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RESEARCH PAPER

Determination of pharmaceuticals in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry

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Abstract A method for the quantitative determination of seven pharmaceuticals in bivalves was developed by QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Both the European Standard Method EN 15662 and the AOAC Official Method 2007.01 for QuEChERS were tested. In addition, several clean-up strategies were evaluated in order to clean the matrix previous to the LC-MS/MS analyses. Dispersive solid-phase extraction with silica gel and modification of the chromatographic separation were the clean-up strategies that gave the best results. The optimized method was validated in mussels (*Mytilus galloprovincialis*) and allowed the determination of pharmaceuticals at nanograms per gram levels (dry weight (d.w.)). Limits of quantification ranged from 5 to 100 ng/g. Apparent recoveries ranged from 35 to 77 %. The application of this method to bivalves revealed the presence of salicylic acid at concentrations up to 103 ng/g (d.w.) in mussel samples.

Keywords Pharmaceuticals · QuEChERS · Liquid chromatography-tandem mass spectrometry · Bivalves

Introduction

Pharmaceuticals are widely used in human as well as animal applications. They are introduced into the aquatic system due to the incapability of wastewater treatment plants to eliminate them completely and also as a consequence of agricultural run-off and aquaculture applications [1]. They are considered to be emerging organic contaminants (EOCs), and most of them are still unregulated or not commonly regulated [2]. Several studies have determined the presence of pharmaceuticals in different waterbodies, such as surface [3] and groundwaters [4] from nanograms per litre to micrograms per litre concentrations, and in estuarine [5] and marine waters [6] at levels of nanograms per litre.

In the last few years, several studies have started to focus on the determination of EOCs in aquatic organisms. Living organisms are complex sample matrices which contain a high amount of interfering compounds that complicate the determination of the target compounds. According to the review by Huerta et al. [1], several studies describe the presence of pharmaceuticals in aquatic organisms, mainly in fish. Included among the aquatic organisms, bivalves are sessile filter-feeding organisms which interact with water and sediment and filter large volumes of water [7]. They are, therefore, particularly susceptible to environmental contaminants and are an interesting group of organisms to be monitored. In a recent publication, Martínez Bueno et al. [8] developed an analytical method for the determination of two anticonvulsants and some of their transformation products in mussels (*Mytilus galloprovincialis*), enabling the detection of the target compounds at low nanograms per gram concentration levels.

The complexity of the biotic matrices normally requires time-consuming sample preparation. The most commonly used extraction techniques in biotic samples include ultrasonication [9], rotary extraction [10], microwave-assisted micellar extraction (MAME) [7] and pressurized liquid extraction (PLE) [11]. Usually, the extraction methods based on these techniques are followed by solid-phase

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extraction (SPE) as a subsequent clean-up step. An alternative extraction technique is QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which was introduced by Anastassiades et al. [12] in 2003 to determine pesticide residues in fruit and vegetables. The QuEChERS methods involve a first step based on salting-out extraction with a solvent (mainly acetonitrile) followed by dispersive SPE (dSPE). Besides its original application, this technique has already been employed for the extraction of pharmaceuticals in different matrices such as sediments [13, 14], sewage sludge [15, 16], soil [17–19], blood [20–22], milk [23], molluscs [8, 24] and fish [25], among others [8, 26].

To determine the presence of pharmaceuticals, gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are the most common techniques, due to their selectivity, specificity and sensitivity. In LC-MS and LC-MS/MS, atmospheric pressure ionization (API) sources are the most commonly used interfaces. In a study conducted by Schlüsener and Bester [27], the two ionization modes, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were compared for the analysis of steroid hormones in influents and effluents of sewage treatment plants and a high matrix effect was observed in the use of ESI. However, only a few pharmaceuticals can efficiently be ionized by APCI and, for this reason, most of them have to be determined using ESI [1, 27].

The aim of this study was to develop a rapid method based on QuEChERS extraction followed by LC-MS/MS to determine the presence in different bivalve species of seven relevant pharmaceuticals from different therapeutic classes: salicylic acid, the metabolite of an analgesic; clofibric acid, the metabolite of a lipid regulator; bezafibrate, another lipid regulator; and four non-steroidal anti-inflammatory drugs (NSAIDs): ketoprofen, naproxen, diclofenac and ibuprofen. Of these bivalve species, the mussel *M. galloprovincialis* was selected to validate the method and, later, this was applied to evaluate the occurrence of these pharmaceuticals in various bivalve species.

Materials and methods

Reagents and chemicals

Salicylic acid, clofibric acid, ketoprofen, naproxen, bezafibrate, diclofenac and ibuprofen were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of individual standards at 1000 mg/L were prepared in methanol and stored at 4 °C.

The citrate buffer packet was obtained from Scharlab (Sentmenat, Spain) and contained 4 g magnesium sulphate, 1 g sodium chloride, 0.5 g sodium hydrogencitrate sesquihydrate and 1 g sodium citrate, whereas the acetate buffer packet was obtained from Supelco (Sigma-Aldrich) and contained 6 g of magnesium sulphate and 1.5 g of sodium acetate.

Various materials for dSPE were tested: primary secondary amine (PSA)/magnesium sulphate, PSA/magnesium sulphate/C_{18} and silica gel from Scharlab and PSA/magnesium sulphate/graphitized carbon black (GCB), Z-Sep$^+$, Florisil and alumina from Supelco.

Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallès, Spain). Acetonitrile (ACN) and methanol were of HPLC grade and supplied by Prolabo (Llinars del Vallès, Spain). Acetic acid (LC-MS grade) was purchased from Sigma-Aldrich, and nitrogen gas (N_2) was sourced from Carburos Metálicos (Tarragona, Spain).

Sampling and sample pre-treatment

All bivalve species were bought in the local market including lagoon cockle (*Cerastoderma glaucum*), coquina clam (*Donax trunculus*), manila clam (*Ruditapes philippinarum*), striped venus clam (*Chamelea gallina*), sword razor clam (*Ensis* sp.) and mussel (*M. galloprovincialis*) from different locations, such as Galicia, Atlantic Coast (NW, Spain), and the Ebro River Delta, Mediterranean Coast (NE, Spain), and (*Mytilus edulis*) from the Atlantic Coast of France.

The sample pre-treatment was the same for all of the mentioned species. All organisms were removed from the shell and homogenized with a Taurus Robot 300, and the composite biotic samples obtained were frozen for 24 h before being freeze-dried with Labconco FreeZone 4.5 (Kansas City, MO, USA).

QuEChERS extraction

The European Standard Method EN 15662 was adapted to the dried matrix. One gram of the frozen-dried sample was weighed in a 50-mL polypropylene centrifuge tube from Scharlab. Ten millilitres of water was added, and the tube was shaken manually for 1 min. Then, 10 mL of ACN was added and the tube was shaken vigorously also for 1 min. Subsequently, the citrate buffer packet (EN method) was added and the tube was first shaken manually for 15 s and then using a vortex (Heidolph Reax 2000) for 45 s. Afterwards, the tube was centrifuged for 5 min at 7000 rpm in a centrifuge from Hettich Zentrifugen (Germany). The supernatant (ACN layer) was transferred into a 15-mL centrifuge tube from Supelco containing 1 g of silica gel as a dispersive sorbent. After this step, the extract was shaken manually for 15 s and then using a vortex for 45 s, before finally being centrifuged for 5 min at 7000 rpm. One millilitre of the supernatant was transferred into a glass tube and evaporated to dryness under a gentle N_2 stream. The residue obtained was redissolved in 1 mL of 0.5 % acetic acid in

ultrapure water/ACN (70:30, *v*/*v*) and filtered through a 0.22-μm PTFE syringe filter from Scharlab before injection.

Liquid chromatography-tandem mass spectrometry analysis

Chromatographic analyses were performed with Agilent 1200 series HPLC (Waldbronn, Germany) coupled to triple quadrupole (QqQ) 6410 series MS with an ESI interface from Agilent Technologies. The HPLC system was equipped with a degasser, a binary pump, an automatic injector and a column oven, all from Agilent Technologies. The chromatographic separation was achieved with an Ascentis Express C_{18} Fused-Core® column (5 cm×4.6 mm i.d.; 2.7 μm) from Supelco. The mobile phase was 0.5 % acetic acid in ultrapure water (A) and ACN (B). The separation was performed with the following gradient: initially, 30 % B, which was maintained for 2 min, then raised to 39 % B in 6 min and to 100 % B in 17 min, which was maintained for 2 min, before finally being decreased back to initial conditions in 3 min. Between injections, the column was allowed to equilibrate under the initial conditions for 5 min. The flow rate was 0.6 mL/min, the oven temperature was set at 25 °C, and the injection volume was 25 μL.

Injections of individual standards of 1 ppm dissolved with a mobile-phase composition of 50:50 (A/B) were used to optimize MS/MS parameters. For each compound, one or two precursor ion/s was/were selected and the cone voltage was then optimized for each precursor ion (Table 1). The optimized ionization source parameters were as follows: capillary voltage of 3000 V in the negative mode, nebulizer pressure of 60 psi, drying gas (N_2) flow of 7 L/min and drying gas temperature of 250 °C. Collision energies were optimized in order to select, when possible, three characteristic multiple reaction monitoring (MRM) transitions for each compound (Table 1). Moreover, MRM ratios (the relation between the abundance of each qualifier transition and the quantifier transition) were calculated (Table 1). Chromatograms and spectra were recorded and processed using the Agilent Mass Hunter Qualitative Analysis software.

Results and discussion

Liquid chromatography-tandem mass spectrometry

Chromatographic separation was achieved with the following gradient: 30 % B was maintained for 2 min, before being raised to 39 % B in 6 min, and then to 100 % B in 7 min, and it was maintained 100 % B for 3 min. Subsequently, it was decreased back to the initial conditions in 2 min. However, diclofenac and ibuprofen showed a poor signal when the bivalve samples were analyzed and, for that reason, the gradient was slightly modified in order to improve their signals, as will be discussed in the "Modifications of chromatographic conditions" section.

For MS detection with a QqQ analyzer, several parameters were optimized and the optimum values are described in the "Liquid chromatography-tandem mass spectrometry analysis" section. The following ESI source parameters were optimized, and the values in parentheses were assayed: capillary voltage (2000–4500 V in increments of 500 V); nebulizer pressure (30, 45 and 60 psi); drying gas (N_2) flow (7, 9 and 12 L/min) and temperature (250, 300 and 350 °C). Deprotonated $[M-H]^-$ molecules were selected as precursor ions for all compounds, except for bezafibrate and diclofenac, in which cases both $[M-H]^-$ and $[(M+2)-H]^-$ were selected as precursor ions. For both compounds, the selected $[M-H]^-$ gave only two product ions, and in selecting $[(M+2)-H]^-$ as a second precursor ion, a third MRM transition could be obtained. The cone voltages tested were 60, 80, 100 and 120 V, and collision energies for each precursor ion tested were between 5 and 40 eV in increments of 5 eV. For all of the compounds, three MRM transitions were monitored, with the exception of salicylic acid and clofibric acid, with which just two product ions were generated, and ketoprofen and ibuprofen, with which just one product ion could be monitored. Common fragmentation pathways are based on the loss of the carboxyl group and methyl group. The proposed formulas for the product ions obtained and their respective cone voltage and collision energies are shown in Table 1. Ion ratios detailed in Table 1 were calculated as described in the "Liquid chromatography-tandem mass spectrometry analysis" section.

QuEChERS extraction

The original QuEChERS method described by Anastassiades et al. [12] is based on the extraction with ACN and the addition of anhydrous $MgSO_4$ and NaCl, in an aqueous matrix followed by a clean-up step using PSA as dSPE. However, this method underwent several modifications in the subsequent years and other solvents, such as ethyl acetate, dichloromethane and acetone, have been used as extraction solvents. In the present study, 10 mL of water was added to the dried matrix to promote the salting-out extraction. ACN was chosen as the extraction solvent as it can be easily separated from water [28]; it does not extract as much lipophilic material, such as waxes, fat and lipophilic pigments [29], and it is the solvent of preference in the QuEChERS methodology.

With respect to the salt composition, Lehotay et al. [30] modified the original method using acetate buffer which went on to become AOAC Official Method 2007.01. The original method [31] was also modified by using citrate buffer, being registered as European Standard Method EN 15662 [31]. The two standard methods mentioned (AOAC and EN) were tested using the mussel samples.

t1.1 **Table 1** MRM transitions and MS/MS parameters

Compound	CV (V)	CE (eV)	Precursor ion (*m/z*)	Product ions (*m/z*)	Ion ratio (%)
Salicylic acid	80	10	137 $[M-H]^-$	93 $[M-H-CO_2]^-$	7.7
		35		65 $[M-H-CO_2-CO]^-$	
Clofibric acid	80	10	213 $[M-H]^-$	127 $[C_6H_4ClO]^-$	13.9
		5		85 $[C_4H_5O_2]^-$	
Ketoprofen	60	5	253 $[M-H]^-$	209 $[M-H-CO_2]^-$	
Naproxen	60	30	229 $[M-H]^-$	169 $[M-H-C_2H_4O_2]^-$	
		10		170 $[M-H-CHO]^-$	78.7
		5		185 $[M-H-CO_2]^-$	31
Bezafibrate	100	10	360 $[M-H]^-$	274 $[M-H-C_4H_6O_2]^-$	
		25	360 $[M-H]^-$	154 $[M-H-C_{12}H_{14}O_3]^-$	36.8
		10	362 $[(M+2)-H]^-$	276 $[(M-2)-H-C_4H_6O_2]^-$	30.5
Diclofenac	80	5	294 $[M-H]^-$	250 $[M-H-CO_2]^-$	
		5	296 $[(M+2)-H]^-$	252 $[(M+2)-H-CO_2]^-$	57.7
		20	294 $[M-H]^-$	214 $[M-H-CO_2-HCl]^-$	6.5
Ibuprofen	60	5	205 $[M-H]^-$	161 $[M-H-CO_2]^-$	

t1.17 *CV* cone voltage, *CE* collision energy

Recoveries of the extraction process (REs) were calculated for both standard methods by comparing the peak area of spiked mussel samples at 1000 ng/g in dry weight (d.w.) and the peak area of mussel samples that were spiked after the extraction process [32]. Thus, REs show the yield of the extraction process and do not take into account any losses caused by matrix interferences. REs were very similar, with values ranging from 61 to 95 % in both methodologies for all compounds, except for salicylic acid and clofibric acid, which showed recoveries around 20 % higher when using citrate buffer than when using acetate buffer (data not included). For this reason, subsequent experiments were just conducted with the citrate buffer (EN method).

Clean-up strategies

One of the greatest drawbacks of LC-MS is the perturbation of the signal by co-extracted substances from the sample matrix, particularly in complex matrices such as biota [33]. For this reason, several strategies were evaluated in order to clean the matrix and reduce its effect on the response.

The use of dSPE and modification of the gradient profile of the chromatographic separation were strategies that provided satisfactory results, and they will be described in detail in the subsequent sections. Moreover, three other strategies were assayed, although none of them provided satisfactory results. The first one was freezing out the ACN extract of QuEChERS in order to precipitate lipids, waxes, sugars and other matrix co-extractives with low solubility in ACN [34]. The second one was dilution of the ACN layer, with the dilution factors based on 0.5 % acetic acid in ultrapure water/ACN at 1:1 and 2:1 being tested. However, the limits of detection (LODs) and limits of quantification (LOQs) were significantly affected since they increased as the dilution factor rose. Finally, precleaning of the solid sample with 10 mL of hexane ultrasonicated for 10 min prior to extraction was also evaluated, without any improvement.

dSPE

QuEChERS method involves a second step where the extract is cleaned up by using a dSPE with the main objective to remove interfering compounds present in the matrix. Mussels are fat or lipid-containing matrices, although fats are not very soluble in ACN, a certain quantity of them might co-extract, so they have to be removed prior to the final determination step [12].

PSA is used as the sorbent for dSPE in the original QuEChERS method [12] to remove various polar organic acids, polar pigments, some sugars and fatty acids [28]. Other commonly used dSPE sorbents in the QuEChERS methodology cited in the literature are C_{18}, used to remove non-polar interfering substances like lipids [28]; EnvC, for removing sterols and chlorophylls [26]; and Z-Sep$^+$, for removing fats and non-polar compounds [8]. In the present work, several commercial dSPE sorbents already combined were tested: PSA (100/600 mg PSA/$MgSO_4$), PSA/C_{18} (100/600/100 mg PSA/$MgSO_4$/C_{18}), PSA/EnvC (150/15/900 mg PSA/$MgSO_4$/GCB) and Z-Sep$^+$ (500 mg). Moreover, Florisil (500 mg), alumina (1000 mg) and silica (1000 mg) were also tested.

To optimize the dSPE, several extractions of non-spiked mussel samples were conducted using the procedure described in the "QuEChERS extraction" section and the supernatant of different extractions was mixed in order to avoid any difference in the extraction process. Later, different aliquots were spiked and transferred into 15-mL tubes containing the

different sorbents mentioned above. In addition, an aliquot spiked after the extraction process without any dSPE sorbent was evaporated and reconstituted. For each compound, the effectiveness of the clean-up was evaluated by comparing the signal obtained with each dSPE sorbent with the signal obtained without any dSPE sorbent. The results obtained are shown as a percentage in Fig. 1, where values higher than 100 denote an improvement in retaining interfering substances, while values below 100 denote no improvement, and thus, the clean-up procedure was not effective. As can be seen in Fig. 1, none of the commercial dSPE sorbents tested resulted in an improvement in terms of retaining interfering substances and consequently increasing the signal response for all compounds and, in most cases, they even retained the target analytes. This is the case of Z-Sep^{+}, which was supposed to remove fats and non-polar compounds [8], but in the present study, it completely retained four of the seven studied analytes. Only silica improved the signal response for most of the compounds, and it was selected for the clean-up step. In fact, silica has been used for clean-up in different solid matrices [35].

Modifications of chromatographic conditions

Another strategy to reduce the effects of matrix compounds on the analytes response, described in the literature, is the modification of the chromatographic separation between the analytes and the co-eluting substances that interfere with the analysis [36]. According to Gosetti et al. [37], special attention should be paid towards the analytes eluting in the solvent front (highly polar and not retained compounds) or during the end of an elution gradient. With the initial gradient described in the "Liquid chromatography-tandem mass spectrometry" section, chromatographic separation was achieved in 13.5 min. However, diclofenac and ibuprofen, the last eluted compounds, showed poor signals. For this reason, analysis time was increased, with the gradient proposed in the "QuEChERS extraction" section, where the slope of the gradient was more gradual. With this strategy, diclofenac and ibuprofen were eluted more than 1 min later than with the initial gradient proposed in the "Liquid chromatography-tandem mass spectrometry" section. In addition, the peak signal was slightly increased, by around 10 % for these two compounds. Thus, this gradient was incorporated in the method and all of the compounds eluted in less than 14.5 min. Another strategy would be to use a longer column; however, it was ruled out as the analysis time would increase.

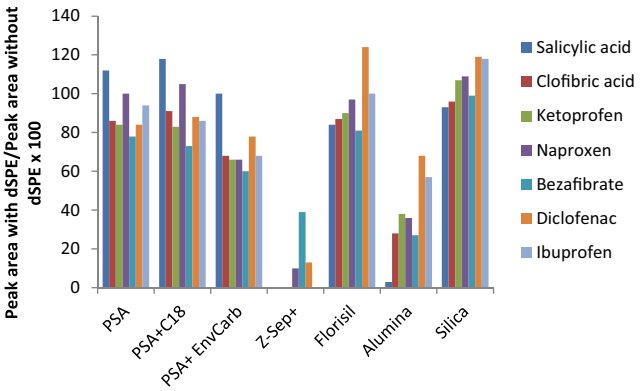

Fig. 1 Effectiveness of the clean-up evaluated by comparing the signal obtained with each dSPE sorbent with the signal obtained without any dSPE sorbent

Method validation

Once the method had been optimized, mussel *M. galloprovincialis* was selected to carry out the validation study in order to demonstrate the performance of the method. Apparent recoveries (App REs), matrix effect (ME) and REs were calculated, matrix-matched calibration curves were plotted for each analyte, and linear ranges, LODs and LOQs were calculated. Moreover, repeatability (n=5) and reproducibility between days (n=5) were conducted. All of the results are shown in Table 2. One compound, salicylic acid, was found in the blank samples. For this reason, the peak area obtained in the blank was taken into account for validation. The method proved also to be applicable to the other bivalve species, providing comparable validation data.

App REs (which include the overall method) were calculated at two different concentration levels. The highest level studied was 1000 ng/g (d.w.) for all of the compounds, and the lowest levels were 50 ng/g (d.w.) for clofibric acid, naproxen, bezafibrate and diclofenac and 250 ng/g (d.w.) for salicylic acid, ketoprofen and ibuprofen. This distinction in the low concentration level was due to the difference in signal response between compounds. App REs were calculated by interpolation with an external standard calibration curve of the peak area obtained for each analyte from a sample spiked before extraction. App REs were very similar at both concentration levels. Table 2 shows the apparent recoveries for the highest level, ranging between 35 and 77 %. Any loss of signal observed in the samples may be attributable to the extraction process or to ion suppression, as the App RE includes the entire method procedure.

Another parameter assayed in the validation process was the ME, which was assessed as follows:

$$\mathrm{ME}(\%) = -\left[100-\left(\mathrm{B}\Big/\mathrm{A}\times 100\right)\right]$$

where A is the instrumental response for standards injected directly and B is the response of a mussel sample spiked before LC-MS/MS injection. When analytes were

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t2.1 **Table 2** Validation data with *Mytilus galloprovincialis* samples

t2.2		Apparent recovery (%)	ME (%)	RE (%)	Linear range (ng/g)	LOD (ng/g)	Repeatability (%RSD; n=5)		Reproducibility (%RSD; n=5)		t2.3
							1000 ng/g	50 ng/g	1000 ng/g	50 ng/g	
t2.4	Salicylic acid	46	−25	61	10–2000	5	8	17[a]	15	19[a]	
t2.5	Clofibric acid	77	−11	86	10–2000	1	3	5	10	17	
t2.6	Ketoprofen	48	−47	91	100–2000	50	3	13[a]	6	21[a]	
t2.7	Naproxen	46	−52	95	10–2000	2.5	2	7	6	20	
t2.8	Bezafibrate	48	−47	89	5–2000	2.5	3	9	9	19	
t2.9	Diclofenac	35	−63	93	10–2000	5	2	14	7	14	
t2.10	Ibuprofen	39	−57	90	100–2000	50	4	8[a]	4	12[a]	

Samples were spiked at 1000 ng/g (d.w.) to calculate apparent recoveries, ME and RE

ME matrix effect, *RE* recovery of the extraction process, *LOD* limit of detection

[a] Spiked at 250 ng/g (d.w.)

present in a blank sample, the peak area of the blank was subtracted from B in order to calculate the ME. If the ME=0, no matrix effect is present; if the ME>0, there is signal enhancement; and if the ME<0, signal suppression is present. All of the studied compounds showed signal suppression, as their ME<0. The most affected compounds were diclofenac and ibuprofen, with values of signal suppression of 63 and 57 %, respectively.

REs of the extraction process were calculated as described in the "QuEChERS extraction" section by comparing the instrument response from a certain amount of compound added to samples before extraction and the same amount of compound added to samples after QuEChERS extraction. REs varied between 61 and 95 % which confirms that most of the losses are due to ME.

The optimized method provided good linearity since the coefficients of determination (R^2) of the matrix-matched calibration curves were acceptable for all analytes, as they were between 0.9961 and 0.9997.

LODs and LOQs were calculated as the analyte concentration that produced a peak signal of three and ten times the background noise, respectively. LOQs were considered to be the first point included in the calibration curve. For salicylic acid that was present in the blank samples, LOD and LOQ were estimated from an external standard calibration curve using its App RE factor. LODs were between 1 and 5 ng/g (d.w.), with the exception of ibuprofen and ketoprofen, which were 50 ng/g (d.w.). LOQs were between 5 and 10 ng/g (d.w.) in all of the studied compounds except for ibuprofen and ketoprofen, which were 100 ng/g (d.w.). The present LOQs are in agreement with those found by Wille et al. [11] in a study conducted in 2011 for salicylic acid and diclofenac in mussel samples when these samples were analyzed using PLE and SPE followed by ultra-high performance liquid chromatography (UHPLC)-MS/MS using QqQ as an analyzer. Ramírez et al. [10] reported LODs of 45.9 ng/g (d.w.) for ibuprofen and 2.69 ng/g (d.w.) for clofibric acid in fish muscle tissue, which are also in accordance with those obtained in the present study. Huerta et al. [33] reported LOD of 0.5 ng/g (d.w.) and LOQ of 1.66 ng/g (d.w.) for diclofenac in fish homogenate, which are 1 order of magnitude lower than those obtained in the present study. However, these limits were achieved using a tedious method based on PLE as an extraction technique and gel permeation chromatography (GPC) as a later clean-up followed by UHPLC coupled to a hybrid triple quadrupole linear ion trap mass spectrometer.

Figure 2 shows a MRM chromatogram of a mussel sample spiked at 1000 ng/g (d.w.) of each studied analyte. For each compound, the quantification transition is plotted.

Repeatability and reproducibility were calculated by means of percentage of relative standard deviation (%RSD) at both levels of concentration used for calculating the App RE. The results, as detailed in Table 2, were always below 21 % for all of the compounds at both concentration levels.

Application

The method developed was applied to analyze different bivalve samples since it was tested that the validation data was similar to mussel. *M. edulis* from the Atlantic Coast of France and the species *M. galloprovincialis* collected from two different locations the Ebro River Delta, Mediterranean Coast (NE, Spain), and Galicia, Atlantic Coast (NW, Spain), were bought in the local market together

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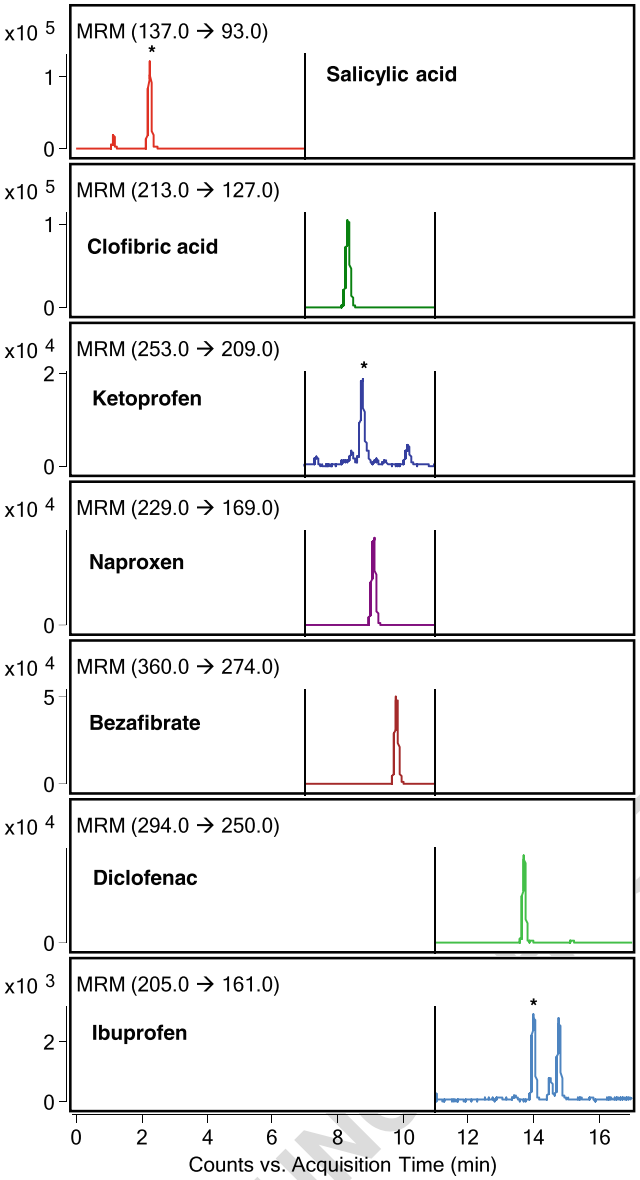

Fig. 2 MRM chromatogram of a mussel sample spiked at 1000 ng/g (d.w.)

with lagoon cockle (*C. glaucum*), coquina clam (*D. trunculus*), manila clam (*R. philippinarum*), striped venus clam (*C. gallina*) and sword razor clam (*Ensis* sp.) which are another widely consumed bivalve species. The confirmation criteria were retention time, MRM transitions and ion ratios, including its percentage of variation accepted, as described in the European Directorate [38].

One compound, salicylic acid, was found in all of the mussel samples analyzed at maximum concentrations of 103.26 ng/g (d.w.). Of the other bivalve species analyzed, salicylic acid was determined in the lagoon cockle (*C. glaucum*), coquina clam (*D. trunculus*) and striped venus clam (*C. gallina*). All of the results are shown in Table 3, where maximum concentration values

Table 3 Concentration of salicylic acid and percentage of ion ratio

Species	Salicylic acid	
	Conc. (ng/g)	Ion ratio[a] (%)
Mytilus galloprovincialis[b]	95.88	7.3
Mytilus galloprovincialis[c]	98.47	7.4
Mytilus edulis	103.26	6.1
Cerastoderma glaucum	35.73	6.7
Donax trunculus	59.02	7.8
Chamelea gallina	65.17	8.2

[a] Relation between the abundance of the qualifier transition (137→65) and the quantifier transition (137→93)

[b] From the Ebro River Delta, Mediterranean Coast (NE, Spain)

[c] From Galicia, Atlantic Coast (NW, Spain)

from each species analyzed and percentage of ion ratio are reported. These ion ratios are within the percentage of variation described by the European Directorate [38]. As an example, Fig. 3 shows two MRM chromatograms (quantification transition) where the top figure (a) refers to mussel from the Ebro River Delta and the lower one (b) belongs to the lagoon cockle (*C. glaucum*).

Wille et al. [11] found concentrations of salicylic acid in *M. edulis* in the Belgian coastal waters, in some cases similar to those found in the present study. However, in their work, they detected concentrations up to 490 ng/g, which are higher than those found in the present study. Huerta et al. [33] found diclofenac at concentrations up to 8.8 ng/g in fish homogenate in different species. This compound was not detected in the bivalve species evaluated in the present study.

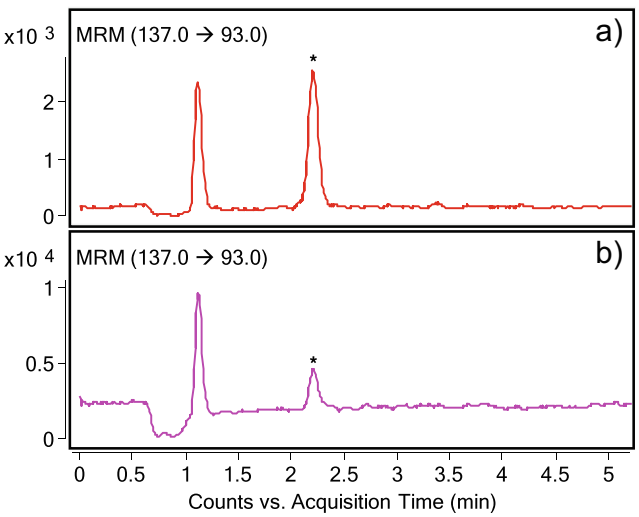

Fig. 3 MRM chromatograms (quantification transition) of **a** the mussel from the Ebro River Delta and **b** the lagoon cockle

Conclusions

A rapid and reproducible method was successfully developed for the quantification of seven pharmaceuticals belonging to different therapeutic classes in the bivalve samples. Several strategies were evaluated in order to clean the matrix, and dSPE using silica gel and modification of the chromatographic separation provided the best results. The method based on QuEChERS extraction and dSPE clean-up followed by LC-MS/MS was validated in mussels (*M. galloprovincialis*). The optimum method provided App RE between 35 and 77 % and LODs between 1 and 50 ng/g. The method was applied to analyze the samples of different bivalve species, and salicylic acid was found in mussels from three different locations and also in some of the bivalve species at nanograms per gram (d.w.) levels.

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