Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 1146–1156

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Enzymatic-microwave assisted extraction and high-performance liquid chromatography–mass spectrometry for the determination of selected veterinary antibiotics in fish and mussel samples

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ARTICLE INFO

Article history:
Received 25 June 2010
Received in revised form 1 December 2010
Accepted 3 December 2010
Available online 9 December 2010

Keywords:
Antibiotics
Fish
Mussel
Enzymatic-microwave extraction
HPLC–MS

ABSTRACT

A new method based on enzymatic-microwave assisted extraction prior to high performance liquid chromatography (HPLC) has been developed for the determination of 11 antibiotics (drugs) and the main metabolites of five of them in fish tissue and mussel samples. The analysed compounds were sulfadiazine (SDI), N^4-acetylsulfadiazine (NDI), sulfamethazine (SMZ), N^4-acetylsulfamethazine (NMZ), sulfamerazine (SMR), N^4-acetylsulfamerazine (NMR), sulfamethoxazole (SMX), trimetroprim (TMP), amoxicillin (AMX), amoxicilloic acid (AMA), ampicillin (AMP), ampicilloic acid (APA), chloramphenicol (CLF), thiamphenicol (TIF), oxytetracycline (OXT) and chlortetracycline (CLT).

The main factors affecting the extraction efficiency were optimized in tissue of hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*), *mussel* (*Mytilus* sp.) and wedge sole (*Solea solea*). The microwave extraction was carried out using an extraction time of 5 min with 5 mL of water at 50 W and posterior clean up with dichloromethane.

High-performance liquid chromatography (HPLC)–mass spectrometry was used for the determination of the antibiotics. The separation of the analysed compounds was conducted by means of a Phenomenex® Gemini C_{18} (150 mm × 4.6 mm I.D., particle size 5 μm) analytical column with LiChroCART® LiChrospher® C_{18} (4 mm × 4 mm, particle size 5 μm) guard-column. Analysed drugs were determined using formic acid 0.1% in water and acetonitrile in gradient elution mode as mobile phase. Under the optimal conditions, the average recoveries of all the analysed drugs were in the range 70–100%. The proposed method was applied to samples obtained from Mediterranean sea and also evaluated by a laboratory assay consisting in the determination of the targeted analytes in samples of *Cyprinus carpio* that had been previously administered the antibiotics.

1. Introduction

The occurrence of antimicrobials in fish tissues has received broad interest over the last years [1–4]. Reports recently published have demonstrated that continuous exposures to these compounds may result in accumulation of the parent compound, their metabolites or both in tissues of aquatic organisms [5].

Antimicrobials are widely prescribed for therapeutic and prophylactic reasons against microbial infections and also in animal farms as growth promoting agents. The presence of these drugs in animal tissues can have undesirable effects on consumer health, such as allergies, but that is not the main problem because there is a low incidence of such cases; the main problem is that uncontrolled ingestion of antimicrobials by consumer causes the development of bacterial resistance, which translates into a much bigger problem for consumers health when dealing with infections.

The recognition of the risks associated with direct and indirect effects on human health due to both active and passive consumptions of antibiotics has led to set regulations on the use of some antibiotics in food production of animal origin (specially antibiotics of which cannot be determined safe levels of residues) and the establishment of maximum residue limits (MRLs) for those involving known risks. The European Union (EU) establishes these limits in its Council Directive 2377/90 EC [6].

Analytical procedures for the determination of antibiotics in animal tissues have been summarized in several reviews [7,8], but to date relatively few references on determinations in fish or bivalve tissues have been published. Most recent papers are focussed on HPLC and CE, some LC–MS methods have been developed for the determination of antibiotic groups such as sulfonamides [9,10], tetracyclines [11,12] and quinolones [13,14], but very few related to the simultaneous determination of antibiotics belong-

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doi:10.1016/j.jpba.2010.12.002

ing to different classes [15,16]. A liquid chromatography–tandem mass spectrophotometry (LC–MS–MS) screening method has been developed targeting 23 pharmaceuticals and two metabolites, with different physicochemical properties, in fish tissues [5], a simultaneous determination of selected veterinary antibiotics in gilthead sea bream, using liquid chromatography–mass spectrometry [17], a paper that includes a multiresidue screening of 38 drugs [18] and two very recent papers have been published, one of them for the simultaneous determination of 17 sulfonamides and 5 tetracyclines in fish tissue using advance mass spectrometry [19] and another based on micellar liquid chromatography, technique that permits the direct injection of physiological samples reducing considerably pretreatment steps [20]. Most methodologies imply extraction procedures based on solid–liquid extractions with relatively polar solvents and subsequent clean up step by solid-phase extraction prior to chromatography analysis [18,21–23], but there is a highly interest in improving chromatographic detection sensitivity not paying much attention to sample treatment procedures. In general, sample preparation is crucial and supposes the most time consuming step when using HPLC methods applied to complex matrices such as food samples.

One of the main problems that the quantitative analysis of pharmaceuticals in biological samples presents is that the analyte usually is bound to proteins and peptides, with the consequent need for cleavage of these structures before analysis. Enzymatic digestion is widely accepted as sample preparation method for the analysis of compounds in biological matrices; however, these methods are labour-intensive and greatly lengthen the time of analysis.

Since early 1990s, the use of microwave assisted extraction (MAE) has been promoted as a suitable alternative to different extraction techniques [24,25]. It has been reported to be economical, reliable and environmentally friendly and a technique that offers comparable or better extraction efficiencies than more conventional techniques [26,27]. MAE for analytical purposes have found widespread use in several areas including environmental analysis, food and clinical determinations, but few reports have been published on analysis of bioactive compounds and drugs in animal tissues [26–28]. There are no papers to our knowledge about the combination of enzymes and microwaves to determine drugs in animal tissues. Despite resulting a strange combination because the enzymes lose their enzyme activity to microwave irradiation, in this paper it is shown that at low powers the extraction efficiency increases, possibly due to the drugs get free from protein binding sites.

The objective of this work was to develop and validate an affordable method for the simultaneous determination of selected veterinary antibiotics that are widely used in veterinary medicine for marine origin food. The analysed drugs include two penicillins and their main metabolites (amoxicillin (AMX) and ampicillin (AMP) and metabolites amoxicilloic acid (AMA), ampicilloic acid (APA)), two tetracyclines (oxytetracycline (OXT) and chlortetracycline (CLT)), two amphenicols (chloramphenicol (CLF) and thiamphenicol (TIF)) and five sulfonamides (sulfadiazine (SDI), sulfamethazine (SMZ), sulfamerazine (SMR), sulfamethoxazole (SMX) and trimetroprim (TMP)) and their metabolites (N^4-acetylsulfadiazine (NDI) metabolite, N^4-acetylsulfamethazine (NMZ) and N^4-acetylsulfamerazine (NMR)). The method involves enzymatic-microwave assisted extraction, followed by a simple liquid extraction procedure of clean-up combined with liquid chromatography for the determination of the cited antibiotics in several marine origin food tissues: hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*), *mussel* (*Mytilus galloprovincialis*) and wedge sole (*Solea solea*) by mass spectrometry detection.

The appropriateness of the method was also evaluated by a laboratory assay consisting in the determination of the targeted analytes in samples of *Cyprinus carpio* that were previously administered controlled doses of the antibiotics. Finally, the method was applied to samples of wild fishes from The Mediterranean Sea.

2. Materials and methods

2.1. Chemical and reagents

AMX, AMP, SDI, SMR, SMZ, SMX, TMP, CLF, TIF, OXT and CLT (97–99.9% purity) were purchased from Sigma–Aldrich Quimica, S.A. (Madrid, Spain). NMR, NMZ, NDI, AMA and APA were synthesized in our laboratory according to the protocols described by Pfeifer et al. [29] and Baker [30] and purity was checked by thin layer chromatography and mass spectrometry. Methanol (HPLC grade), acetonitrile (HPLC grade), ethyl acetate, 2-propanol (HPLC grade) and formic acid (98–100% purity), all analytical grade, were purchased from VWR (Barcelona, Spain). Dichloromethane for analysis (HPLC grade) was obtained from Romil Ltd. (Waterbeach, Cambridge, UK). Deionised water was obtained from a Milli-Q plus water system Millipore (Billerica, MA, USA). Lipase from *Candida rugosa* and Protease from *Bacillus licheniformis* were purchased from Sigma–Aldrich Quimica, S.A. (Madrid, Spain) and Proteinase-K 20.2 mg mL^{-1} recombinant PCR grade was obtained from Roche Diagnosis (Barcelona, Spain).

Individual standard solutions of AMX, AMP, OXT, TIF, CLT, CLF, AMA and APA at a concentration of 100 μg mL^{-1} and 50 μg mL^{-1} of SDI, SMR, SMZ and SMX were prepared by dissolving the drugs in deionised water. Individual standard 100 μg mL^{-1} solutions of TMP, NDI, NMR and NMZ were prepared by dissolving the drugs in methanol. All standard solutions were stored at 4 °C and AMX, AMP, TIF, CLF, SDI, SMR, SMZ, SMX, TMP, NDI, NMR and NMZ solutions were stable for at least one month; AMA and APA solutions were daily prepared before used and were stable for 24 h; OXT and CLT stock solutions were stable for one week.

2.2. Equipment

Chromatographic separation was performed using a PerkinElmer Series 200 LC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP® LC/MS/MS (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQLIT) mass spectrometer equipped with an electrospray ion source. Separations were carried out using a reversed-phase Phenomenex® Gemini C_{18e} 110 Å analytical column (150 mm × 4.6 mm, particle size 5 μm) preceded by a guard-column LiChroCART® 4-4 LiChrospher® 100 RP-18 (4 mm × 4 mm, particle size 5 μm) (Merck, Darmstadt, Germany).

A Sigma centrifuge Laborzentrifugen 4–10 (Osterode, Germany) was used to centrifuge samples. A microwave system Ethos 900 (Milestone, Sorisole, Italy) with programmable power and irradiation time was used to carry out the extractions.

2.3. LC and MS–MS conditions

A modification of a chromatographic separation optimized in a previous paper [31] was used. The mobile phase consisted of a mixture of 0.1% formic acid in water pH 2.6 (component A) and acetonitrile (component B). A gradient elution program at a 0.4 mL min^{-1} flow rate was used to achieve the separation. The program begins with an isocratic step at 99% A for 7 min followed by a linear elution gradient from 99% to 70% A in 30 min more, and returned to initial conditions. Multiple Reaction Monitoring (MRM) was applied where the parent ions and fragment ions were monitored at Q1 and Q3, respectively. Both positive and negative ionisation modes were employed. In general, the precursor ion was $[M+H]^+$ for most of the analysed compounds except for TIF and CLF

Table 1
LC–MS/MS conditions.

Analyte	Retention time (min)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)		Collision energy (eV)	Collision cell exit potential (V)
			Quantitation ion	Confirmation ion		
AMA	15.1	384.2	367.1	323.1	20.0	17.0
AMX	16.1	366.2	349.1	208.1	15.0	10.0
APA	20.1	368.2	324.2	307.2	20.0	15.0
SDI	21.3	251.2	156.1	108.0	20.0	7.0
TMP	21.9	291.1	261.1	230.2	25.0	12.0
AMP	22.2	350.1	192.1	106.0	20.0	9.0
OXT	23.6	461.0	426.1	443.1	20.0	18.0
SMR	24.3	265.2	172.1	108.1	20.0	8.0
NDI	26.3	293.2	198.0	134.0	20.0	8.0
SMZ	26.4	279.1	186.1	124.1	20.0	8.0
NMR	27.5	307.2	172.0	198.1	25.0	5.0
CLT	28.8	479.0	462.0	444.0	25.0	2.0
NMZ	30.0	321.2	255.1	186.1	20.0	10.0
SMX	33.9	254.2	108.1	156.1	30.0	5.0
TIF	31.0	321.1	275.0	165.1	10.0	11.0
CLF	37.4	354.1	338.0	308.0	10.0	15.0

that was $[M-H]^-$. Nitrogen was used as collision gas at 4 psi. The ion source and curtain gases were set at 30 psi in both cases. The electrospray voltage was −4.5 kV. The optimisation of MS parameters (precursor ions, collision energy, collision cell exit potential and quantitation and confirmation transitions) was performed by flow injection analysis for each compound dissolved in mobile phase and infused at 10 μL min^{-1}. Table 1 shows the values of the parameters optimised and the MRM transitions selected.

2.4. Sample pre-treatment

Fish and mussels were purchased from different supermarkets in the city of Seville (Spain), around 2000 g were shipped to our laboratory under cool conditions in a portable refrigerator. The muscle tissue was extracted and frozen at −20 °C upon lyophilising. Samples were lyophilised before being analysed by a freeze dry system (FreeZone 2.5, Labconco, Mo, USA). Then they were homogenized using a grinder and kept at −20 °C until analysis.

To optimise the method, lyophilised tissue samples were spiked with all compounds, which were dissolved in 5 mL methanol. After spiking, the samples were shaken intensively so that the compounds spread throughout the spiking solution in the sample and were in sufficient contact with the matrix. They were then evaporated to dryness at room temperature after 24 h contact at 4 °C.

2.5. Carp (C. carpio) assays

This experiment was carried out to evaluate the method in samples, which we were sure, that had been exposure to the antibiotics. Carp was selected for this experiment because they are species that can live for a long period of time in an aquarium and they are easy to acquire alive in pet shops. Before the assay, the animals were maintained in a tank of 1.0 m^3 in the laboratory (filled with 700 L of water and equipped with a filtration and aeration system for aquariums) for a period of two months to assure the elimination of antibiotics possibly administered in the pet shop as prophylactic treatment.

Four different assays were carried out using different combinations of antibiotics in order to assure that the doses of the antibiotics and the combinations used were not lethal for the animals: assay 1 (TIF, TMP and CLF); assay 2 (SDI, SMR, SMZ and SMX); assay 3 (SDI, SMX, OXT and AMX) and assay 4 (OXT, CLT, AMX and AMP).

Three animals (30 g weight each approximately) were isolated into a small aquarium of 20 L before the administration of the antibiotics. 100 mg of each antibiotic (according to recommended doses [32]) were mixed in a small ball of bread to assure the animals ingested the entire dose. A first dose of the antibiotic mixture was administered and 24 h later a second dose. After 1 h, the animals were sacrificed and the tissues submitted to the procedure described bellow in Section 2.6. Each assay was carried out with three animals whose tissue was obtained and treated as described in previous section. Three carps were used for blank analysis.

2.6. Wild fish samples

In order to verify whether the presence of pharmaceuticals is significant in aquatic ecosystems, we proceeded to capture a variety of marine specimens on the coast closest to a town with some importance. Samples were obtained from the fishing town of Aguilas located at the southwest corner of the region of Murcia (Spain), with an area of 251.77 km^2 and 28 km of Mediterranean coastline. It maintains a stable population of 28,800 inhabitants, which in summer reaches 150,000. The capture of the specimens was carried out by using artisan fishing with a net at a distance between 100 and 200 m from the beach, in the month of August 2009. The specimens obtained were: *Trachynotus ovatus*, *Sarpa salpa*, *Oblada melanura* and *Liza ramada*. The animals were then sacrificed and the tissues submitted to the procedure described bellow in Section 2.6.

2.7. Sample extraction

2.0 g of lyophilised tissue was weighted into the extraction vessels. 5 mL of deionised water and 50 μL of Proteinase-K solution were added to the samples. Extraction was carried out in 5 min at 50 W of irradiation power. The resulting mixture was centrifuged at 8000 × *g* for 5 min and the liquid phase treated with 100 μL of formic acid plus 5 mL of dichloromethane, manually agitated for 1 min and the organic phase reserved for posterior evaporation under a nitrogen stream. The dichloromethane addition was repeated and all the liquid extracts were evaporated. The residue was reconstituted with 1 mL of deionised water, filtered through 22-μm nylon membranes and 20 μL was injected into the chromatographic system.

3. Results and discussion

3.1. Stability of the analytes

A study of stability was realised to fix the maximum limits of microwave irradiation power and time that could be applied to the analytes before suffering any degradation. A standard solution

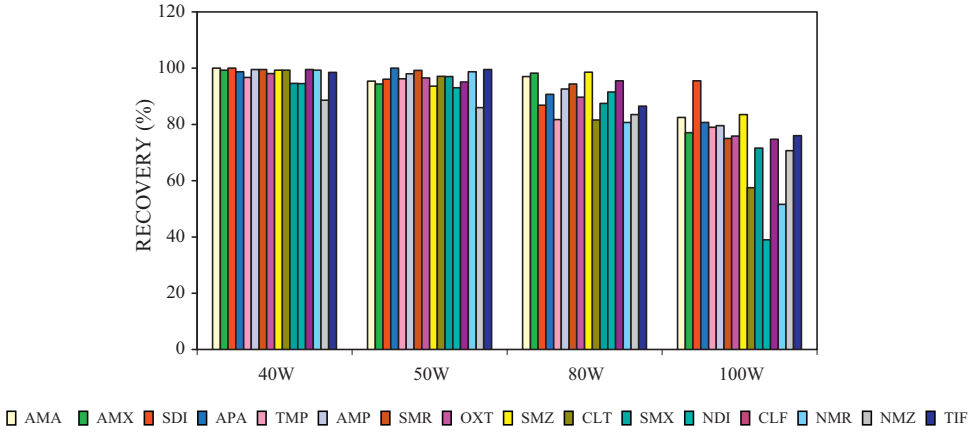

Fig. 1. Stability of a 0.5 μg mL^{-1} standard solution at different irradiation powers.

containing 200 ng mL^{-1} of each analyte was submitted to powers of 40, 50, 80, 100 W for 6 min. Results obtained showed (Fig. 1) that at powers over 50 W some of the analytes begin to suffer a degradation so 50 W was selected for further optimisation. The effect of the time of application of microwaves on stability of the analytes was also evaluated. Times between 1 and 10 min were applied finding that at times over 7 min a slightly degradation was observed for SDI, NDI, AMP and SMR.

3.2. Optimisation of classical MAE

Spiked samples, according to Section 2.4, of 250 ng g^{-1} of each analyte were used to optimise all the MAE parameters. The parameters extracting agent, volume of extracting agent, irradiation power and time of application were optimised.

Different solvents were assayed and results are shown in Fig. 2. As it can be observed, formic acid aqueous solution (0.2%, w/v) was the solvent that gave the best results in terms of recovery. The volume was also evaluated (from 2.5 mL to 10 mL). As it can be seen in Fig. 3, a volume of 5 mL gave the best extraction efficiencies. It could be surprising that higher volumes gave worst results when, in general, in conventional extraction techniques the recoveries increase with higher volumes of solvent. This may be due to an inadequate movement or heating of the extracting agent when microwaves are applied to larger volumes of solvent, or that matrix of samples containing proteins and fats exhibit this behaviour when microwaves are applied [33].

The effect of the irradiation power was also optimised; powers of 25, 50, 70, 80 and 100 W were tested obtaining the best recoveries at 50 W as it is shown in Fig. 4. The last parameter to optimise was the time of application. Fig. 5 shows that 5 min gave the best results. Times above 5 min not only affect the stability of the analytes but also allow the extraction of undesired substances so it led to worse recoveries. Under these conditions recoveries of all analytes were in the range 50–90%, as it can be seen in Fig. 5.

3.3. Optimisation of enzymatic-MAE

In order to improve extraction efficiencies, a combination between an enzymatic digestion and a subsequent inhibition of enzyme activity by means of microwaves was assayed finding that at low irradiation powers not only enzyme activity remains stable but also recoveries of almost all analytes increased. Taking into account this fact a procedure using a combination between enzymes and microwaves was studied and optimised. The parameters to optimise for the enzymatic treatment were: type and quantity of enzyme and time and power applied. A previous bibliographic search of published papers led us to select three different enzymes to study: Protease, Proteinase-K and a combination Protease-Lipase. In preliminary studies the different enzymes were tested on the four types of tissues setting the conditions at 5 mL of water, 50 W of irradiation power and 5 min. The amounts of enzyme tested were 100 μL of a commercial solution of Proteinase-K of 20.2 mg mL^{-1}; 50 mg of Protease and 100 mg of a mixture Protease: Lipase (1:1, w/w). Fig. 6 shows the results obtained and as it can be seen, Proteinase-K gave the best recoveries. The amount of enzyme was also optimized looking for the smallest amount that gave the best results because the reagent is costly. 25, 50 and 100 μL of the commercial solution of Proteinase-K of 20.2 mg mL^{-1} were checked and no substantial difference was obtained between 50 and 100 μL so a volume of 50 μL was selected. Under these conditions recoveries (see Fig. 6) of nearly all analytes, except for the metabolites, were approximately 90% and higher which justifies the use of the enzyme. This fact is quite interesting because enzymatic digestion procedures usually require times of treatment of several hours, even days in some cases. When lower times and irradiation powers than 5 min and 50 W were tested, in general, worse recoveries were obtained and at higher times and irradiation powers, as it is described in Section 3.2, the extraction of undesirable substances and degradation of analytes arose.

3.4. Matrix effects

One drawback that electrospray ionisation techniques present is that the presence of matrix components may affect the ionisation of the target analytes. For assessment of matrix effects, signal suppression/enhancement was studied by analysing standard solutions 100 ng mL^{-1} of the analytes and extracts of the different tissues spiked at the same concentration level. MS responses were compared and the relative responses (response matrix/response solvent) were calculated obtaining results ranging from 0.80 for ampicillin in anchovy matrix and 1.12 for oxytetracycline, being the rest of the results within that range. Although no significant matrix effects were observed, matrix-matched calibration was selected for quantitation purposes to avoid slight variations that could arise in the MS signal in wild fish samples.

3.5. Validation study

The proposed method for antibiotics determination in fish samples was validated checking the quality parameters of the analytical method as indicated in the Commission Decision 2002/657/EC [34]. We have mainly considered the items related to the estimation of the well known performance characteristics parameters. The fol-

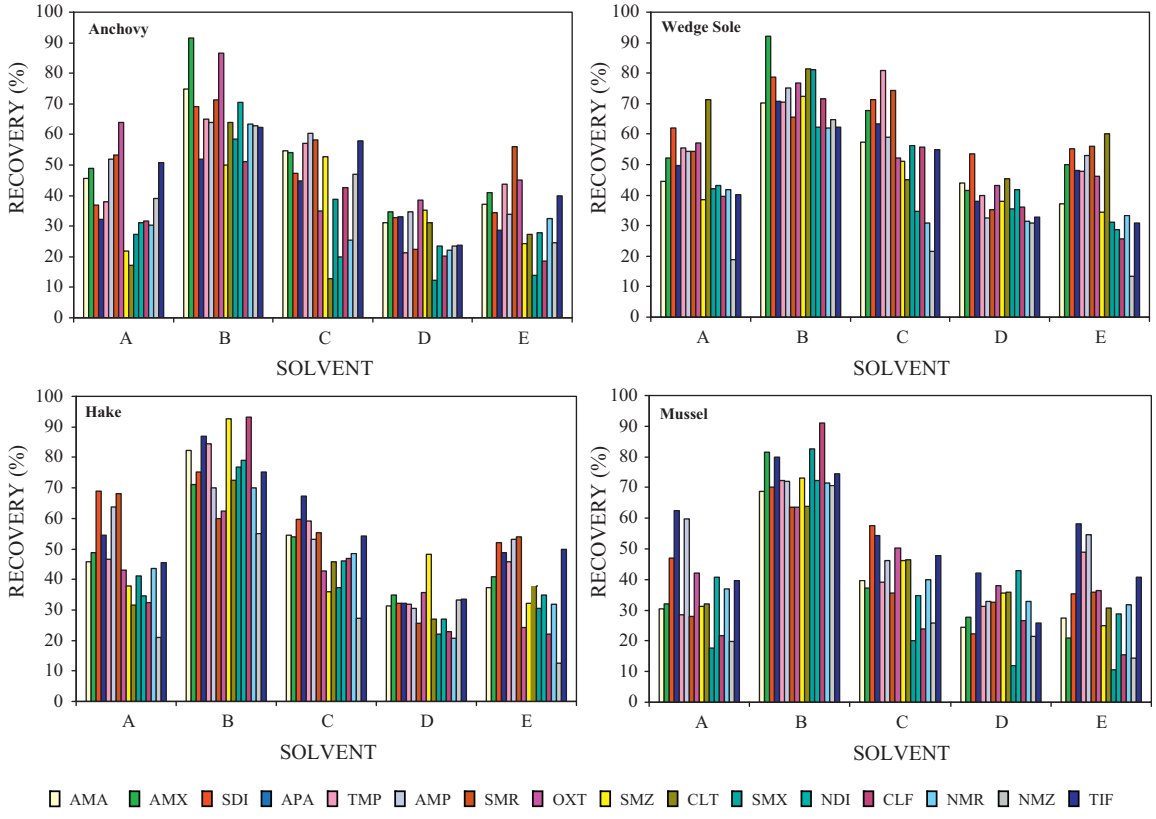

Fig. 2. Effect of type of extractant on extraction recovery.

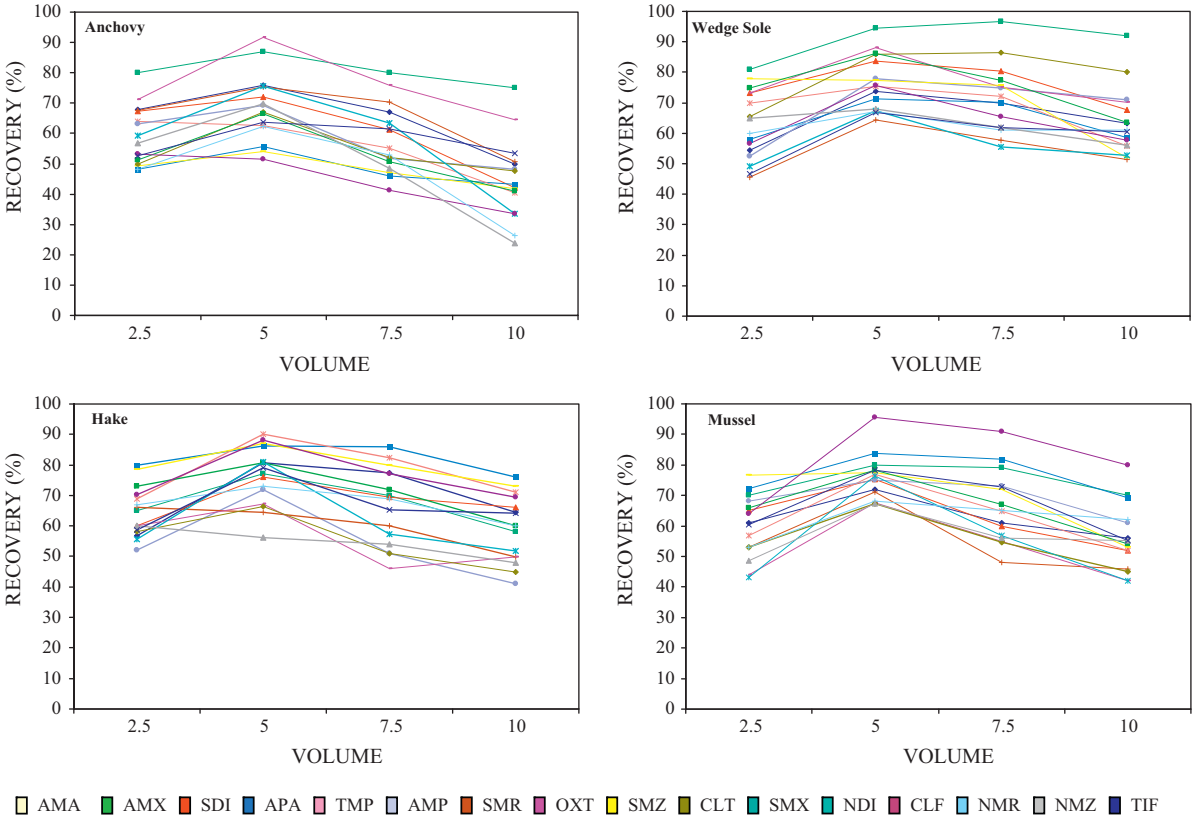

Fig. 3. Effect of volume of extractant on extraction recovery.

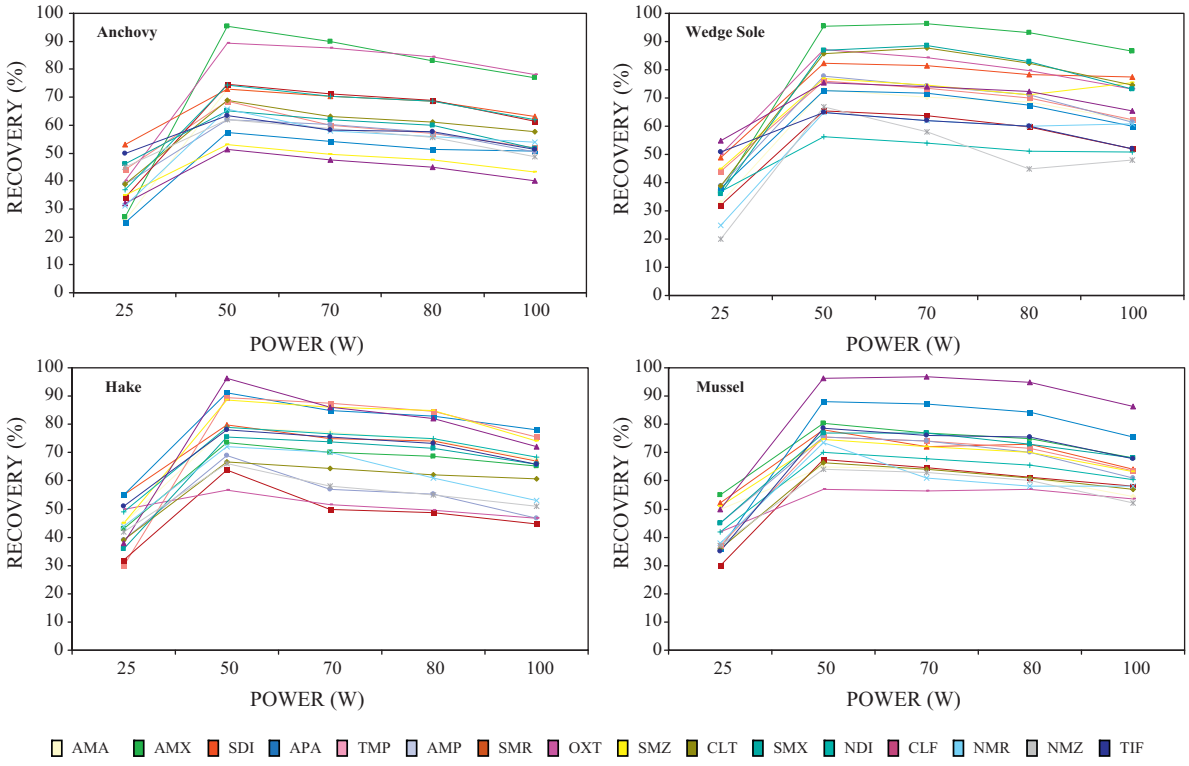

Fig. 4. Effect of irradiation powers on extraction recovery.

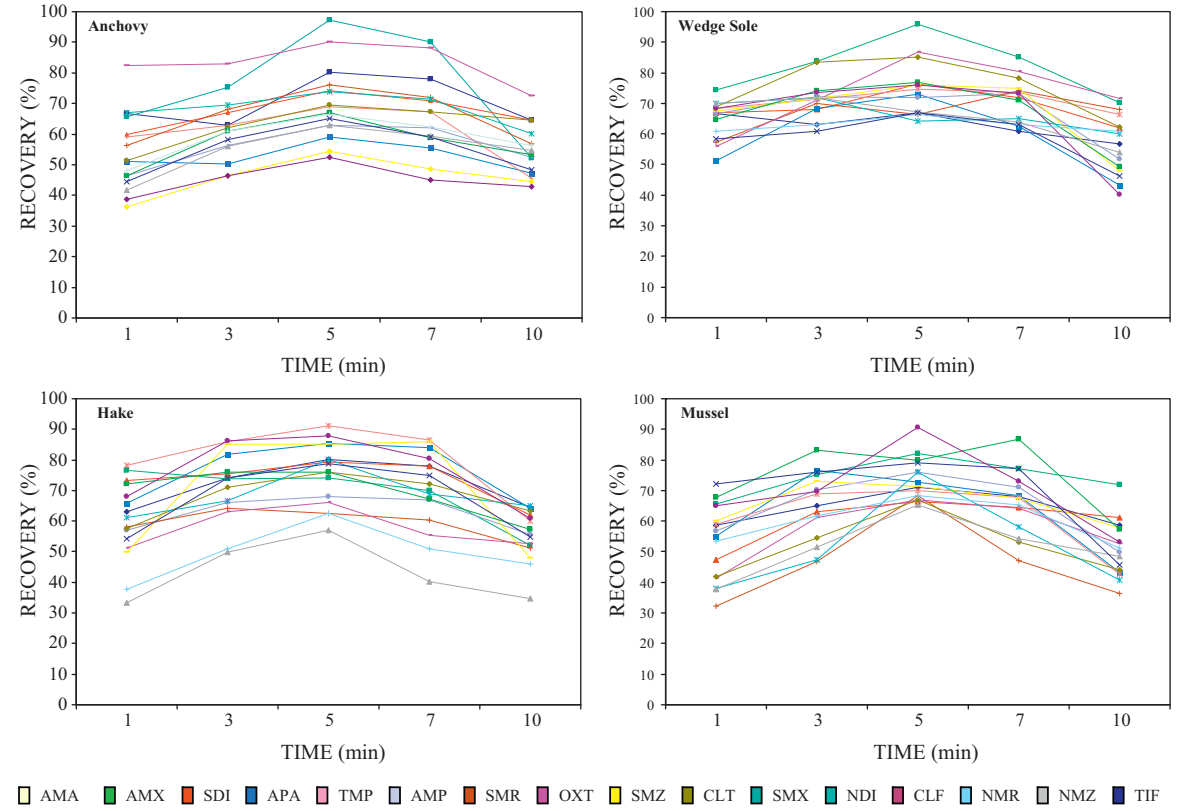

Fig. 5. Effect of time of irradiation power on extraction recovery.

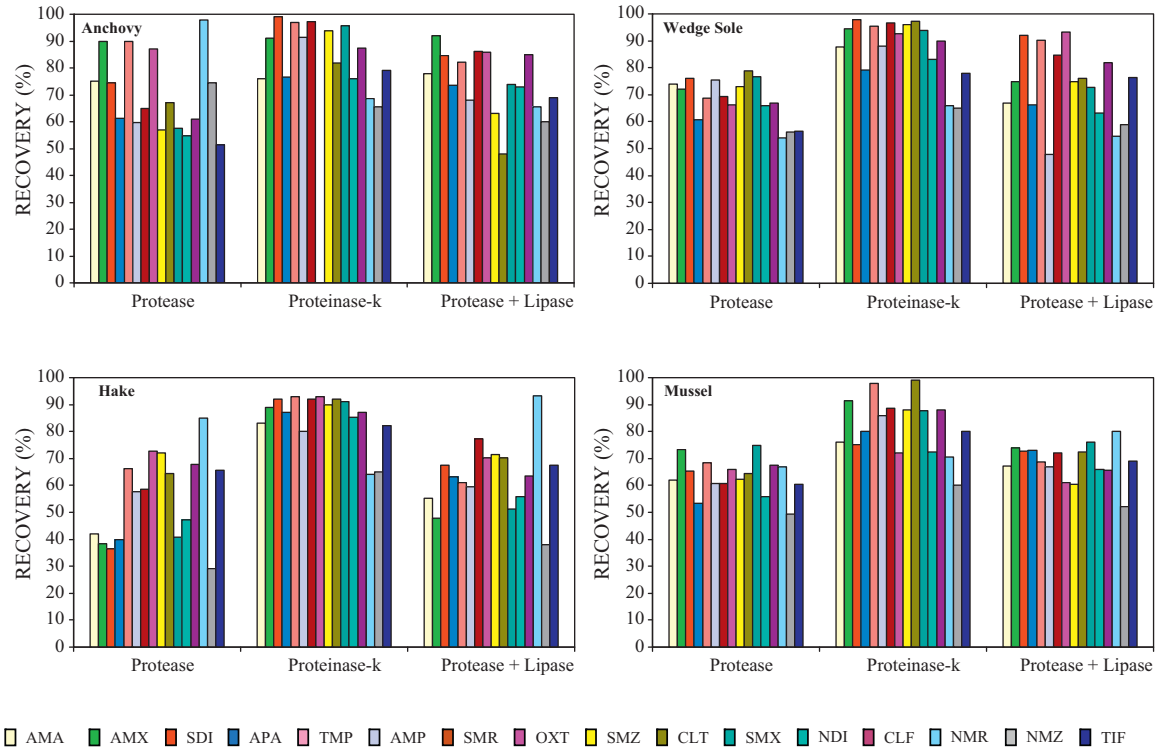

Fig. 6. Effect of type of enzyme on extraction recovery.

lowing criteria were considered: selectivity, sensitivity (limits of detection and quantification), linearity of the response function, precision, recovery and robustness.

The selectivity of the method was evaluated using blank samples of each matrix ($n = 3$); no interference peaks were found at the retention times of the analytes studied.

Linearity was studied from matrix-matched calibration, spiking blank extracts at five concentration levels (from 25 to 500 ng g^{-1}). A 5-point (in triplicate) calibration curve, based on peak areas, was constructed using a least-square linear regression analysis at the different concentrations. Linear relationships over the studied range were obtained with correlation coefficients $r \geq 0.999$ and the calibration curves obtained showed no changes over the course of one month. Limit of detection and quantitation were calculated based on the standard deviation of the intercept (σ) and slope (S) obtained from the calibration curve of each analyte. LOD = $3\sigma/S$ and LOQ = $10\sigma/S$ (figures depicted in Table 2). CCα and CCβ (Table 2) were calculated by analysing 20 blanks per matrix spiked with the analytes at the permitted limit. The concentration at the permitted limit plus 1.64 times the corresponding standard deviation equal the decision limit ($\alpha = 5\%$) and the CCα plus 1.64 times the corresponding standard deviation equals the CCβ ($\alpha = 5\%$). When no MRL is established CCα was calculated analysing 20 blanks and calculating the signal to noise ratio at the time window in which the analyte was expected and CCβ by analysing 20 blanks spiked at CCα, the values at CCα plus 1.64 times the corresponding standard deviation equals the CCβ ($\alpha = 5\%$).

The whole procedure was applied to 10 blank samples of the four different tissues to verify the specificity of the method showing that no interference was detected around the retention times

Table 2
Limits of detection (LOD) and quantitation (LOQ), decision limit (CCα) and detection capability (CCβ).

Analyte	Anchovy				Wedge sole				Hake				Mussel			
	LOD	LOQ	CCα	CCβ	LOD	LOQ	CCα	CCβ	LOD	LOQ	CCα	CCβ	LOD	LOQ	CCα	CCβ
AMA	5	17	3	5	5	15	2	4	5	16	3	4	5	17	3	5
AMX	16	55	9	15	16	53	9	14	16	57	9	15	16	55	9	15
SDI	2	6	1	1	2	6	1	2	2	6	1	2	2	7	1	2
APA	5	18	3	5	5	17	3	5	5	16	3	4	5	17	3	5
TMP	2	5	1	1	2	5	1	1	2	5	1	1	2	5	1	1
AMP	2	8	1	2	2	9	1	2	2	9	1	3	2	9	1	2
SMR	2	6	1	2	2	6	1	2	2	7	1	2	2	7	1	2
OXT	4	14	2	4	4	15	2	4	4	15	2	4	6	19	3	5
SMZ	2	7	1	2	2	7	1	2	2	7	1	2	2	7	1	2
CLT	5	18	3	5	5	15	3	4	5	16	3	4	5	15	2	4
SMX	3	10	2	3	3	11	2	3	3	11	2	3	3	11	2	3
NDI	6	20	3	5	5	18	3	5	5	18	3	5	6	21	3	6
CLF	3	10	2	3	3	9	2	3	3	10	2	3	3	10	2	3
NMR	5	18	3	5	5	19	3	5	5	20	3	5	5	18	3	5
NMZ	8	27	4	7	8	27	4	7	8	27	4	7	8	29	5	8
TIF	2	6	1	2	2	6	1	2	2	6	1	2	2	6	1	2

Data expressed in ng g^{-1}.

Table 3
Recoveries of spiked samples ($n = 3$) at three concentration levels.

	Anchovy			Wedge sole			Hake			Mussel		
	50 ng g^{-1}	100 ng g^{-1}	200 ng g^{-1}	50 ng g^{-1}	100 ng g^{-1}	200 ng g^{-1}	50 ng g^{-1}	100 ng g^{-1}	200 ng g^{-1}	50 ng g^{-1}	100 ng g^{-1}	200 ng g^{-1}
AMA	71 ± 4	70 ± 2	74 ± 4	83 ± 2	82 ± 2	84 ± 1	80 ± 3	81 ± 2	81 ± 3	80 ± 3	82 ± 2	73 ± 1
AMX	86 ± 3	82 ± 4	89 ± 4	92 ± 4	92 ± 6	93 ± 4	84 ± 5	84 ± 9	89 ± 5	94 ± 4	92 ± 4	86 ± 3
SDI	95 ± 5	94 ± 5	92 ± 5	93 ± 6	90 ± 6	92 ± 5	95 ± 8	96 ± 5	90 ± 8	92 ± 4	90 ± 9	92 ± 4
APA	79 ± 3	70 ± 3	72 ± 3	75 ± 4	72 ± 5	79 ± 8	87 ± 6	88 ± 6	83 ± 6	80 ± 4	73 ± 10	78 ± 3
TMP	96 ± 4	95 ± 7	95 ± 7	89 ± 3	90 ± 6	93 ± 3	89 ± 5	90 ± 5	97 ± 4	97 ± 3	90 ± 7	89 ± 3
AMP	93 ± 2	91 ± 3	88 ± 2	95 ± 5	89 ± 2	88 ± 3	87 ± 2	88 ± 6	82 ± 12	86 ± 4	89 ± 3	85 ± 3
SMR	96 ± 3	95 ± 3	92 ± 2	95 ± 6	95 ± 5	93 ± 4	96 ± 4	97 ± 6	90 ± 9	91 ± 6	95 ± 5	85 ± 7
OXT	94 ± 7	95 ± 6	96 ± 7	90 ± 5	83 ± 4	87 ± 4	81 ± 5	85 ± 2	91 ± 5	90 ± 3	83 ± 7	82 ± 3
SMZ	97 ± 2	95 ± 1	93 ± 2	92 ± 6	92 ± 8	94 ± 2	98 ± 4	99 ± 5	90 ± 3	92 ± 3	92 ± 9	91 ± 6
CLT	88 ± 6	80 ± 6	78 ± 5	95 ± 6	95 ± 6	95 ± 6	94 ± 4	94 ± 3	90 ± 3	95 ± 3	95 ± 6	75 ± 6
SMX	99 ± 3	90 ± 2	95 ± 2	87 ± 5	81 ± 5	89 ± 5	88 ± 5	87 ± 4	93 ± 4	94 ± 6	81 ± 4	85 ± 4
NDI	80 ± 5	75 ± 5	80 ± 4	86 ± 5	86 ± 5	88 ± 9	71 ± 6	68 ± 4	69 ± 9	67 ± 5	70 ± 7	68 ± 2
CLF	85 ± 4	86 ± 3	86 ± 3	96 ± 4	87 ± 3	88 ± 9	87 ± 6	88 ± 5	85 ± 6	82 ± 5	88 ± 5	82 ± 5
NMR	67 ± 7	68 ± 6	70 ± 6	63 ± 4	63 ± 4	68 ± 4	65 ± 3	65 ± 2	63 ± 5	64 ± 6	70 ± 2	62 ± 4
NMZ	65 ± 5	69 ± 5	64 ± 5	63 ± 4	65 ± 4	68 ± 4	66 ± 4	66 ± 4	63 ± 4	64 ± 5	67 ± 10	61 ± 9
TIF	80 ± 6	83 ± 5	85 ± 5	77 ± 5	75 ± 2	76 ± 8	77 ± 6	78 ± 2	83 ± 5	79 ± 2	82 ± 4	88 ± 7

of the analytes in any of the samples analysed. Recoveries (Table 3) were assessed by performing test on spiked samples at three concentration levels 50, 100 and 200 ng g^{-1} (these levels corresponds approximately to 0.5× MRL, 1× MRL and 2× MRL, according to EU guidelines recommendation). Fig. 7a and b shows a chromatogram of a spiked sample of hake at 50 ng g^{-1} of each analyte.

To evaluate the precision of the method, the repeatability and intermediate precision were studied. Precision study was performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the $m \times p \times n$ design (m = analytical levels, p = days and n = replications). From the corresponding ANOVA, the intermediate precision as well as the presence of significant bias is computed. To evaluate the repeatability and the intermediate precision, spiked samples at three concentrations levels 50, 100 and 200 ng g^{-1} of each analyte in triplicate ($n = 3$) were subjected to the entire analytical procedure and measured in one single day and one day per week during two months, respectively. Results obtained for the four matrices studied were similar and the data obtained for intermediate precision for wedge sole samples are shown in Table 4. The repeatability also expressed as relative standard deviation, was in the range 1.8–7.4% for anchovy matrix, 2.6–9.6% for wedge sole matrix, 2.4–6.8% for hake matrix and 1.9–5.8% for mussel matrix.

The robustness of the HPLC method was evaluated by the study of the effect of slight changes on the main chromatographic parameters: concentration of formic acid of the mobile phase, flow rate and pH. Small variations in formic acid concentration (±0.01%) did not significantly affect the separations in terms of resolutions and peak shapes. When flow rate was slightly varied (±0.02 mL min^{-1}) no significant variations in neither retention times nor resolution nor peak shapes were observed (<10%). Changes (±0.1) in pH of the mobile phase did not affect the efficiency of the separation. None of the studied variables were critical for the method which would allow using this method by different laboratories, analyst or instrument without any appreciable error.

Table 4
Intermediate Precision.

% RSD			
Analyte	50 ng g^{-1}	100 ng g^{-1}	200 ng g^{-1}
AMA	7	10	1
AMX	3	1	2
SDI	8	4	2
APA	5	5	2
TMP	4	2	2
AMP	8	2	1
SMR	7	4	2
OXT	2	1	1
SMZ	2	6	3
CLT	7	3	1
SMX	2	6	3
NDI	4	2	3
CLF	5	7	3
NMR	4	2	2
NMZ	3	2	2
TIF	10	5	4

3.6. Applications

Samples of mussels and each kind of fish studied in this work were purchased from local groceries in five different days, extracted and analysed according to the optimised method to determine the presence of some of the analytes studied in this work. No antibiotic were found in the samples.

Alternatively, the presence of the antibiotics administered to carps (*C. carpio*) samples was evaluated. First, control tissue samples were analysed and none of the analytes were detected. Results obtained from the assays described in Section 2.5 are shown in Table 5. As it can be seen, the analytes administered were in most cases detected by the procedure but in several case levels were not quantifiable. In several cases the corresponding metabolites were also detected and/or quantified. In all quantified samples the levels are above MRL, which highlights that it is necessary to elapse an adequate time from administration of the antibiotic to commercialisation. Additionally, the viscera of the corresponding carps used for the assay were also analysed following the same procedure, as the presence of the analytes was also expectable. Data obtained reflects this fact, although levels in fish viscera are not submitted to legislation, these are higher than the obtained in muscle samples.

Finally, in order to verify whether the presence of pharmaceuticals is significant in the aquatic ecosystems, we proceeded to capture a variety of marine specimens on the coast closest to a town with some importance. Samples were obtained from the fishing town of Aguilas located at the southwest corner of the region of Murcia (Spain), with an area of 251.77 km^2 and 28 km of Mediterranean coastline. It maintains a stable population of 28,800 inhabitants, which in summer reaches 150,000. The capture of the specimens was carried out using artisan fishing with a net at a distance between 100 and 200 m from the beach, in the month of August 2009. The specimens analysed were: *T. ovatus*, *S. salpa*, *O. melanura* and *L. ramada*. Results obtained are shown in Table 6; as it can be seen, levels obtained for OXT in samples of *T. ovatus* are really

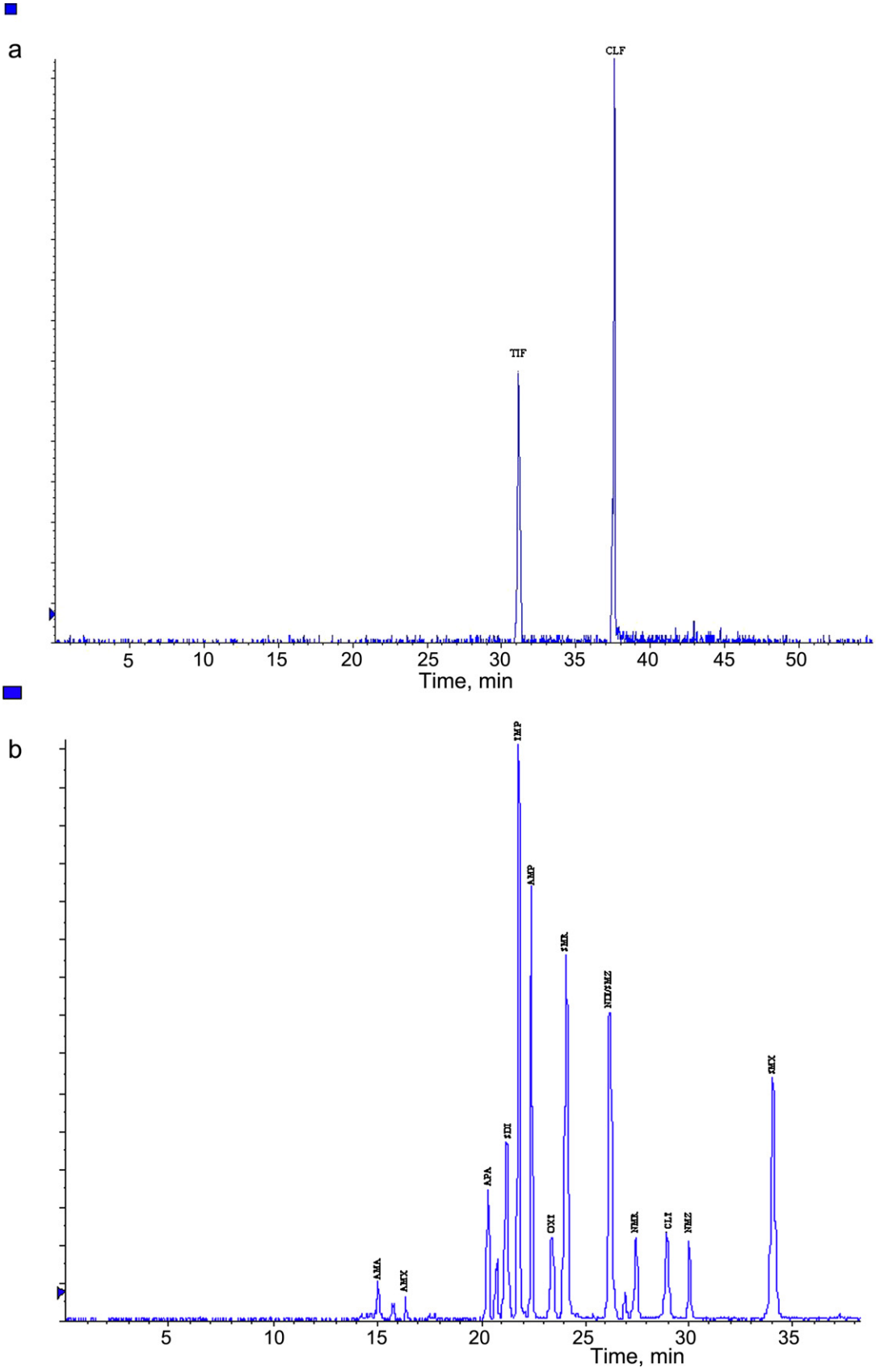

Fig. 7. MRM chromatograms of a spiked (50 ng g^{-1}) sample of hake. (a) Positive mode ionisation and (b) negative mode ionisation.

Table 5
Carp samples results.

	Assay 1 ($ng\,g^{-1}$)		Assay 2 ($ng\,g^{-1}$)		Assay 3 ($ng\,g^{-1}$)		Assay 4 ($ng\,g^{-1}$)	
	Viscera	Tissue	Viscera	Tissue	Viscera	Tissue	Viscera	Tissue
AMA	–	–	–	–	*D*	D	D	D
AMX	–	–	–	–	320 ± 16	D	470 ± 6	D
SDI	–	–	380 ± 14	190 ± 3	880 ± 18	100 ± 2	–	–
APA	–	–	–	–	–	–	390 ± 8	20 ± 5
TMP	–	–	–	–	–	–	–	–
AMP	–	–	–	–	–	–	280 ± 12	D
SMR	–	–	430 ± 12	340 ± 3	–	–	–	–
OXT	–	–	–	–	D	50 ± 7	D	D
SMZ	–	–	290 ± 9	250 ± 8	970 ± 12	90 ± 10	–	–
CLT	–	–	–	–	–	–	D	D
SMX	–	–	150 ± 11	100 ± 9	–	–	–	–
NDI	–	–	290 ± 13	D	70 ± 7	ND	–	–
CLF	660 ± 12	670 ± 13	–	–	–	–	–	–
NMR	–	–	670 ± 12	ND	–	–	–	–
NMZ	–	–	D	ND	D	D	–	–
TIF	D	ND	–	–	–	–	–	–

(–) Non administered; ND: not detected; D: detected.

Table 6
Results of wild fish samples.

	Liza ramada ($ng\,g^{-1}$)		*Trachynotus ovatus* ($ng\,g^{-1}$)		*Oblada melanura* ($ng\,g^{-1}$)		*Sarpa salpa* ($ng\,g^{-1}$)	
	Viscera	Tissue	Viscera	Tissue	Viscera	Tissue	Viscera	Tissue
AMA	–	–	–	–	–	–	–	–
AMX	–	–	–	–	–	–	–	–
SDI	180 ± 10	–	190 ± 9	–	–	–	10 ± 1	50 ± 2
APA	–	–	–	–	–	–	–	–
TMP	–	–	–	–	–	–	–	–
AMP	–	–	–	–	–	D	–	–
SMR	–	–	40 ± 9	D	D	–	70 ± 3	–
OXT	50 ± 5	–	–	–	–	–	–	–
SMZ	D	–	70 ± 8	–	–	–	–	–
CLT	160 ± 7	–	590 ± 6	580 ± 13	–	–	–	–
SMX	–	–	–	–	–	–	–	–
NDI	–	–	D	–	–	–	D	–
CLF	–	–	–	–	–	–	–	–
NMR	–	–	–	–	–	–	20 ± 7	–
NMZ	–	–	–	–	–	–	–	–
TIF	–	–	–	–	–	–	–	–

(–) Not detected; D: detected.

surprising and unexpected, this could imply a long exposure of the animal to the compound. In general, the results obtained show that some of the specimens have been exposure to antibiotics in some way but levels found are bellow MRL fixed by the Council Directive 2377/90 EC.

4. Conclusions

A method for the simultaneous determination of several antibiotics belonging to different classes in fish and mussel was developed and validated. The extraction procedure implies an enzymatic-microwave assisted extraction step and a solvent extraction clean-up. The method is simple and provides good validation parameters in terms of linearity, precision and limits of detection and quantification, which allows its application to the quantitative analysis on real samples. The method was applied to samples of *C. carpio* after administration of the antibiotics via feed, obtaining quantitative results, which demonstrate its applicability in control analysis of fish samples derived from the Council Directive 2377/90 EC. The procedure was also applied to samples captured by means of artisan fishing obtaining low levels of some of the antibiotics in some samples.

Acknowledgements

This work was supported by the Project CTM2009-12858-C02-01 from the "Dirección General de Investigación y Gestión del Plan Nacional de I+D+i (Ministerio de Educación y Ciencia, Spain)".

We are grateful to Dr. Maria Isabel García Moreno from the Department of Organic Chemistry, University of Seville, for their valuable help in the metabolites synthesis. MRP is grateful to University of Seville for personal funding through the "Fundación Cámara" program.

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