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Multi-residue method for trace level determination of UV filters in fish based on pressurized liquid extraction and liquid chromatography–quadrupole-linear ion trap-mass spectrometry☆

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ABSTRACT

So far, the very few studies addressing the occurrence of UV filters (UV F) in biota showed important limitations in the analysis of the so complex biological matrices. In order to improve the knowledge on the bioaccumulation of UV F by fish, a simple and highly sensitive method was successfully developed and validated for the simultaneous determination of eight extensively used UV F and transformation products with a wide range of physicochemical properties. The present study demonstrated that liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) using a QqLIT mass analyser was applicable to the simultaneous analysis of UV F in fish. Pressurized liquid extraction (PLE) was chosen for the sample pretreatment due to the good extraction efficiency provided. An additional SPE clean-up step was added in order to minimize matrix effects and to improve the sensitivity. The method allowed recovery efficiencies in the range 70–112% for most compounds at the three spike levels. The low limits of detection (MLOD) achieved (0.1–6.0 ng/g dw) allowed the reliable quantification of UV F residues in fish samples. The developed methodology was applied to assess the occurrence of UV F in different fish species from the Guadalquivir river basin (Spain). Results confirmed the bioaccumulation of benzophenone-3 (BP3), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) in the fish samples. The maximum concentration of 240 ng/g dw corresponded to EHMC, which was also the most ubiquitous compound. The reported concentrations constitute the first occurrence data of UV F residues in fish from Iberian rivers.

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1. Introduction

UV filters (UV F) or sunscreen agents are chemical compounds that mitigate the deleterious effects of sunlight. This group of additives is worldwide used in many personal care products as well as in many industrial goods to protect skin from chronic (skin cancer) or acute (photoaeging, sunburn) exposure to UV radiation [1] and to protect products from photodegradation (yellowing) [2].

UV F enter the aquatic environment continuously through two principal pathways: by direct inputs from aquatic recreational activities, and mainly by indirect inputs through sewage waters. Once discharged from industrial and urban sources, they ultimately enter surface waters and, as they are only partly removed in wastewater treatment plants (WWTPs), act as pseudo-persistent pollutants.

The increasing use of UV F may constitute a potential risk for the environment since most of them have multiple hormonal activities in fish [3], even at environmentally relevant concentrations [4], and in rodents. [5]. Besides, a recent study indicates that exposure to benzophenone type UV F may be associated with oestrogen-dependent diseases such as endometriosis in women [6].

Because of the high lipophilicity and poor biodegradability of many UV filters they have been detected at high concentrations in sewage sludge [7–11]. UV F have also been observed in surface water [12–17], seawater [15,17–19] and wastewater [13,15–17,20,21] and in sediments [11,22–24]. UV F also accumulate in humans being detected in milk [25,26], semen [27] and placental tissues [28].

The accumulation of UV F residues in biota has scarcely been studied. The current knowledge on the bioaccumulation and analytical methodology applied for the determination of UV F in

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biota was recently reviewed by Gago-Ferrero et al. [29]. Fish are important bioindicators of the occurrence of persistent lipophilic contaminants. Values from 9 to 2400 ng/g lipids have been reported in fish samples in a few studies [2,13,30–33] and concentrations over 7000 ng/g were detected in mussels [34]. Fent et al. [2] detected EHMC in crustacean and mollusks in the range 22–50 ng/g lipids, and in fish at values up to 337 ng/g lipids. The higher concentration, above 700 ng/g lipids, was reported for fish-eating birds (*Phalacrocorax* sp.), which suggests that biomagnification occurs through the food web.

Determination of UV F in the aqueous environment has been mainly performed by gas chromatography coupled to mass spectrometry (GC–MS). Matrix effects are not critical for the ionization modes typically used and good method limits of detection (MLODs) are achieved. However, these methods have important limitations. They solely can be applied to substances that are volatile and of low polarity or can be derivatized (where differences in matrix components may result in quite different derivatization efficiencies which compromise precision and accuracy of the analysis). If the objective is to perform the simultaneous determination of several UV F, with a wide range of physicochemical properties, liquid chromatography (LC) offers better features than GC. LC allows the analysis of a wide range of compounds and significantly increases the potential for the analysis of transformation products and metabolites, which are usually more hydrophilic than the parent compounds, without the need of derivatization. Thus, LC coupled to tandem mass spectrometry (LC–MS/MS) is the technique of choice for a multiclass UV F determination in environmental samples. Gago-Ferrero et al. have recently reviewed the LC–MS/MS methods published up to date for the determination of UV F in the environment [35]. So far there are only two approaches for the LC–MS/MS determination of UV F in biota. In the first one, Meinerling and Daniels developed a method for the analysis of four UV F in the muscle of rainbow trout. Soxhlet extraction was used followed by gel permeation chromatography (GPC) and by a clean-up step with a Florisil column [31]. In the second one, a method for the simultaneous determination of nine UV F was reported by Zenker et al. [32]. Mid-polar and lipophilic UV F were extracted by solvent extraction and further purified by reversed phase chromatography (RP-HPLC). The fraction containing mid-polarity UV F was analysed by HPLC–MS, whereas the fraction containing the lipophilic ones was determined by GC–MS.

The present study aims to develop and validate a robust, simple, fast, environmentally friendly, sensitive and selective analytical method based on HPLC–MS/MS for the quantitative determination of eight UV F in fish. Pressurized liquid extraction (PLE) was chosen as the extraction method because it is an automated technique, highly reproducible, and of low solvent and time consumption, especially compared with other conventional methods such as Soxhlet or ultrasound extraction.

Analyte identification and confirmation were performed using a hybrid quadrupole-linear ion trap-mass spectrometer (HPLC–QqLIT-MS/MS). The new method was applied to the determination of UV F in fish samples collected along the Guadalquivir river basin (Spain), constituting the first study on UV F bioaccumulation in fish from Iberian rivers.

2. Materials and methods

2.1. Standards and reagents

The most commonly used UV F were selected for the study covering a wide range of physicochemical properties. Table 1 shows their structures, CAS numbers and other properties.

Benzophenone-3 (BP3), octocrylene (OC), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA), 2,4-dihydroxybenzophenone (BP1), 4-hydroxybenzophenone (4HB), 4,4′-dihydroxybenzophenone (4DHB) and the isotopically labelled compound benzophenone-C_{13} (BP-C_{13}, were of the highest purity (>99%) and were obtained from Sigma–Aldrich (Steinheim, Germany); 4-methylbenzylidenecamphor (4MBC, 99% purity) was supplied by Dr Ehrenstorfer (Augsburg, Germany); and EHMC (98%) by Merck (Darmstadt, Germany). The isotopically labelled compounds 2-hydroxy-4-methoxy-2′,3′,4′,5′,6′-d_5 (BP3-d_5) and 3-(4-methylbenzylidene-d_4)camphor(4MBC-d_4, used as internal standards (>99%), were obtained from CDN isotopes (Quebec, Canada). Methanol (MeOH), acetone, dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (AcEt) and HPLC grade water (Lichrosolv), as well as formic acid (98% purity), aluminium oxide and Florisil were provided by Merck. N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Pressurized liquid extraction cellulose filters used were obtained from Dionex Corporation (Sunnyvale, CA, USA). Isolute C18 (500 mg, 3 mL) and Isolute aluminium oxide AL-N (500 mg, 6 mL) cartridges used for solid phase extraction (SPE) were obtained from Biotage (Uppsale, Sweden). Cartridges Oasis HLB (200 mg, 3 mL) were obtained from Waters Corporation (Milford, MA, USA), and basic alumina cartridges (5 g, 25 mL) were obtained from Interchim (Montluçon Cedex, France).

Individual stock standard solutions as well as the isotopically labelled internal stock standard solution were prepared on a weight basis in MeOH at 200 mg/L. The solutions were stored in the dark at −20 °C. A mixture standard solution at 20 mg/L in MeOH of each compound was prepared weekly. Working solutions were prepared daily by appropriate dilution of the mixture stock standard solution in MeOH.

2.2. Sample collection

Fish samples analysed in this study were collected along the Guadalquivir river basin (south of Spain) in 2010. Fish of the species *Luciobarbus sclateri* and *Cyprinus carpio* were captured at the selected sampling points. Fish were sampled by DC electric pulse. Next, the fish were killed, frozen, thawed, homogenized and lyophilized. The samples were made up from a pool of each fish species from each sampling point. The lyophilized samples were stored in sealed containers at −20 °C until analysis.

2.3. Sample preparation

Aliquots of the sample (1 g) were placed in different beakers, completely covered with acetone and spiked with 50 ng of the surrogate standard solution of BP-C_{13}. The acetone was left to evaporate at room temperature until the fish samples were dry.

Background contamination is a common problem in the determination of UV filters at environmental levels. Therefore, several measures were taken in order to prevent this problem. All glassware used was previously washed and heated overnight at 380 °C, and further sequentially rinsed with a collection of organic solvents and HPLC grade water, and immediately used. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware were used. Since many of the compounds analysed undergo photo-degradation stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

Lipid content in fish was determined by PLE following the method developed by Spiric et al. [36].

Table 1
UV F abbreviations, structures and other relevant data.

Abbreviation	Name (INCI nomenclature)[a]	CAS no.	Structure and molecular weight (g/mol)	Log K_{ow}
BP3	Benzophenone-3	131-57-7	228.24	3.79[b]
BP1	Benzophenone-1	131-56-6	214.22	3.15[c]
4HB	4-Hydroxybenzophenone	1137-42-4	198.2	2.92[c]
4DHB	4,4′-Dihydroxy benzophenone	611-99-4	214.22	2.19[b]
4MBC	4-Methylbenzylidene camphor	36861-47-9	254.37	4.95[b]
EHMC	Ethylexyl methoxycinnamate	5466-77-3	290.4	5.8[b]
OC	Octocrylene	6197-30-4	361.5	6.88[b]
OD-PABA	2-Ethylhexyl 4-dimethylaminobenzoate	21245-02-3	277.4	5.412[c]

[a] INCI (International Nomenclature for Cosmetic Ingredient) elaborated by CTFA and Cosmetics Europe (former COLIPA).
[b] Experimental values from database of physicochemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm.
[c] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1999–2011 ACD/Labs).

2.3.1. Extraction and clean up

Fish samples were extracted using an ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA) in 11 mL stainless steel cells. Solvent composition, adsorbent material, extraction temperature, extraction time and number of cycles of the PLE procedure were optimized. The experiments were conducted as follows: one cellulose filter followed by 1 g of Florisil (previously heated at 130 °C, for 24 h) was placed at the bottom of the cells. Aliquots of 1 g of freeze dried fish spiked with 50 ng of the surrogate standard BP-C_{13} were mixed in the extraction cells with Florisil in order to perform in-cell purification. Extraction was implemented in 2 cycles of 5 min of static time at 100 °C and 1500 psi using AcEt/DCM (1:1, v/v) as extracting solvent.

The PLE extract obtained (~25 mL) was diluted to 200 mL with HPLC water (MeOH < 5%), and further purified by solid phase extraction (SPE) using Isolute C18 (500 mg, 3 mL) cartridges, from Biotage. The cartridges were conditioned with 5 mL of AcEt/DCM (1:1, v/v) followed by 5 ml of MeOH and 5 mL of HPLC water at neutral pH. Then the PLE diluted extract was loaded onto the cartridges using a Baker vacuum system (J.T. Baker, The Netherlands). Finally, the compounds were eluted sequentially with 7 mL of AcEt/DCM (1:1, v/v) and 2 mL of DCM at 1 mL/min flow rate. Finally, the SPE extracts

were evaporated under a gentle nitrogen stream and reconstituted with 1 mL of ACN containing the isotopically labelled internal standards at a concentration of 20 ng/mL. Analysis was carried out by triplicate.

2.4. LC–MS/MS analysis

Analyses were performed by liquid chromatography–tandem mass spectrometry using a 4000 Q TRAP™ MS/MS system from Applied Biosystems–Sciex (Foster City, CA, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) from Merck, preceded by a guard column of the same packaging material. In the optimized method, the mobile phase consisted of a mixture of HPLC grade water and ACN, both with 0.15% formic acid. The adopted elution gradient started with 5% of ACN, increasing to 75% in 7 min, and then to 100% in the following 3 min. Pure organic conditions were kept constant for 2 min and finally initial conditions were reached in the next 2 min. The total run time for each injection was 20 min, the injection volume was set to 20 μL and the mobile phase flow-rate to 0.3 mL/min.

MS/MS detection was performed in positive (PI) electrospray (ESI) ionization mode under selected reaction monitoring (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M+H]^+$ were monitored per analyte to enhance method sensitivity and selectivity. The most abundant transition was used for quantification, while the second most abundant was used for confirmation. Fragmentation voltage and collision energy were optimized for each transition. The optimized values were selected as a compromise using the optimum values for the majority of the analytes. This procedure was in compliance with the European Council Directive 2002/657/EC, that although it was initially conceived for food residue analysis, it has been accepted by the scientific community for environmental analysis. Chromatographic retention times (t_R), SRM transitions, cone voltages, collision energies and the proposed product ions for the transitions are shown in Table 2. The mass spectrometer was controlled by Analyst 1.4.2 software from Applied Biosystems/MDS Sciex and the Symbiosis from the Symbiosis Pico for Analyst software.

2.5. Validation

The developed method was evaluated under the optimized conditions in terms of linearity range, sensitivity, accuracy, repeatability, reproducibility and matrix effects.

Blank tests were carried out to rule out possible contamination from the sampling, storage or instrumentation. In order to comply with internal quality control procedures, two control spiked samples, two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis was in a state of statistical control.

3. Results and discussion

3.1. Optimization of LC–MS/MS

According to the literature, for the efficient separation of UV F organic solvents such as MeOH and ACN with buffers and other organic modifiers are commonly used. In the current study, several combinations were tested using MeOH and ACN as organic phase and different concentrations of formic acid as organic modifier to determine the mobile phase that offered short retention time and sufficient resolution with little if any signal suppression. The best results were obtained using water and ACN, both with 0.15% formic acid. The formic acid significantly improved the peak shape of BP3 and its derivatives, whereas the other compounds showed equal or slightly better performance.

MS/MS operational parameters were optimized by using UV F individual standard solutions at 0.5 mg/L. ESI conditions were obtained as a compromise using the optimum values for most compounds. For all target UV F, ESI operating under positive conditions showed the best performance. Optimum conditions were: capillary voltage, 5000 V; source temperature, 700 C; curtain gas, 30 psi; ion source gas 1, 50 psi, ion source gas 2, 60 psi; entrance potential 10 V. Cone voltage was optimized for each compound in order to obtain maximum response for the protonated molecular ion $[M+H]^+$ and to prevent in-source fragmentation. Data acquisition was performed in SRM mode, and different collision energies were tested to obtain the optimum response. Two transitions per compound (including internal standards), for quantification and confirmation, were selected.

Table 2 summarizes the optimized values of MS/MS parameters for the target compounds and the proposed product ions. For BP3 and its derivatives the loss of 77 amu occurs, which corresponds to the $[M+H]^+ \rightarrow [M-C_6H_5]^+$, transition. We observed also another fragment corresponding to the complementary fragment of the molecule $[C_6H_5C{=}O]^+$. In the case of 4DHB, with one symmetric hydroxyl group on each side of the ketone, besides the loss of 77 amu, we considered the transition 215 → 93, which corresponds to $[C_6H_4OH]^+$.

Other compounds, including EHMC, OC and OD-PABA contain a relatively long chain branched alkyl group at the ester group and hence their collision induced dissociation mainly occurs through a McLafferty rearrangement, where the corresponding alkenes are lost, leaving the charge back at the ester group. Subsequently, the formed cation continues losing other fragments mainly H_2O [15].

3.2. Optimization of the extraction

3.2.1. PLE extraction

The optimization of the PLE conditions was performed by analysing fish samples spiked at 100 ng/g dw. Different blanks of fish were analysed by LC–MS/MS under the initial conditions and the chromatograms showed some peaks of target analytes at the same retention time. In each experiment a blank and three spiked samples were analysed and the signal of the blank was subtracted.

Fish is a complex matrix which can contain high percentages of fats that may hinder the analysis leading to strong matrix effects. Considering these facts and previous works conducted with UV F in complex environmental matrices [8,10,23], a simultaneous in-cell clean-up step was incorporated by including adsorbents other than diatomaceous earths together with the sample in the extraction cell. Aluminium oxide and Florisil were tested in order to obtain cleaner extracts to facilitate further analysis. The combination of solvent, temperature, number and time of extraction cycles were investigated in order to determine optimum extraction conditions for the target analytes.

The initial conditions were selected from our previous study [10,23], and were as follows: temperature of 100 °C, pressure of 1500 psi, 5 min of static extraction time, two cycles, 90 s of purge time, 30% of flush volume and 1 g of lyophilized fish sample. Extraction pressure was set up to 1500 psi for all PLE experiments since no significant impact on the extraction efficiency was expected [37].

The use of Florisil showed better results than aluminium oxide in terms of extraction efficiency, extract cleaning and chromatographic peak shape under all the tested conditions.

Since UV F constitutes a family of compounds with a wide range of physicochemical properties it was necessary to reach a compromise which provided good recovery rates for most compounds. The tested solvents and mixture solvents were DCM/AcEt (1/1, v/v),

Table 2
SMR experimental conditions used in the HPLC–MS/MS determination of UV F and proposed products ions.

Compound	Retention time (min)	SRM transition[a]	Cone (V)	Collision energy (eV)	Proposed ion
4DHB	5.65	215 → 121 215 → 93	45	27 45	$[M-C_6H_5OH]^+$ $[C_6H_4OH]^+$
4HB	7.04	199 → 121 199 → 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
BP1	7.63	215 → 137 215 → 105	40	27 29	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
BP3	9.27	229 → 151 229 → 105	40	25 27	$[M-C_6H_5]^+$ $[C_6H_5{=}O]^+$
4MBC	10.92	255 → 212 212 → 105	61	29 41	$[M+H-C_3H_7]^{\bullet+}$ $[MeC_6H_4CH_2]^+$
OC	11.56	362 → 250 362 → 232	71	15 27	$[M+H-C_8H_{16}]^+$ $[M+H-C_8H_{16}-H_2O]^+$
EHMC	11.81	291 → 161 291 → 179	51	25 13	$[M+H-C_8H_{16}-H_2O]^+$ $[M+H-C_8H_{16}]^+$
OD-PABA	12.00	278 → 166 278 → 151	86	27 43	$[M+H-C_8H_{16}-H_2O]^+$ $[i\text{-}BuC_6H_4C{=}O]^+$

[a] All compounds were determined in positive electrospray mode. Precursor ions correspond in all cases to $[M+H]^+$.

AcEt, AcEt/MeOH (1/1, v/v), MeOH and MeOH/H_2O (1/1, v/v). Fig. 1 shows the recovery rates obtained for each compound as a function of the extracting solvent. H_2O was not suitable to extract the most lipophilic compounds, whereas the use of MeOH resulted in poorly clean extracts. Best conditions were observed using the organic mixture DCM/AcEt (1/1, v/v) which allowed good recovery rates for all the target compounds.

Temperature is a very important parameter in PLE extraction. Application of higher temperature in PLE decreases the viscosity of solvents, thus allowing their better penetration into the sample matrix [38]. Temperatures over 100 °C were discarded since a big increase in matrix effects and interferences were previously observed (data not shown). The recoveries obtained at low temperatures were lower for most of the analysed compounds, thus 100 °C was selected as the optimum temperature for the extraction. Each static cycle introduce fresh solvent, which is very useful for complex matrix as fish, whereas the longer the time of a cycle the better the diffusion of analytes into the extraction solvent. For a more exhaustive extraction process it is recommended to divide the extraction into more than one cycle [39]. To check this, the extracts of individual cycles of 5 min were collected as well as the extracts from 2 and 3 cycles. Results showed that two cycles were sufficient to satisfactorily extract the compounds.

Despite the Florisil in-cell purification, the direct injection of the PLE extracts leads to high matrix effects and interferences. LODs obtained were above 40 ng/g dw for most compounds, too

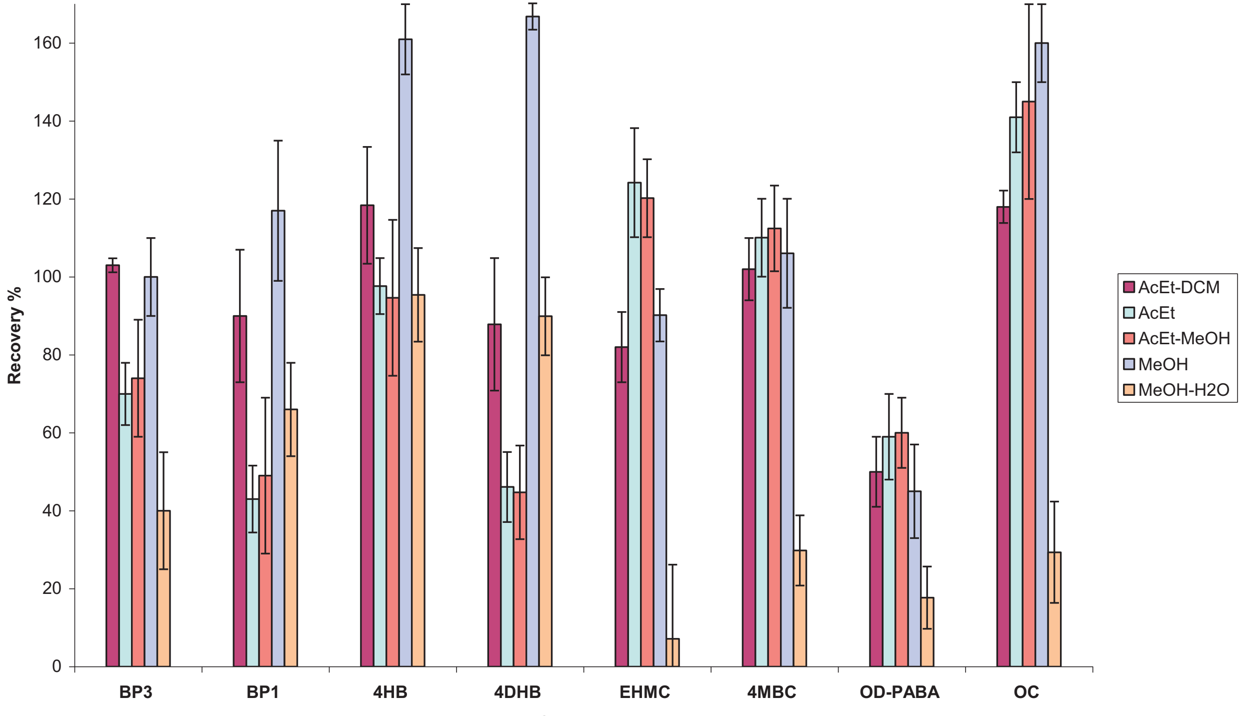

Fig. 1. The influence on the recovery efficiency of the different solvent combinations tested. AcEt, ethyl acetate; DCM, dichloromethane; MeOH, methanol; H_2O, water.

high for the determination of these compounds in fish samples. In consequence an additional clean-up step was found to be required.

3.2.2. SPE purification

The optimization of the SPE process was conducted by analysing the PLE extracts of fish samples spiked at 100 ng/g dw and diluted to 200 mL with HPLC grade water (MeOH < 5%). As in the optimization of the PLE conditions, in each experiment a blank and three spiked samples were analysed and the signal of the blank was further subtracted. The cartridge type and the sample extraction volume were optimized. Higher recovery rates and lower MLODs were the criteria applied in the optimization process. Fig. 2 compares the recovery rates achieved with the different cartridges tested. Polymeric cartridges Oasis HLB, silica bonded Isolute C18, alumina Isolute AL-N and a tandem combination of Isolute C18 and basic alumina Interchim ALB cartridges were the four approaches tested. SPE experiments were performed as described previously in Section 2.3.1. The tandem approach achieved good recoveries for the most lipophilic compounds, but could not retain BP3 and its transformation products. The alumina cartridge provided rather low recoveries for most compounds. The Oasis HLB cartridge achieved good results for the less lipophilic compounds, however for the most lipophilic ones were poor, in particular compared with the Isolute C18 cartridge. In conclusion, further experiments were carried out with Isolute C18 cartridges.

A breakthrough study was then carried out to determine the best sample volume. PLE extracts were diluted to 100, 200 and 500 mL with HPLC grade water. Differences among these volumes were not significant. Since low volumes may lead to clogging in the loading step when extracts are very dirty, 200 mL was selected as the optimum volume.

3.3. Validation

Instrumental analytical parameters, linearity ranges, correlation coefficients (r^2), instrumental limits of detection (ILOD) and quantification (ILOQ) and inter-day and intra-day precisions expressed as relative standard deviation (RSD) are summarized in Table 3. The calibration curves obtained for the SRM transitions were linear for all the compounds in a wide range of concentrations, typically from 0.5 to 500 ng/g with $r^2 > 0.9978$ for all compounds. ILODs and ILOQs were estimated for each compound. ILOD, defined as the lowest analyte concentration with a signal to noise ratio (S/N) of three, and ILOQ, defined as the concentration with S/N ratio of 10 and imprecision lower than 20% were evaluated by injecting 5 μL of diluted UV F solutions. ILODs ranged from 0.2 to 4 pg injected and ILOQs from 0.7 to 47 pg injected. The intra-day instrumental precision was determined by analysing seven replicates of a mixture standard solution at 20 μg/L within a given day. Inter-day instrumental precision was also estimated by analysing seven replicates of the same solution on seven different days. Good precision was obtained with RSD values in the range 3–5% (intra-day) and 5–8% (inter-day).

The identification and confirmation criteria for the analysis of the target compounds were based on the Commission Decision 2002/657/EC. Retention times of UV F in standards and in the samples were compared at a tolerance of 2.5% and the relationship between the two transitions was compared with the relative ion intensities of UV F standards. A difference of less than 20% was considered acceptable according to the EU directive.

MLODs (lowest analyte concentration with a signal to noise ratio (S/N) of three) and MLOQs (concentration with S/N ratio of 10 and imprecision lower than 20%) were evaluated by spiking fish samples, extracting and analysing several times. Low MLOD and MLOQ values, ranging from 0.1 to 6.0 ng/g dw and 0.3 to 20.0 ng/g dw, respectively were achieved as summarized in Table 4. MLODs were improved by almost one order of magnitude as a result of the addition of the SPE clean-up step. These MLOQs were below the reported concentrations in fish [35] and suitable to be used in routine screening and quantification of UV F in biota samples.

Since no certified reference materials were available for the analysis of UV F in fish, the accuracy assessment was performed with relatively uncontaminated fish. Seven fish samples were spiked with the target UV F and the surrogate standard at three levels (10 (or 20 when MLOQ > 10), 50 and 100 ng/g dw). The isotopically labelled internal standards BP3-d_5 and 4MBC-d_4 were added before the injection in order to compensate the instrumental variability and matrix effects. Different blanks of fish were processed by each tested procedure. The chromatograms showed some small peaks corresponding to the target analytes. In each experiment one blank and seven spiked samples were analysed and the signal of the blank was subtracted. Mean recovery rates were calculated for each analyte and for spike level. Table 4 shows that recovery rates were between 70% and 111% except for OD-PABA (36–42%) with RSD < 15%. The low extraction efficiency reported for OD-PABA can be the consequence of the high matrix effects observed at its retention time that could not be compensated by the internal standards. The surrogate standard should exceed 75% recovery to meet the quality criterion for an efficient extraction. Otherwise, the analysis was considered invalid and the sample was prepared and analysed again. Fig. 3 shows the extracted ion chromatograms for the studied UV F in a spiked fish sample at 50 ng/g dw.

The extent of matrix effects was estimated during analysis. They were determined by comparing the analytical response given by a standard in pure solvent and in fish extract at the different spike levels. Although the addition of a SPE clean-up step considerably decreased the matrix effects, their suppression values comprised between 15% and 65%. The use of the isotopically labelled internal standards compensated the matrix effects almost completely for all compounds except for OD-PABA, for which compensation was solely partial.

Considering the aforementioned, quantification was carried out following the internal standard calibration approach.

Finally, to ensure a good accuracy of the developed method, the samples analysed in Section 3.4 were further quantified by standard addition showing no significant differences in the obtained concentration values.

3.4. Application to real samples

To demonstrate the suitability of the developed method for the determination of UV F in biota, some fish samples from the Guadalquivir river basin were analysed. Table 5 summarizes the results of this study, showing solely the detected compounds. Three out of the eight target UV F: BP3, EHMC and OC, were present. The UV F detection frequency was about 80%. The highest levels (above 290 ng/g dw) were detected in fish of the species *L. sclateri*, endemic of the Iberian Peninsula. EHMC was the most ubiquitous and at the highest concentration. EHMC is extensively used in several personal care products and has shown estrogenic activity [3] and effects on the global gene expression in fish [4]. One factor to consider in the high levels found for this compound in *L. sclateri* fish, a predator species, is biomagnification. Fent et al. suggested that biomagnification occurs for EHMC in the aquatic environment [2]. In this study biomagnification is suggested in the predator/prey pairs cormorant and fish (barb, chub and brown trout) and between the omnivorous barb feeding on *Gammarus*. The herein reported values are in agreement with studies performed in other European river basins but constitute the first data on UV F bioconcentration in fish from Iberian rivers [2,13,31–33].

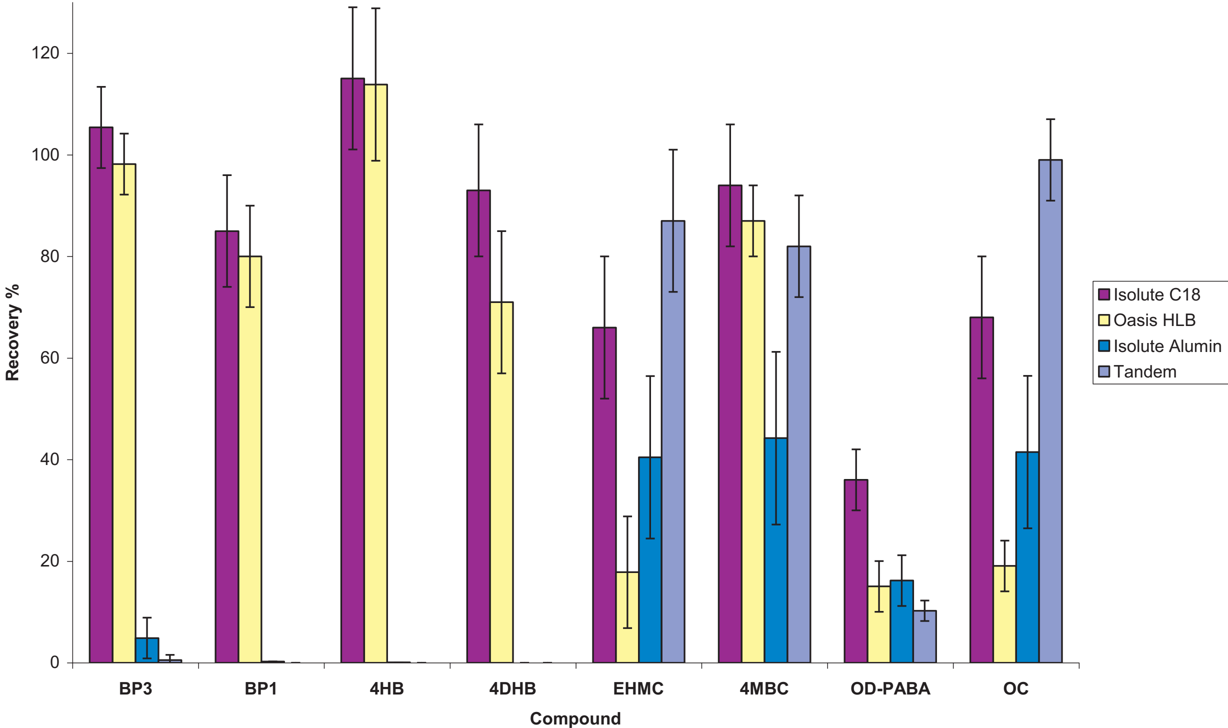

Fig. 2. The influence on the recovery efficiency of the different cartridges tested (Isolute C18, Oasis HLB, alumina Isolute AL-N and a tandem combination of Isolute C18 and basic alumina Interchim ALB).

Table 3
Instrumental quality parameters obtained for the LC–QqLIT-MS/MS method for the analysis of UV F in fish.

	Linearity range (μg/L)	r^2	ILOD (pg)	ILOQ (pg)	Precision[a] (%RSD) $n=7$	
					Intraday	Interday
BP3	0.5–500	0.9997	4	13	3	5
BP1	0.5–500	0.9999	10	33	3	6
4HB	0.5–500	0.9998	6	20	4	5
4DHB	0.5–500	0.9998	14	47	3	5
4MBC	0.5–500	0.9997	6	20	3	6
EHMC	0.5–500	0.9994	10	33	5	7
OC	2.5–500	0.9993	10	33	5	8
OD-PABA	0.1–100	0.9978	0.2	0.7	4	7

[a] Injections of 100 pg.

Table 4
Performance of the HPLC–ESI-MS/MS developed method for the analysis of UV filters in fish.

	Conc. (ng/g dw)	Rec. (%) ±RSD	Conc. (ng/g dw)	Rec. (%) ±RSD	Conc. (ng/g dw)	Rec. (%) ±RSD	MLOD (ng/g dw)	MLOQ (ng/g dw)
BP3	10	112 ± 14	50	106 ± 7	200	107 ± 6	1.2	4.0
BP1	20	91 ± 7	50	90 ± 7	200	92 ± 6	4.0	13.3
4HB	20	110 ± 12	50	112 ± 6	200	110 ± 5	6.0	20.0
4DHB	20	94 ± 10	50	92 ± 9	200	96 ± 8	5.0	16.7
4MBC	10	109 ± 10	50	99 ± 7	200	95 ± 5	0.7	2.3
EHMC	20	70 ± 10	50	72 ± 10	200	66 ± 7	5.0	16.7
OC	20	70 ± 11	50	80 ± 10	200	75 ± 9	6.0	20.0
OD-PABA	10	36 ± 12	50	40 ± 11	200	42 ± 11	0.1	0.3

Conc., concentration; Rec., recovery; RSD, relative standard deviation; MLOD, method limit of detection; MLOQ, method limit of quantification.

Table 5
Concentration of the detected UV F in fish (ng/g dw).

Sample	Common name	Scientific name	% lipid	BP3	EHMC	OC
F1	Andalusian Barbel	*Luciobarbus sclateri*	27	n.d.	n.d.	n.d.
F2			29	n.d.	19.0	<LOQ
F3			41	24.3	241.7	30.4
F4			34	16.5	63.0	n.d.
F5	Common Carp	*Cyprinus carpio*	9	11.2	<LOQ	n.d.

n.d., not detected.

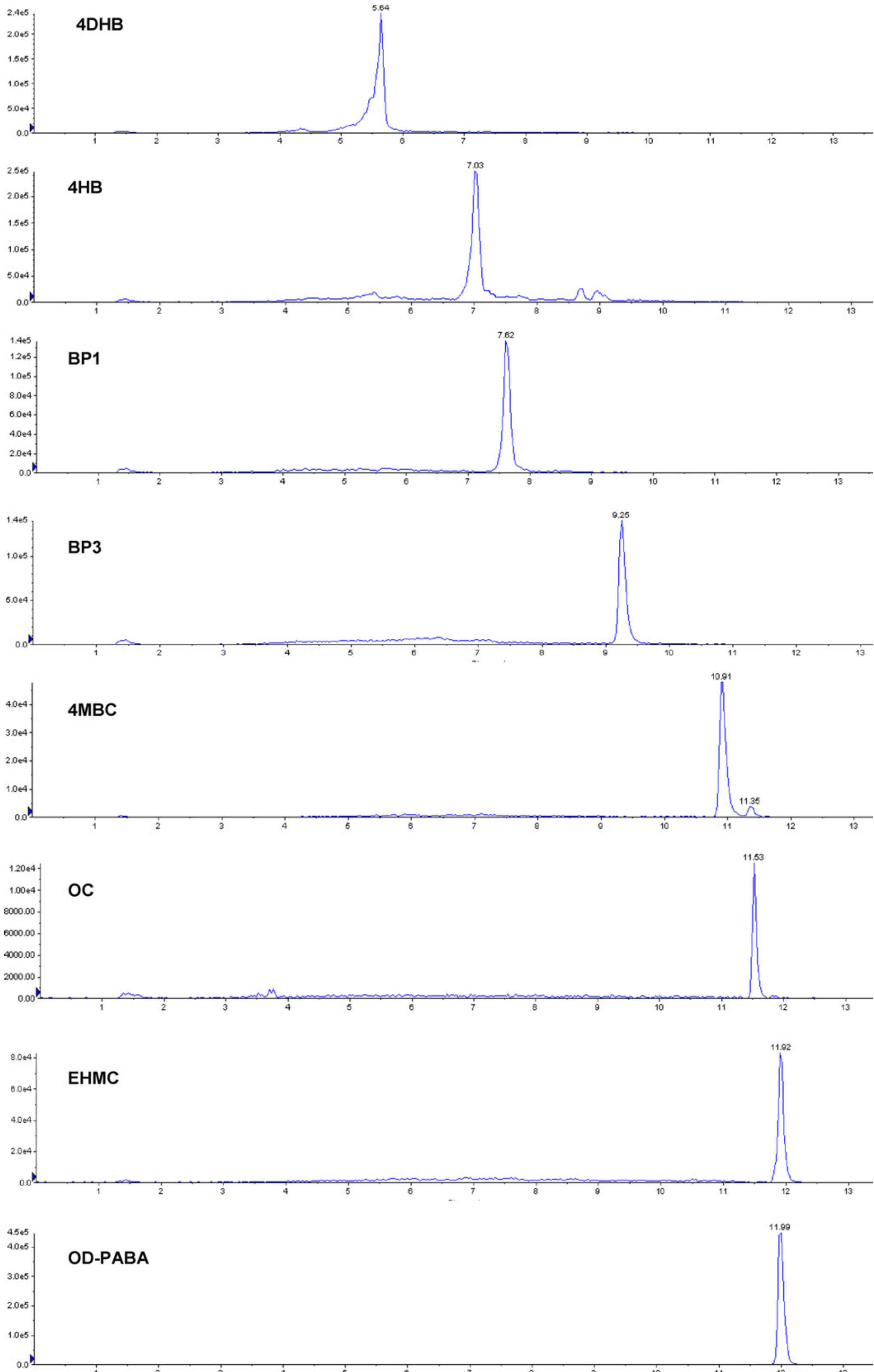

Fig. 3. Reconstructed ion chromatograms showing the SRM transitions for the studied UV F in a spiked fish sample at 50 ng/g dw.

4. Conclusions

This study involved the development and validation of a LC–MS/MS method for the simultaneous analysis of eight UV F compounds, including three BP3 transformation products, with a wide range of physicochemical properties, in fish. Especial efforts were devoted to optimize an efficient purification process together with an exhaustive extraction procedure. Results indicated that a two-step purification procedure, involving in-cell PLE and further SPE purifications, was required to obtain a clean fish extract.

The multi-residue method developed was efficient, with high sensitivity and accuracy allowing its use for monitoring the bioaccumulation potential of sunscreen agents in fish. In particular, the sample preparation step developed allowed a considerable reduction in time, solvents and personnel effort when analysing very complex sample matrix such as fish.

The performance of the proposed method was satisfactory in the determination of the target UV F in real fish samples from the Guadalquivir river basin (Spain). BP3, EHMC and OC were detected at high concentrations, up to 240 ng/g dw. These data constitute the first determination of UV F residues in fish from Iberian rivers.

The detection of these emerging pollutants also in fish from Spain evidences their widespread distribution in the environment, and therefore the need to carry out further studies in order to better understand their occurrence and fate. To carry out such research simple, fast and robust methodology with the appropriate sensitivity and selectivity as the one presented here is required.

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References

[1] F.P. Gasparro, Environ. Heath Perspect. 108 (2000) 71.
[2] K. Fent, A. Zenker, M. Rapp, Environ. Pollut. 158 (2010) 1817.
[3] P.Y. Kunz, K. Fent, Aquat. Toxicol. 79 (2006) 305.
[4] S. Zucchi, D.M. Oggier, K. Fent, Environ. Pollut. 159 (2011) 3086.
[5] A.M. Calafat, L.Y. Wong, X. Ye, J.A. Reidy, L.L. Needham, Environ. Health Perspect. 116 (2008) 893.
[6] T. Kunisue, Z. Chen, G.M. Buck-Louis, R. Sundaram, M.L. Hediger, L. Sun, K. Kannan, Environ. Sci. Technol. 46 (2012) 4624.
[7] C. Plagellat, T. Kupper, R. Furrer, L.F. De Alencastro, D. Grandjean, J. Tarradellas, Chemosphere 62 (2006) 915.
[8] A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 5619.
[9] R. Rodil, S. Schrader, M. Moeder, J. Chromatogr. A 1216 (2009) 8851.
[10] P. Gago-Ferrero, M.S. Díaz-Cruz, D. Barcelo, Chemosphere 84 (2011) 1158.
[11] Z. Zhang, N. Ren, Y.F. Li, T. Kunisue, D. Gao, K. Kannan, Environ. Sci. Technol. 45 (2011) 3909.
[12] T. Poiger, H.R. Buser, M.E. Balmer, P.A. Bergqvist, M.D. Müller, Chemosphere 55 (2004) 951.
[13] M.E. Balmer, H.R. Buser, M.D. Müller, T. Poiger, Environ. Sci. Technol. 39 (2005) 953.
[14] P. Cuderman, E. Health, Anal. Bioanal. Chem. 387 (2007) 1343.
[15] R. Rodil, J.B. Quintana, P. López-Mahía, S. Muniategui-Lorenzo, D. Prada-Rodríguez, Anal. Chem. 80 (2008) 1307.
[16] N. Negreira, I. Rodríguez, M. Ramil, E. Rubi, R. Cela, Anal. Chim. Acta 654 (2009) 162.
[17] E. Magi, M. Di Carro, C. Scapolla, K.T.N. Nguyen, Chromatographia 75 (2012) 973.
[18] K.H. Langford, K.V. Thomas, J. Environ. Monitor. 10 (2008) 894.
[19] I. Tarazona, A. Chisvert, Z. Leon, A. Salvador, J. Chromatogr. A 1217 (2010) 4771.
[20] A. Wick, G. Fink, T.A. Ternes, J. Chromatogr. A 1217 (2010) 2088.
[21] W. Li, Y. Ma, C. Guo, W. Hu, K. Liu, Y. Wang, T. Zhu, Water Res. 41 (2007) 3506.
[22] H.H. Jeon, Y. Chung, J.C. Ryu, J. Chromatogr. A 1131 (2006) 192.
[23] P. Gago-Ferrero, M.S. Díaz-Cruz, D. Barcelo, Anal. Bioanal. Chem. 400 (2011) 2195.
[24] R. Rodil, M. Moeder, Anal. Chim. Acta 612 (2008) 152.
[25] J. Hany, R. Nagel, Deut. Lebensm. Rundsch. 91 (1995) 341.
[26] M. Schlumpf, P. Schmid, S. Durrer, M. Conscience, K. Maerkel, M. Henseler, M. Gruetter, I. Herzog, S. Reolon, R. Ceccatelli, O. Faass, E. Stutz, W. Lichtensteiger, Toxicology 205 (2004) 113.
[27] Z. León, A. Chisvert, I. Tarazona, A. Salvador, Anal. Bioanal. Chem. 398 (2010) 831.
[28] F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, J.L. Vílchez, M.F. Fernández, N. Olea, Talanta 85 (2011) 1848.
[29] P. Gago-Ferrero, M.S. Diaz-Cruz, D. Barcelo, Anal. Methods 5 (2013) 355.
[30] M.A. Mottaleb, S. Usenko, J.G. O'Donell, A.J. Ramirez, B.W. Brooks, C.D. Chambliss, J. Chromatogr. A 1216 (2009) 815.
[31] M. Meinerling, M. Daniels, Anal. Bioanal. Chem. 386 (2006) 1465.
[32] A. Zenker, H. Schmutz, K. Fent, J. Chromatogr. A 1202 (2008) 64.
[33] M. Nagtegaal, T.A. Ternes, W. Baumann, R. Nagel, UWSF-Z. Umweltchem. Ökotoxikol. 9 (1997) 79.
[34] M. Bachelot, Z. Li, D. Munaron, P. Le Gall, C. Casellas, H. Fenet, E. Gomez, Sci. Total Environ. 420 (2012) 273.
[35] P. Gago-Ferrero, M.S. Díaz-Cruz, D. Barceló, Anal. Bioanal. Chem. 404 (2012) 2597.
[36] A. Spiric, D. Trbovic, J. Djinovic, R. Petronijevic, V. Matekalo-Sverak, Anal. Chim. Acta 672 (2010) 66.
[37] S.B. Hawthorne, Y. Yang, D.J. Miller, Anal. Chem. 66 (1994) 2912.
[38] A. Jelic, M. Petrovic, D. Barcelo, Talanta 80 (2009) 363.
[39] Technical note-DIONEX; http://www.dionex.com/en-us/webdocs/4736_TN208.pdf