Different sample treatments for the determination of ICM-XR in fish samples followed by LC-HRMS

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ABSTRACT

Iodinated X-ray contrast media (ICM-XR) are a group of pharmaceuticals widely used in medicine. Due to their low biodegradation rate, which makes their removal at wastewater treatment plants difficult, and the high doses at which they are administered, they have been detected in aquatic environments. In the present paper, a method for the quantitative determination of a group of ICM-XR in different fish species was developed and validated for the first time. Two extraction techniques were compared: pressurised liquid extraction (PLE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), with PLE being selected, followed by liquid chromatography-high resolution mass spectrometry. In addition, several clean-up strategies were evaluated. The optimised method provided PLE recoveries ranging from 60% to 88% and limits of detection ranging from 5 ng/g to 25 ng/g (dry weight). The method was applied in order to evaluate the presence of the selected ICM-XR in different fish species.

1. Introduction

Iodinated X-ray contrast media (ICM-XR) are a group of pharmaceuticals that are applied in clinical diagnosis in order to obtain radiographic images of soft tissues, such as blood vessels or organs. They are administered to patients in an aqueous solution at a high dose (200 g/application) and are designed to be inert and not to interact within the human body [1]. For this reason, they are made with structural and physicochemical features that provide them high stability, high solubility and high polarity. Consequently, they are not metabolised and are excreted through urine and faeces, within only 24 h after administration. Due to the mentioned characteristics, ICM-XR have a low biodegradation rate, which makes their removal at wastewater treatment plants (WWTPs) difficult. Therefore, they can reach other environmental compartments through effluents and the reuse of sewage sludge from WWTPs. One study conducted by Carballa et al. [2] investigated the behaviour of several organic contaminants through the processes of a WWTP and also their removal efficiency. They found that the ICM-XR iopromide was not removed and remained in the aqueous phase. In addition to the high concentrations at which they are administered, this fact means that these compounds can easily reach the water system and, therefore, the biota with which it is in contact. For this reason, several methods have been described to determine these compounds in environmental matrices, mainly in water bodies [1]. ICM-XR have been found in different aquatic environments, such as effluents from WWTPs at a maximum concentration of iopromide up to 20 μg/L [3], in the groundwater at a concentration of diatrizoic acid up to 1.1 μg/L [4], and in surface waters and drinking waters at low ng/L [5]. However, one study reported concentrations of diatrizoic acid up to 4 μg/L in surface waters and 1.2 μg/L in drinking waters [6].

For the determination of ICM-XR, the most commonly used analytical techniques in the literature are liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) [1]. Among the methods developed for aqueous matrices, solid-phase extraction (SPE) is the most widely used extraction technique [1]. As regards solid matrices, such as sludge, not many studies have been performed. In one study conducted by Ternes et al. [7], ultrasound-assisted solvent extraction (USE) was employed as the extraction technique, followed by SPE as the clean-up. In this study, none of the ICM-XR studied was detected above its limit of quantification (LOQ), which was 50 ng/g. In another study performed by Echeverría et al. [8], pressurised liquid extraction (PLE) was used as the extraction technique. In this case, the ICM-XR found were also below their LOQs (25 ng/g). To date, there is no study on the bioaccumulation of these compounds in aquatic organisms. Although one study conducted in 1999 by Steger-Hartmann et al. [9] revealed no toxic effects produced by the administration of iopromide in short-term toxicity tests performed on bacteria, algae, crustacean and fish, as well as no long-term toxic effects on the crustacean *Daphnia magna*, it must be

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taken into account that continuous exposure to contaminants and their products may lead to changes over time, even though no ecotoxicological effects can be observed in acute toxicity tests [1].

Therefore, it is important to develop analytical methods to determine ICM-XR in biological matrices. In recent years, some methods to determine pharmaceuticals using fish as an indicator organism have been published [10], but none of them has focused on ICM-XR. However, this type of matrix usually involves long purification steps, which makes these studies more challenging.

The aim of this study was to develop an analytical method to determine a group of ICM-XR in different fish species. Two extraction techniques were compared: QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and PLE. Moreover, different clean-up strategies were evaluated. The determination was performed by LC coupled with high resolution mass spectrometry (HRMS). Once the method had been developed and validated, it was applied to evaluate the occurrence of these compounds in different fish species. This was the first time that an analytical method was developed to determine these compounds in fish. Because of the high complexity of the matrix, HRMS may be advantageous for its analysis [11].

2. Experimental

2.1. Materials, reagents and standards

Iopamidol (97.5%), diatrizoic acid (92.4%), iomeprol (98.0%) iohexol (99.0%) and iopromide (97.0%) were supplied by Dr. Ehrenstofer (Augsburg, Germany) being the two latter in form of racemates. Individual stock solutions of 1000 mg/L were prepared in methanol (MeOH) and stored at −20 °C. A mix solution of 50 mg/L in MeOH was prepared weekly and stored, also at −20 °C. Deuterated compounds iopamidol-d8 and diatrizoic acid-d6 with an isotopic purity of 99.4% and 98.5%, respectively, were purchased from LGC Standards (Wesel, Germany) and were used as surrogate internal standards, which from now on they will be abbreviated as internal standards (I.S).

The organic solvents MeOH and acetonitrile (ACN) were of HPLC grade and provided by J.T Baker (Deventer, the Netherlands). Acetone and the solvents tested for the on-cell clean-up (hexane, ethyl acetate, isooctane and dichloromethane) were also of HPLC grade and purchased from Prolabo (Llinars del Vallès, Spain). Formic acid, acetic acid, sulphuric acid and the sorbents tested for the in-cell clean-up (C_{18}, Florisil, silica and alumina) were supplied by Sigma-Aldrich (St. Louis, USA).

Ottawa sand was purchased from Fisher Scientific (Waltham, MA, USA) and Oasis® HLB cartridges (500 mg/6 cc) and Oasis® MCX cartridges (150 mg/6 cc) used in SPE were obtained from Waters (Milford, MA, USA).

The ultrapure water was produced by an ultrapure water system from Veolia Water (Barcelona, Spain). The nitrogen gas (N_2) was obtained from Carburos Metálicos (Tarragona, Spain).

The three QuEChERS methods were evaluated. The European Standard Method EN 15662 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. The AOAC Official Method 2007.01 packet was obtained from Waters, and contained 6 g of magnesium sulphate and 1.5 g of sodium acetate. To perform the original QuEChERS method, 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride, both from Sigma-Aldrich, were mixed in the laboratory.

2.2. Sampling and sample pretreatment

The species *Cyprinus carpio* (common carp), *Silurus glanis* (wels catfish) and *Perca fluvialitis* (perch) were taken from the Ebro River (NE, Spain). The widely consumed marine species, *Merluccius merluccius* (European hake), *Sparus aurata* (gilt-head bream), *Mullus surmuletus* (striped red mullet), *Scomber scombrus* (Atlantic mackerel), *Thunnus thynnus* (Atlantic bluefin tuna), *Solea solea* (common sole) and *Psetta maxima* (turbot) were bought in the local market. For all species, side fillets were separated and subsequently homogenised and frozen at −20 °C for 24 h. Once frozen, the samples were lyophilised using the freeze-drying system Genevac miVac Duo Concentrator (Ipswich, Suffolk, UK). Eventually, the lyophilised samples were ground to obtain a homogeneous powder and sieved (500 μm) to obtain particles of the same size.

The percentage of lipid content of the abovementioned species was determined gravimetrically by the evaporation of the extract obtained by PLE extraction according to [12]. These PLE extractions were performed on an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA) using hexane:dichloromethane (1:1, v:v) as extraction solvent. The other extraction parameters were: pressure 1500 psi, temperature 100 °C, preheating time 5 min, static time 10 min, number of cycles 2, purge time 90 s and flush volume 80%.

In order to optimise the extraction procedure and obtain efficient extractions, 1 g of freeze-dried fish sample was weighed and then it was wetted with acetone and, later, spiked with the analytes at the desired concentration. The mixture was homogenised and left under a hood overnight allowing the solvent to evaporate. Of the species mentioned, *Mullus surmuletus* was selected and several individuals were pooled to perform the optimisation of the method as it is one of the species with highest lipid content.

2.3. Extraction

Although the three QuEChERS methods were evaluated adapting procedures to the dry matrix, the best results were obtained by the AOAC Official Method 2007.01. To do so, 1g of freeze-dried fish sample was weighed in a 50 mL polypropylene centrifuge tube, then 15 mL of ultrapure water was added, and the mixture was shaken manually for 1 min. Afterwards, 15 mL of ACN containing 1% acetic acid was added and it was also mixed by manual shaking for 1 min. After that, the buffer (AOAC packet) was added and the mixture was homogenised again for 15 s by manual shaking and for 45 s using a Heidolph Reax 2000 vortex. At the end, the tube was centrifuged for 5 min at 7000 rpm in a centrifuge from Hettich Zentrifugen (Germany). 1 mL of the ACN layer was transferred into a glass vial, then evaporated to dryness and re-dissolved in 1 mL of ultrapure water, which was filtered through a 0.45 μm syringe filter before being injected into the LC-HRMS system.

PLE extractions were performed with the same equipment used to determine the lipid content. A cellulose filter from Teknokroma (Sant Cugat del Vallès, Spain) was placed at the bottom of an 11 mL stainless steel cell. 3 g of Ottawa sand was placed on top, followed by 1 g of the freeze-dried sample, which had previously been mixed with 2 g of Ottawa sand. Then, Ottawa sand was added again to fill up the cell and, finally, another cellulose filter was placed on top. MeOH was used as the optimised extraction solvent and the optimal extraction parameters were: temperature 40 °C, preheating time 5 min, static time 5 min, number of cycles 1, purge time 60 s and flush volume 50%.

The extract obtained from the PLE (~17 mL) was cleaned by SPE using an Oasis® MCX cartridge. In the present work, this cartridge was used to retain interfering substances instead of concentrating the extract. For this reason, the loading was collected and no elution step was performed. The SPE protocol was as follows: the cartridge was conditioned with 5 mL of ultrapure water followed by 5 mL of MeOH. Then, it was loaded with the PLE extract, which was collected in a vial and evaporated to dryness in a miVac concentrator and, finally, the dried extract was re-dissolved in 1 mL of ultrapure water that was filtered through a 0.45 μm syringe filter and injected into the LC-HRMS.

2.4. LC-(Orbitrap)HRMS analysis

Chromatographic analyses were performed with an Accela 1250 HPLC system connected to an Exactive Orbitrap™ mass spectrometer, all from Thermo Scientific (Bremen, Germany). The chromatograph was equipped with a quaternary pump (1250 bar), an Accela Autosampler automatic injector, kept at 10 °C, and a column oven, which was maintained at 25 °C. The interface employed was a heated electrospray ionisation (HESI-II) source, operating in positive ionisation mode. The instrument was equipped with a high energy collisional dissociation cell (HCD) in order to fragment the analytes for confirmation purposes. The chromatographic separation was performed with an Ascentis Express C_{18} Fused-Core® column (5 cm × 4.6 mm i.d.; 2.7 μm particle size) from Supelco (Sigma-Aldrich). The mobile phase composition was a mixture of ultrapure water with formic acid (pH 2.6) as solvent A and ACN as solvent B. The gradient used started with 2% B which was increased to 4% B within 2 min, and then raised to 25% B within 12 min. It was then increased to 100% B in 2 min and maintained at 100% B for 4 min. Finally, it returned to initial conditions within 2 min. The flow-rate was 0.2 mL/min and the injection volume was 25 μL. The chromatographic analysis took place within 10 min and the time between the runs was 5 min

Optimal ionisation source parameters were: spray voltage 4.0 kV; sheath gas 60 AU (arbitrary unites); tube lens voltage 140 V; auxiliary gas 5 AU; skimmer voltage 35 V; capillary voltage 60 V; heater temperature 400 °C; capillary temperature 280 °C; and probe position adjustments: 0 as side to side position; D as vertical position and micrometer 0.75.

The data was acquired in one single window by continuously alternating two scan events: one without fragmentation at 50000 full width at half maximum (FWHM) resolution with an injection time of 250 ms, and one with fragmentation at 10000 FWHM with 50 ms injection time using 30 eV in the HCD. The diagnostic ions were measured for quantification (with a mass error of 5 ppm) and fragments and the corresponding ion ratios were used for confirmation purposes.

3. Results and discussion

3.1. LC-(Orbitrap)HRMS

The separation of the compounds was slightly challenging, since ICM-XR compounds are highly polar and two of them (iohexol and iopromide) present stereoisomers. For these compounds the signal of the isomers was added for quantification. The chromatographic separation described by Echeverría et al. [13] was used as a starting point and was slightly modified by testing different initial %B. Despite optimising the chromatographic separation (described in Section 2.4), some compounds could not be completely separated. This is the case of iohexol stereoisomer, which co-elutes with diatrizoic acid. It was not possible either to separate iohexol completely from iomeprol, although they can be distinguished by their masses. However, the final separation prevents overlapping between iopamidol and iomeprol, compounds that have the same m/z and which cannot be separated by MS.

To optimise the HRMS parameters continuous infusion of standard compounds was used, prepared with a mobile phase composition of 15% ACN and 85% water at pH 2.6 with HCOOH. The exact m/z was recorded in full scan at 50000 FWHM for each compound in positive and negative mode. As expected, the signal obtained for all of the compounds was higher in positive mode. For all of the compounds, $[M+H]^+$ was selected for quantification (Table 1). Once the exact m/z had been recorded, each ionisation source parameter was optimised individually and a compromise was chosen for all of the compounds. For the spray voltage, values between 2 and 5 kV were evaluated, while for the capillary voltage, values from 10 to 100 V were tested. The tube

lens voltage was measured between 50 and 200 V, and the skimmer voltage from 5 to 50 V. In addition, all the gas parameters and temperatures were assayed: sheath gas was evaluated between 50 and 100 AU, and the auxiliary gas from 0 to 50 AU. Capillary and heater temperature were measured between 250 °C and 450 °C. Finally, the probe position was evaluated, horizontal position (side to side) from −1 to 1, vertical position from A to D and the micrometer from 0 to 1. The optimal parameters can be found in Section 2.4.

Moreover, fragment ions for each compound were obtained for confirmation purposes. To do so, the signal intensity was monitored while applying different collision energies (from 5 to 60 eV) in the HCD. It was observed that 30 eV could be adopted as a compromise value of fragmentation for all the studied compounds, as at least one fragment ion could be obtained with the higher response. The selected fragment for each compound can also be found in Table 1. These fragments are in agreement with those reported in the literature using MS/MS [1,3,13,14]. The fragment ion from iopamidol might correspond to the cleavage of the amide bond and the loss of $C_3H_9NO_2$ and HI. The fragment ion from diatrizoic acid might correspond to the loss of 2I and HI. The fragment ion from iohexol can be assigned to the loss of a water molecule. The fragment ion from iomeprol might correspond to the loss of $C_3H_9NO_2$ due to the cleavage of the amide bond. Finally, the fragment ion from iopromide, as in the case of iopamidol, can be attributed to the loss of $C_3H_9NO_2$ and HI.

Once the LC-HRMS was optimised, instrumental limits of detection (LODs) and LOQs were experimentally evaluated (n = 3). The LODs were determined in line with [11,15], when a signal intensity higher than 1×10^3 of the precursor ion was accomplished. LOQs were defined as the lowest point of the calibration curve. LODs (2 and 3 μg/L) and LOQs (5 μg/L) were achieved. The instrumental limits obtained in the present study are in accordance with those obtained with MS/MS when QqQ was employed as the analyser [13].

3.2. Extraction

Two different techniques, QuEChERS and PLE, were evaluated to extract the five selected ICM-XR from fish. QuEChERS is a cheap technique that does not require any analytical equipment and it has been recently employed to extract pharmaceuticals from biota samples [16]. PLE has been extensively used to extract a wide range of contaminants from different solid matrices and, although it requires analytical equipment, its robustness has been demonstrated [8,17,18]. For QuEChERS extraction, three different methods were evaluated: the original QuEChERS method [19], the AOAC Official Method 2007.01 [20] and the European standard method EN 15662 [21]. To select the best QuEChERS method, apparent recoveries (App REs) were compared, which were calculated by comparing the peak signal of the analytes from samples spiked at 1500 ng/g (d.w.), before the extraction and the peak signal of the analytes in standard solutions directly injected into the LC-HRMS system. The AOAC method provided the highest App REs, which were between 11% and 48%, while they were below 14% for the other methods. Then, the extraction recoveries (REs) and the ME were also calculated for the AOAC method. REs were calculated by comparing the peak signal of the analytes in a sample spiked at 1500 ng/g (d.w.) before the extraction and the peak signal of the analytes that were spiked after extraction at the same concentration. The ME was evaluated as the formula described below, where B is the peak signal of the analytes in a sample spiked after the extraction and A is the peak signal of the analytes in standard solution directly injected into the LC-HRMS.

$$ME\ (\%) = -[100 - (B/A * 100)]$$

When these parameters were evaluated, low REs were obtained, with values ranging from 15% to 40% with the exception of iopromide with an RE of 64%. In all the analytes, the ME was in the form of ion

Table 1
Chemical stucture, pk$_a$, log Kow and accurate masses of the studied ICM-XR..

Compound	Chemical formula	Structure	pk$_a$[a]	log Kow[b]	Precursor ion [M + H]$^+$	Fragment ion
					(m/z)	(m/z)
Iopamidol	C$_{17}$H$_{22}$I$_3$N$_3$O$_8$		6.9	− 2.4	777.86383	558.88678
Diatrizoic acid	C$_{11}$H$_9$I$_3$N$_2$O$_4$		1.1	1.8	614.77808	233.05626
Iohexol	C$_{19}$H$_{26}$I$_3$N$_3$O$_9$		10.6	− 3.0	821.8891	803.87891
Iomeprol	C$_{17}$H$_{22}$I$_3$N$_3$O$_8$		10.6	− 2.3	777.86285	686.79895
Iopromide	C$_{17}$H$_{24}$I$_3$N$_3$O$_8$		6.6	− 2.1	791.87885	572.9035

[a] Values calculated using Sparc (http://archemcalc.com/sparc).
[b] Values obtained from PubChem Compound Database (pubchem.ncbi.nlm.nih.gov).

suppression with values ranging from 24% to 29%. The low REs were probably due to the high polarity of the analytes and they were not even improved when more polar mixtures of solvents such as MeOH/ACN were used. For this reason, QuEChERS was rejected and PLE was assayed.

According to Runnqvist et al. [22] and also based on our previous experience, initial conditions were fixed as: 1 g of sample, preheating time 5 min, static time 5 min, 1 cycle, extraction temperature 80 °C, flush volume 50% and purge time 60 s

The first parameter optimised was the extraction solvent. Water, acidified water with HCOOH (pH 2.6), MeOH, ACN, acetone and a mixture of MeOH:water (1:1; v:v) were tested. To achieve suitable conditions for the injection to LC, water extracts (17 mL) were diluted to 25 mL with ultrapure water, while extracts containing organic solvents were evaporated and re-dissolved with 25 mL of ultrapure water. Table 2 shows the PLE REs and as can be seen, MeOH showed the highest PLE REs followed by the mixture of MeOH:water (1:1; v:v). With ultrapure water diatrizoic acid displayed very low extraction, whereas, with acidified water, PLE REs were generally lower, except for this compound. ACN provided very low PLE REs, which confirms the results achieved with QuEChERS. Acetone provided similar recoveries to ACN. Moreover, the ME values obtained with MeOH were lower than those obtained with the mixture of MeOH:water (1:1; v-v). For these reasons, MeOH was selected as the extraction solvent. In addition, the organic ex-

tract of MeOH can easily be evaporated. The selection of MeOH agrees with previous studies in which the same group of ICM-XR was extracted from sewage sludge [8].

Once the solvent was chosen, the temperature was tested at 40 °C, 60 °C 80 °C and 100 °C PLE REs values were very similar at all temperatures tested, between 60% and 88%, with diatrizoic acid being the compound with the lowest PLE RE (60%), which could not be increased by varying the extraction temperature. For this reason, the extraction temperature was set at 40 °C in order to avoid the co-extraction of interfering substances. Table 2 also details the PLE REs at 40 °C.

Afterwards, different static times (5, 10, 15 and 20 min) were assessed. Although no improvement in PLE REs was observed when the static time was increased. Special attention was paid to diatrizoic acid, whose PLE RE remained at ~60%. For this reason, it was decided to maintain the static time at 5 min

Finally, the number of cycles (one and two cycles) was evaluated. 1 cycle was selected since very similar PLE REs were obtained in both cases. Other parameters, such as preheating time, purge time and % flush volume, are considered of minor influence on the extraction [8,18], so they were not optimised and were kept at the initial levels.

To sum up, the final PLE conditions consisted of 1 g of fish sample, extraction solvent MeOH, temperature 40 °C, preheating time 5 min, static time 5 min, number of cycles 1, purge time 60 s and flush volume 50%.

Table 2

PLE RE and matrix effect (ME) using different extraction solvents and temperatures when the fish sample was spiked at 1500 ng/g (d.w.).

Compound	MeOH			Water		Acidified water		MeOH/water (50:50)		ACN		Acetone	
	40 °C	80 °C											
	PLE RE (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)	PLE RE (%)	ME (%)
Iopamidol	82	81	−40	58	−53	48	−31	67	−54	<10	−47	11	−52
Diatrizoic acid	60	55	−51	<10	−57	33	−34	58	−76	<10	−57	<10	−70
Iohexol	83	80	−45	46	−42	39	−40	63	−63	<10	−46	<10	−52
Iomeprol	79	77	−47	57	−65	49	−59	68	−68	<10	−44	10	−54
Iopromide	88	85	−42	64	−72	38	−64	63	−65	19	−41	23	−53

(% RSD (n=3) < 10%).

3.3. Strategies to reduce the matrix effect

In general, biotic samples are rich in undesirable compounds that might interfere with the analysis. In the present study, high ion suppression was detected. For this reason, several strategies were evaluated in order to clean the matrix and reduce the interferences present in it. Two PLE clean-up strategies were tested: in-cell and on-cell clean-up. Additionally, two different SPE cartridges (Oasis® HLB and Oasis® MCX) were evaluated.

3.3.1. On-cell clean-up

One cleaning strategy that is enabled by the PLE technique is on-cell clean-up once the cell has been assembled by using an appropriate solvent previous to the extraction. Four different apolar or midpolar solvents (hexane, ethyl acetate, isooctane and dichloromethane) and ACN were evaluated. ACN was tested since our previous results obtained with both QuEChERS and PLE confirmed the low affinity of ICM-XR for ACN. The PLE conditions for the clean-up were the same as those used in [18], where interfering substances were removed from sewage sludge. None of the solvents tested resulted in a substantial improvement in terms of increasing App REs. In the case of hexane, ethyl acetate, isooctane and dichloromethane, the App RE of iopamidol was reduced between 7% and 10% approximately, and the other compounds did not show any improvement. In the case of ACN, iohexol and iopamidol showed a slight improvement, at maximum of 5%. For this reason, this strategy was rejected.

3.3.2. In-cell clean-up

Another strategy often used with the PLE technique is in-cell clean-up. Five sorbents: C_{18}, Florisil, silica, alumina and acidic silica, which preparation was adapted from [23,24], were used instead of Ottawa sand in order to perform the in-cell clean-up. As in the case of on-cell clean-up, none of the in-cell sorbents resulted in an improvement in App REs. Only the acidified silica improved the App RE of the diatrizoic acid (10% improvement). However, the App RE of iopamidol was reduced by more than 15%. In addition, florisil slightly improved the App RE of diatrizoic acid, but less than 10%. For this reason, this strategy was also rejected.

3.3.3. Solid-phase extraction

Another strategy often used to clean complex matrices such as biota is SPE [25,26]. Two different cartridges, Oasis® HLB and Oasis® MCX, were evaluated in order to improve App RE. Oasis® HLB sorbent is a hydrophilic-lipophilic balanced reversed-phase sorbent with enhanced retention of polar analytes [13], while Oasis® MCX is a cation-exchanger sorbent based on the Oasis® HLB polymeric structure and modified with sulphonic groups, so that, ionic interactions can be established. In addition, it allows an organic solvent to be loaded, enabling the retention of the interferences in the sorbent [27].

In order to evaluate the cartridges, App REs were also calculated and were compared with a PLE extract that was not passed through any cartridge. 1 g of sample was spiked before extraction at 500 ng/g (d.w). In the case of Oasis® HLB, the conditions proposed in [13] were used as a starting point. In brief, after conditioning the cartridge the PLE extract was loaded, which had been previously evaporated to dryness and reconstituted in 25 mL of ultrapure water adjusted at pH 3 with HCOOH. After that, 5 mL of different clean-up solvents, namely water containing 5% ACN, water at pH 3, hexane and no clean-up were evaluated. Later, the compounds were eluted using 5 mL of MeOH. Finally, the extracts were evaporated to dryness, reconstituted in 5 mL of ultrapure water and filtered before injection.

In the case of Oasis® MCX, the PLE extract was directly loaded without prior evaporation, then the load, which contains the analytes, was collected, evaporated to dryness and reconstituted in 5 mL ultrapure water. Table 3 details the App REs for both sorbents tested as well as without SPE. As can be seen, all the App REs are slightly better for Oasis® MCX. In addition, the protocol is simpler than Oasis® HLB. For all of these reasons, Oasis® MCX sorbent was selected as a clean-up step after PLE.

3.3.4. Calibration approach

Although SPE with Oasis® MCX slightly improved the App REs, two isotopically labelled standards (iopamidol-d8 and diatrizoic acid-d6) were selected to be used as I.S. in order to compensate for the ME. The use of a higher number of isotopically labelled standards was avoided due to the high cost of them. Whereas iopamidol-d8 was used as the I.S. for iopamidol, iohexol, iomeprol and iopromide; diatrizoic acid-d6 was used as the I.S. only in the case of diatrizoic acid. The effectiveness of the deuterated compounds was evaluated by calculating the relative recoveries (REL REs) for each compound. They were calculated by the interpolation of the signal ratio (compound/deuterated compound) of a sample that had been spiked with the analytes and the deuterated compounds before PLE extraction, with a solvent calibration curve with deuterated compounds. REL REs ranged between 83% and 113%, except for iomeprol, which had a REL RE of 57%. Then, the deuterated compounds were incorporated to the method.

At the end, it was decided to concentrate the extract in order to improve the detection limits of the method, thus, the extracts were evaporated to dryness, reconstituted in 1 mL of ultrapure water and filtered before injection. At this point, it should be mentioned that, due to the low response of diatrizoic acid, it was decided to eliminate this compound as well as its corresponding I.S. (diatrizoic acid-d6) from the method and so it was excluded from validation.

3.4. Method validation and application

Finally, the optimised method was validated in order to demonstrate its performance. The final conditions are detailed in Sections 2.3 and 2.4. The species evaluated were divided into three different groups according their % lipid content, which is indicated in brackets for each species. The high lipid content group included: *Mullus surmuletus* (striped red mullet, 23%), *Scomber scombrus* (Atlantic mackerel, 21%), *Sparus aurata* (gilt-head bream, 35%) and *Psetta maxima* (turbot, 31%). The medium lipid content group included: *Cyprinus carpio* (common carp, 15%) and *Silurus glanis* (wels catfish, 12%). The low lipid content group included *Perca fluvialitis* (perch, 3%), *Thunnus thynnus* (Atlantic bluefin tuna, 2%), *Solea solea* (common sole, 5%) and *Merlucius merluccius* (European hake with 3%). From each group, one representative species was selected: *Mullus surmuletus* (high lipid content), *Cyprinus carpio* (medium lipid content) and *Thunnus thynnus* (low lipid content). Matrix-matched calibration curves with the deuterated compounds were plotted for each selected species, while LODs and REL REs were also calculated as described in Sections 3.1 and 3.3.4, respectively. Moreover, blank samples were analysed in order to take into account whether any of the selected compound was present. However, none of the selected compounds was found in blank samples. All of the results

Table 3

% App RE when different SPE sorbents were used for the clean-up and when no SPE was used. For more details see text.

Compound	App RE (%)		
	Without SPE	Oasis® HLB	Oasis® MCX
Iopamidol	30	34	35
Diatrizoic acid	7	10	16
Iohexol	20	27	33
Iomeprol	15	20	27
Iopromide	23	24	38

(% RSD (n = 3) < 12%).

can be found in Table 4. The linear range, for most of the compounds, was between 25 and 500 ng/g (d.w.) in the case of *Cyprinus carpio* and *Thunnus thynnus* and between 50 and 500 ng/g (d.w.) for *Mullus surmuletus*. LODs were 5 ng/g (d.w.) or 10 ng/g (d.w.) in all instances for *Thunnus thynnus* and *Cyprinus carpio*. In the case of *Mullus surmuletus* the LODs were higher (25 ng/g (d.w.)) since it is the species with the highest % lipid content, and therefore a higher ME. REL REs ranged from 88% to 119%. Only in the case of iomeprol they were lower, with values of 56% and 69% for *Mullus surmuletus* and *Cyprinus carpio*, respectively. Iopromide presented the highest REL RE with values up to 125% for *Thunnus thynnus*.

In addition, the repeatability and reproducibility of the method were evaluated for the three species using five replicate extractions of fish sample spiked at 100 ng/g (d.w.), performed on the same day and on different days, respectively. Both were expressed as a percentage of relative standard deviation (%RSD). Table 4 details the %RSD values obtained for the species with highest % lipid content. The values for the two other species were similar or even lower.

An HRMS chromatogram of a fish sample (*Mullus surmuletus*) spiked at 100 ng/g is presented in Fig. 1.

The occurrence of the selected compounds was evaluated in different freshwater species and also in different marine species. The species *Cyprinus carpio*, *Silurus glanis* and *Perca fluvialitis* were taken from the Ebro River. The widely consumed marine species *Merluccius merluccius*, *Sparus aurata*, *Mullus surmuletus*, *Scomber scombrus*, *Thunnus thynnus*, *Solea solea* and *Psetta maxima* were bought in the local market. However, none of the ICM-XR studied was found above the respective LOD in any of the samples analysed. According to Huerta et al. [10] the high-

est levels of pharmaceuticals have been detected in tissues such as liver or brain. For this reason, the liver of *Mullus surmuletus* was also analysed. Nevertheless, none of the studied compounds was detected above the LOD either.

In any case, as this is the first time that these ICM-XR are determined in fish sample, no data to compare whether these findings are as expected was available.

4. Conclusions

An analytical method was developed to determine a group of ICM-XR in different fish species, with PLE as extraction technique. Different approaches were conducted to reduce the high ME encountered in these samples. Of these approaches, SPE with Oasis® MCX was used as a clean-up step and calibration with isotopically labelled compounds was used to compensate this ME.

The method was validated with different fish species, according to their lipid content. The lipid content of the different species analysed slightly modified the figures of merit during the validation of the method. Thus, this content was considered during the application of the method to evaluate the occurrence of the studied compounds. Nevertheless, none of the studied ICM-XR was detected in the analysed samples.

Acknowledgements

Table 4
Method validation data when the samples were analysed by PLE followed by Oasis® MCX clean-up and LC-HRMS.

|	*Thunnus thynnus* (low lipid content)			*Cyprinus carpio* (medium lipid content)			*Mullus surmuletus* (high lipid content)				
	Linear range	LOD	REL RE	Linear range	LOD	REL RE	Linear range	LOD	REL RE	Repeatability	Reproducibility
	(ng/g)	(ng/g)	(%)	(ng/g)	(ng/g)	(%)	(ng/g)	(ng/g)	(%)	% RSD	% RSD
Iopamidol	25–500	10	88	25–500	10	96	50–500	25	109	4	15
Iohexol	25–500	10	106	10–500	5	89	50–500	25	95	12	14
Iomeprol	25–500	10	91	25–500	10	69	50–500	25	56	11	18
Iopromide	50–500	25	125	25–500	10	119	50–500	25	116	12	26
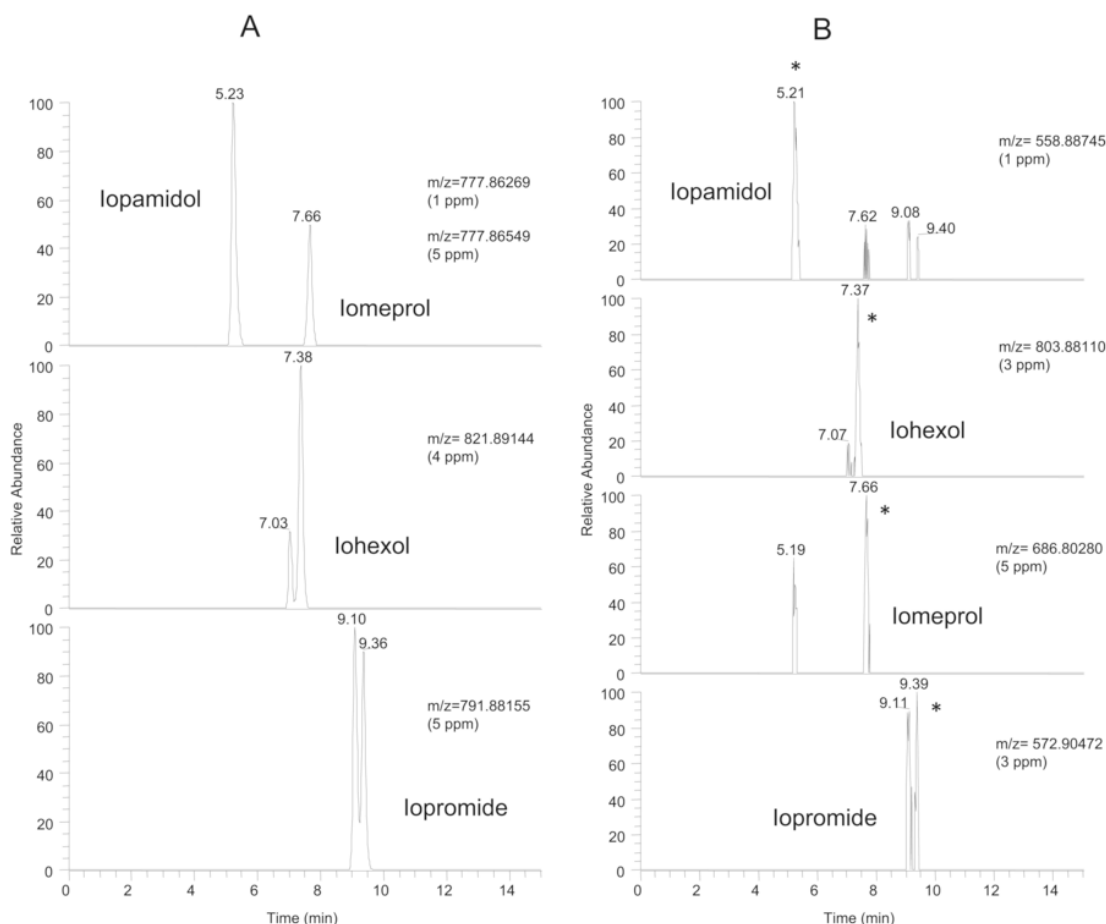
Fig. 1. HRMS chromatogram and mass error in ppm of a fish sample (*Mullus surmuletus*) spiked at 100 ng/g. (A) corresponds to the precursor ions and (B) corresponds to the fragment ions.

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