples

MSPD procedure was performed in fish liver (*Micropogonias furnieri*) and crab hepatopancreas (*Callinectes sapidus*) collected in the *Patos* Lagoon estuary (in Southern Brazil), where agricultural activities are intense.

Animals were collected, kept on ice and transferred to the laboratory, where tissues of interest were dissected, conditioned in calcinated-aluminum foil and frozen (−80 °C) until the analyses.

2.4. Using reused C18-silica material

The reused SPE material used in this study was removed from SPE cartridges that had been used to pre-concentrate pesticides in drinking waters. The procedure to ensure the absence of contamination in the sorbent has been previously described [18]. In each step of the optimization, a procedure which uses only the reagents without any fortification was carried out to evaluate the presence of contamination in the reused material. It is important to highlight that neither background nor variations were found in the chromatograms after the analyses.

2.5. Vortex-assisted MSPD procedure

Aliquots of 0.2 g (liver) or 0.5 g (hepatopancreas) of sample were transferred to a mortar (30 mL capacity), spiked with the standard solution and, after solvent evaporation (approximately one hour), gently homogenized with a pestle with 1 g reused C18 for 5 min to obtain a homogeneous mixture. The mixture was poured into a 15 mL centrifuge polypropylene tube; 5 mL acetonitrile was added and the content was thoroughly vortexed for 1 min. Then, the tubes were placed into a centrifuge at 4000 rpm for 10 min. The extract was collected and 2 μL was injected into the GC–MS (Fig. 1).

3. Method validation

3.1. Limit of detection, limit of quantification and linearity

The limit of detection (LOD) and the limit of quantification (LOQ) of the method for each analyte were obtained considering 3 and 10 times the ratio of signal to baseline (noise), respectively. LOD and LOQ were determined by the injection of different concentrations of analytes diluted with the hepatopancreas and liver extracts and were confirmed experimentally. The LOQ is defined as the lowest validated spike level meeting the method performance acceptability criteria (mean recoveries were ranged from 70 to120%, with an RSDr≤20%).

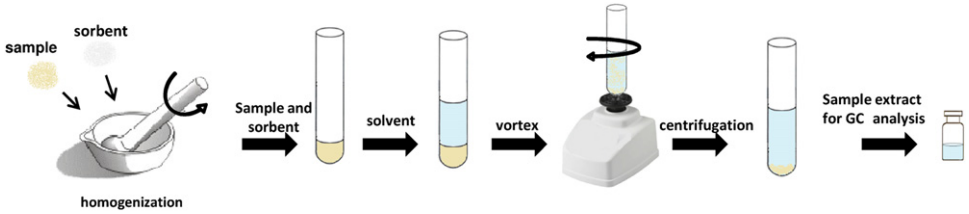

Fig. 1. Scheme of the vortex assisted procedure proposed in this study.

The linearity of the method was evaluated through matrix matched calibration in the concentration range of the LOQ of each compound to a concentration equivalent to 25-fold LOQ value. Three replicates of at least five concentration mixtures of calibration standards were injected. Dilutions of the standard solution of pesticides with the blank extract from the matrix extracted by MSPD were performed. An external calibration, in the same concentrations, was also performed by the dilution of the standard solution of pesticides in acetonitrile.

3.2. Accuracy, precision and matrix effect

The accuracy of the method was evaluated regarding the recovery assays, in compliance with INMETRO [23] and SANCO [24]. Blank tissue samples were fortified by adding a known volume of standard solution containing a mixture of pesticides in 0.5 g (crab hepatopancreas) and 0.2 g (fish liver) samples at the beginning of the process. The levels of fortification were a concentration equivalent to the LOQ, 2-fold LOQ and 10-fold LOQ. Each fortification level was extracted in triplicate and injected three times ($n=9$). The precision of the method was evaluated regarding the repeatability and the intermediate precision. Repeatability was studied in compliance with INMETRO [23], with nine determinations; extraction of the sample by MSPD was performed in three different fortification levels, in triplicate. Intermediate precision was estimated as repeatability, but on different days and by different analysts.

The study of the matrix effect (ME) was performed according to eq. 1, by comparing the slopes in matrix matched calibration solutions prepared in blank tissue extract and calibration solutions prepared in solvent [25]. The extent of the effects due to the matrix components was rated according to the % signal enhancement (+) or suppression (−).

$$ME\% = 100 \times \left(1 - \frac{Sm}{Ss}\right) \quad (1)$$

where Ss is the slope in solvent, Sm is the slope in matrix. No matrix effect is observed when ME(%) is equal to 100%. Values above 100% indicate ionization enhancement, and values below 100% show ionization suppression.

3.3. Quality control

Internal quality controls, such as the use of a blank matrix extract to eliminate false positives due to a possible contamination with pesticides during the extraction procedure, in the instrument or in the chemicals, the extraction with the samples of a spiked blank sample at 10-fold LOQ concentration to check the extraction efficiency and a calibration curve to evaluate sensitivity as well as linearity in the working range of concentrations, were carried out. A reagent blank (acetonitrile) was also injected after every six sample injections to check for carry over and to perform simple cleaning of the chromatographic system. No carry over phenomenon was noticed.

Table 1
Retention time, GC-EI-MS conditions and octanol-water partition coefficient (*Kow*) and vapor pressure.

Pesticides	t_R (min)	Monitored ions (m/z)	Segment	Time window (min)	*Kow*[b]	Vapor pressure (mPa)[b]
dimethoate	10.34	87, 125[a], 93	1	7.5–12.0	0.704	0.25
atrazine	10.86	200, 215[a], 58	1		2.5	0.0385
clomazone	10.97	204, 125[a], 176	1		2.5	19.2
fenitrothion	14.02	277[a], 125, 109	2	12.0–14.5	3.43	18
malathion	14.15	173[a], 127, 93	2		2.75	5.3
fipronil	14.55	367[a], 369, 213	3	14.0–15.0	4.0	0.00037
tebuconazole	16.06	250[a], 125, 163	4	15.0–17.0	3.7	0.0017

[a] íons used for quantification.
[b] [26].

3.4. Statistical analyses

All statistical analyses, including one-way analysis of variance (ANOVA), were performed by the GraphPad InStat software (Version 3.00, 1997). A 95% significance level was adopted for all comparisons.

4. Results and discussion

4.1. GC-EI-MS parameters

The MS system was set in selective ion monitoring (SIM) mode and each compound was quantified based on the peak area using three qualifier ions (Table 1). Identification and confirmation of pesticides were performed as recommended by the European SANCO Guidelines [24].

The optimized injector temperature was 250 °C. The GC oven temperature program was 80 °C, followed by a 50 °C min^{-1} ramp to 160 °C (held for 1 min), then by a 2 °C min^{-1} ramp to 180 °C and a final ramp of 160 °C min^{-1} to 360 °C (held for 4 min). A solvent delay time of 7.0 min was set to prevent instrument damage. The MS was calibrated with perfluorotributylamine (PFTBA). The MS system temperature of the detector interface was set at 280 °C and the source of ionization at 250 °C.

4.2. MSPD

The initial conditions proposed for the extraction method were based on a previously developed and validated MSPD procedure [18].

Some extraction parameters were evaluated to achieve the highest recovery for dimethoate, atrazine, clomazone, fenitrothion, malathion, fipronil and tebuconazole from the crab hepatopancreas.

Since the elution solvent polarity is known to be the key factor in MSPD because, along with the sorbent, it determines the efficiency of the extraction and the purity of the final extracts, the type of solvent and its volume were studied.

Preliminary investigations for the optimization of the MSPD procedure for the extraction of pesticides from hepatopancreas were performed by using 0.5 g hepatopancreas crab samples spiked with pesticides at 10 mg kg^{-1} level, using 1 g reused C18 as solid-phase sorbent. The elution was performed by a vacuum manifold with a flow rate set at 1 mL min^{-1}.

The recovery rates were calculated by matrix-matched calibration; thus, quantification mistakes caused by ion suppression and enhancement were compensated.

To evaluate the influence of the eluting solvent, 10 mL acetonitrile and ethyl acetate was used. The aim was to find a solvent capable of extracting a large number of pesticides with the lowest amount of matrix compounds. Acetonitrile and ethyl acetate are two solvents that are commonly found in pesticide residue analysis. Both solvents present similar polarities; however, the capacity that ethyl acetate has to extract pesticides is higher than the one acetonitrile has, which means that, when ethyl acetate is used, a lot of inferences will be extracted by the eluate [27].

When data were compared (Fig. 2), except in the cases of tebuconazole and clomazone, acetonitrile was observed to provide higher recoveries. Besides, a high matrix effect was observed with ethyl acetate for the compounds dimethoate and atrazine.

Therefore, overall results indicate that the combination of reused C18 as solid-phase and acetonitrile as elution solvent was the most suitable extraction procedure for the combinations tested for the determination of dimethoate, atrazine, clomazone, fenitrothion, malathion, fipronil and tebuconazole in crab hepatopancreas. Recoveries were between 39 and 123% for the target compounds. An advantage of acetonitrile, by comparison with ethyl acetate, is that the former does not extract so much lipophilic material as the latter, e.g., waxes, fat and lipophilic pigments [19,27].

In order to have lower limits of quantification and in an attempt to reduce the solvent volume that was employed, lower volumes of acetonitrile were tested. When 1 mL acetonitrile was used for pesticide elution, the recoveries were lower than when 5 and 10 mL were used (data is not shown). One mL may not be enough to elute all the compounds retained in the sorbent. Higher recoveries were reached when 5 and 10 mL acetonitrile were employed. Five mL was chosen because it presented no significant differences from 10 mL when a statistical test was carried out (t test, $P > 0.05$) and it is cheaper, safer and environmentally friendlier than 10 mL.

After these experiments, a modification during the elution step was performed [28]. After blending the sample (0.5 g) with 1 g reused C18 sorbent in a mortar, the mixture was poured into a 15 mL polypropylene centrifuge tube and 5 mL acetonitrile was added. The tube content was thoroughly mixed for 1 min in a vortex. Then, the tubes were placed into a centrifuge at 4000 rpm for 10 min. This procedure (vortex-assisted) was compared to the traditional MSPD with elution in a vacuum manifold. Results are shown in Fig. 3.

Although the recoveries using the vacuum manifold for the elution were slightly higher, no significant differences were observed when a statistical test was carried out (t test, $P > 0.05$). Besides, this change in the procedure makes it faster, the elution becomes easier and the analysts do not have to be exposed to the organic solvent for long.

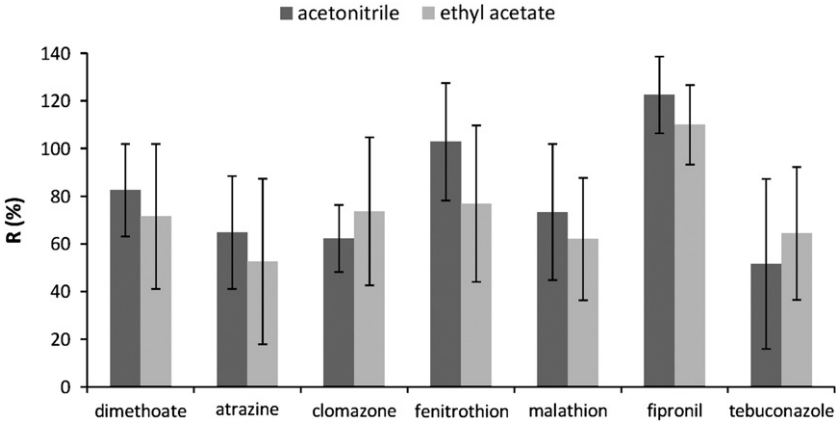

Fig. 2. Effect of the type of extraction solvent on the recoveries of the pesticides (Bars indicate RSD values).

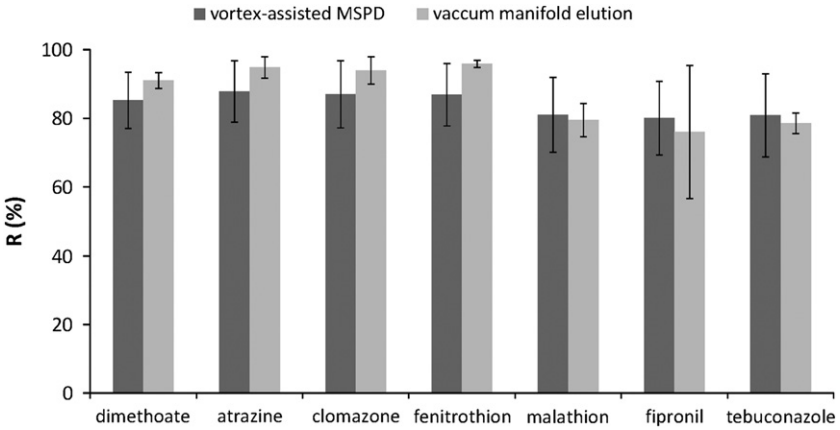

Fig. 3. Effect of the type of elution on the recoveries of the pesticides (Bars indicate RSD values).

5. MSPD validation

5.1. Method validation

Once the factors that affect the MSPD procedure had been optimized, the validation of the method was performed in accordance with SANCO Guidelines [24] for method validation and quality control procedures for pesticide residue analysis in food and feed. The following performance characteristics were studied: linearity, recovery, precision, limits of detection and quantification and matrix effect. The validation process was carried out in crab hepatopancreas and fish liver. For the latter, 0.2 g sample was employed due to the fact that less tissue was available.

5.1.1. Quantification criteria and calibration curves

Quantification of the target compounds was made in the SIM mode. Calibration curves were obtained with standards prepared in blank matrix in order to compensate for the suppression-enhancement of the analyte response due to the presence of certain compounds from the matrix which are not removed in the MPSD step.

Table 2
LOQ and analytical curve parameters in fish liver and crab hepoatopancreas matrices.

Pesticides	Hepatopancreas crab			Fish liver		
	LOQ (mg kg^{-1})	Linearity (mg kg^{-1})	r	LOQ (mg kg^{-1})	Linearity (mg kg^{-1})	r
dimethoate	0.5	0.5–12.5	0.999	1.25	1.25–31.25	0.999
atrazine	0.25	0.25–6.25	0.999	0.625	0.625–15. 63	0.999
clomazone	0.05	0.05–1.25	0.999	0.125	0.05–1.25	0.999
fenitrothion	0.1	0.1–2.5	0.999	0.25	0.25–6.25	0.999
malathion	0.1	0.1–2.5	0.999	0.25	0.25–6.25	0.999
fipronil	0.1	0.1–2.5	0.989	0.25	0.25–6.25	0.996
tebuconazole	0.05	0.05–1.25	0.999	0.125	0.05–1.25	0.999

Table 3
Recovery values ($n=9$) and relative standard deviation (RSD) obtained with the MPSD method at three concentration levels in fish liver and crab hepatopancreas matrices.

pesticide	Hepatopancreas			Liver		
	1LOQ R% ± RSD	2LOQ R% ± RSD	10LOQ R% ± RSD	1LOQ R% ± RSD	2LOQ R% ± RSD	10LOQ R% ± RSD
dimethoate	89 ± 10	74 ± 11	63 ± 2	84 ± 6	73 ± 7	71 ± 5
atrazine	77 ± 9	77 ± 8	69 ± 6	74 ± 10	75 ± 12	68 ± 6
clomazone	84 ± 11	73 ± 21	69 ± 7	65 ± 14	69 ± 8	69 ± 5
fenitrothion	94 ± 10	77 ± 7	67 ± 7	107 ± 9	86 ± 5	81 ± 6
malathion	65 ± 20	56 ± 20	66 ± 3	66 ± 12	65 ± 10	69 ± 8
fipronil	88 ± 18	109 ± 19	122 ± 13	83 ± 19	68 ± 15	72 ± 8
tebuconazole	77 ± 10	61 ± 10	62 ± 5	93 ± 26	58 ± 14	57 ± 8

5.1.2. Linearity and detection and quantification Limits

Linearity was studied by injecting 2 μL spiked blank matrix extracts into the LOQ to 25-fold LOQ range (Table 2). The first calibration level was always equivalent to the LOQ of the compound. Linear calibration curves were plotted by least-squares regression of concentration versus the peak area of the calibrations standards. Adequate linearity in the concentration range from the LOQ to 25-fold LOQ, with correlation coefficients (r) higher than 0.99, was obtained.

The LOQ values for these compounds ranged from 0.05 to 0.5 mg kg^{-1} for crab hepatopancreas, and from 0.125 to 1.25 mg kg^{-1}, for fish liver. The resulting range of LOQ is of the same order as those previously obtained by other authors who used the MSPD technique [29,30].

5.1.3. Accuracy and precision

Recovery data were calculated and compared with the appropriate working standard solutions prepared with the hepatopancreas extracts. The hepatopancreas free from pesticides was fortified at three different concentrations (1, 2 and 10-fold LOQ) and residues were quantified by using the matrix-matched standard. Average recoveries for fish liver ranged from 57 to 107%, with relative standard deviations (RSD) from 5 to 26%, and for crab hepatopancreas, between 56 and 122% with RSDs lower than 21, as can be seen in Table 3. Precision and accuracy were considered adequate to validate the method in accordance with the validation criteria. Fig. 4 shows the chromatogram of a standard solution and a spiked fish liver sample.

5.1.4. Matrix effect

The sample matrix can cause an enhancement in the chromatographic response for pesticide residues in a matrix extract by comparison with the same concentration in a solvent solution. Matrix type, ratio of matrix to analyte concentration, the injector type and the temperature can affect the enhancement values [25]. The slopes of standard curves constructed in acetonitrile and in the extracts may serve as an indicator of the matrix effect (ME (%)). When the slope of the analytical curve prepared by spiking blank matrices extract with known amounts of pesticides was compared with the slope of the analytical curves prepared in acetonitrile, higher enrichment of the signals was found for the fish liver matrix ($>50\%$). For the hepatopancreas matrix, no significant matrix effect ($<10\%$) was found. Results show that the use of a simple external standard calibration method may produce erroneous results in the quantification of pesticides in these samples. In this study, to solve this problem, a matrix matched calibration was used for improving the accuracy of the quantification.

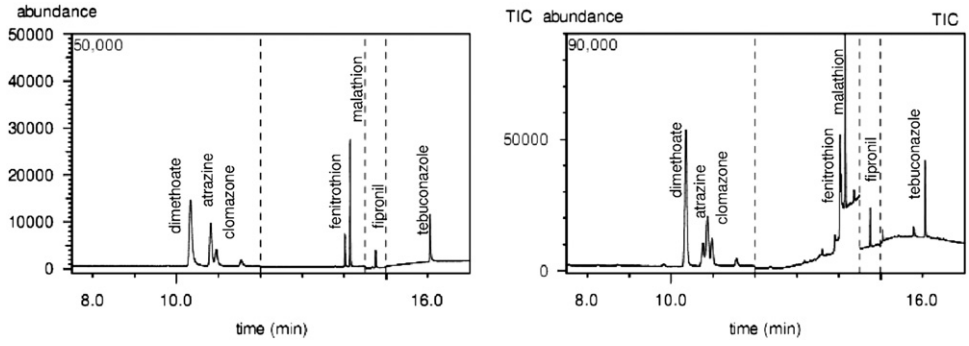

Fig. 4. Total Ion Chromatogram of the standard mixture in solvent (a) and in fish liver extract (b).

5.2. Real sample analysis

The MSPD method was applied to determine the selected pesticides in fish liver and crab hepatopancreas collected in the *Patos* Lagoon in Rio Grande do Sul state (Brazil).

The proposed method was applied to the analysis of 30 fish liver samples and 10 crab hepatopancreas samples. For this purpose, matrix matched calibrations were prepared in each one of the analyzed matrices in order to avoid any matrix interferences.

The results of the real analyzed samples show clomazone in concentrations higher than the LOQ. The compound was found in three samples in the following concentrations: (mg $kg^{-1} \pm$ standard deviation) (0.06 ± 0.011; 0.07 ± 0.005; 0.09 ± 0.003). Clomazone is a herbicide that belongs to the class isoxazolidinone and is widely used in agriculture, especially in paddy rice fields in Southern Brazil. This herbicide is highly effective and can cause contamination due to its high water solubility (1100 mg L^{-1}) and long half-life dissipation, averaging from 28 to 84 days. Clomazone has been reported to contaminate surface waters in the southern region in Brazil [31]. Some studies have reported the effect of clomazone on the metabolic and oxidative parameters of different fish species [32–34].

6. Conclusions

A robust, rapid, simple method was developed for the determination of pesticide residues in biological tissues by GC-EI-MS. The method enables extraction with low solvent consumption and short analysis time. Reused C18, as sorbent material, and elution with acetonitrile efficiently extract the target pesticides. Results demonstrated that accuracy and precision were satisfactory for pesticide determination. In addition, the method requires a small amount of sample and is considerably economical in terms of solvent consumption, cost of material, sample manipulation and time of analysis by comparison with classical procedures. The application of the vortex-assisted MSPD method to the analysis of real samples shows clomazone in some fish liver samples at trace levels.

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