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Influence of pre-treatment process on matrix effect for the determination of musk fragrances in fish and mussel

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ABSTRACT

Musk compounds are widely used as fragrances in personal care products. On account of their widespread use and their low biodegradation, they can be found in environmental samples. In our study two extraction methodologies were compared and different clean-up strategies were also studied in order to develop a reliable analytical method, with minimum matrix effect and good detection limits, to determine synthetic musk fragrances- six polycyclic musks, three nitro musks and the degradation product of one polycyclic musk- in fish and mussel samples. The first extraction technique involves a QuEChERS extraction, a consolidate extraction methodology in the field of food analysis of growing interest over recent years, followed by a dispersive solid-phase extraction (dSPE) as clean-up strategy. The second extraction technique consists of a conventional pressurised liquid extraction (PLE) with dichloromethane and an in-cell clean-up to decrease the matrix effect and remove the undesired components*present in PLE extracts. Large volume injection (LVI) followed by gas chromatography-ion trap-tandem mass spectrometry (GC-IT-MS/MS) was chosen as the separation and detection technique. Validation parameters, such as method detection limits and method quantification limits were found at ng g^{-1} levels for both fish and mussel matrices. Good levels of intra-day and inter-day repeatabilities were obtained analysing fish and mussel samples spiked at 50 ng g^{-1} (d.w.) ($n=5$, RSDs $< 17\%$). The developed PLE/GC-IT-MS/MS method was successfully applied to determine the target musk fragrances present in fish and mussel samples from the local market in Tarragona and fish samples from the Ebro River. The results showed the presence of galaxolide (2.97–18.04 ng g^{-1} (d.w.)) and tonalide (1.17–8.42 ng g^{-1} (d.w.)) in all the samples analysed, while the remaining polycyclic musks such as cashmeran, celestolide and phantolide, were only detected in some of the fish samples analysed. None of the samples analysed contained detectable traces of the nitro musks studied.

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1. Introduction

Musk compounds are a family of cyclic personal care products (PCPs), that include polycyclic musks, nitro musks and macrocyclic musks, widely used as fragrances in consumer products such as cosmetics, toiletries, detergents, soaps, body oils, toothpaste and also as flavours in foods and drinks: in short, they are used in a broad range of everyday products. They belong to the so-called emerging organic compounds (EOCs), which have been of increasing interest, to scientists in recent years [1–8].

Discussions on the toxicology of nitro musks soon arose because of the presence of a nitro aromatic compound in their structure. In this respect, the European Directive 98/62/EEC [9] relating to cosmetic products prohibits the use of musk ambrette, musk moskene and tibetene in cosmetics and limits musk xylene and musk ketone content. Furthermore, nitro musks can be transformed in wastewater treatment plants (WWTPs) - as well as in biota - into amino metabolites [10], and these transformation products can be even more problematic than the parent compounds [11,12]. This has led to a significant decrease in their use, while polycyclic musk production has increased significantly. Polycyclic musks are the musk fragrances that dominate the global market today, and two of them, galaxolide and tonalide, have been included on the EPA's high production list [13]. The use of tonalide in the cosmetic industry has in fact been regulated through European directive 2008/42/EC [14]. Macrocyclic musks, which smell more intensive than polycyclic musk and so less mass is needed to achieve the same performance in perfumery, are not as widely used as polycyclic musks because of the cost of their synthesis. Nevertheless, they are becoming more generally available because of advances made in synthesis methods over the last few years [2,15,16]. It is expected that over the next few years the

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decrease in the price of synthesising macrocyclic musks and their environmentally friendly properties will mean that they will replace polycyclic musk in the market.

On account of their widespread use, musk compounds can be considered ubiquitous throughout the world, and due to their lipophilic characteristics and slow biodegradation, they can be found in surface water [17–19], sewage [20,21], sediments [22] and fish species living in contaminated rivers and estuaries [23–25].

A wide range of analytical methods have been developed to determine musk fragrances in fish tissue. These methods have used a varied assortment of extraction techniques (Soxhlet, microwave assisted extraction (MAE), focused ultrasound-solid liquid extraction (FUSLE), and pressurised liquid extraction (PLE) usually followed by a clean-up step (silica gel, florisil and/or gel permeation chromatography (GPC)) prior to analysis with GC–MS or GC–MS/MS [23,26–29]. In this article, a new extraction methodology of growing interest in the field of food analysis over recent years [30–32] – QuEChERS (quick, easy, cheap, effective, rugged and safe) – was tested and compared in terms of validation parameters with PLE. Special effort was on the reduction of matrix effect.

The QuEChERS methodology was first developed by Anastassiades et al. [33] for the extraction of pesticides from food matrices and involves two basic steps. At first QuEChERS methods use a single step buffered acetonitrile extraction and simultaneously salt out water from the aqueous sample using anhydrous magnesium sulphate to induce liquid-liquid partitioning. Subsequently, a clean-up step using a dispersive solid-phase extraction (dSPE) is often conducted to clean up the mixture, removing any undesired sample components. The main advantages of this extraction methodology are its speed, ease of implementation (instrumentation is not required), minimal solvent requirement and low cost when compared with instrumental extraction techniques.

The aim of this investigation was therefore to develop a rapid, sensitive and accurate analytical method based on GC-IT-MS/MS for determining ten synthetic musk fragrances in fish and mussels. PLE or QuEChERS as extraction procedures were compared and different clean-up strategies as in-cell clean-up sorbent for PLE or dSPE for QuEChERS were assayed to minimise the matrix effect. To the best of our knowledge, this is the first time that QuEChERS has been used to extract musk fragrances present in fish samples.

2. Experimental part

2.1. Reagents and standards

The six polycyclic musks studied were supplied by Promochem Iberia (Barcelona, Spain) and were the following: 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5*H*)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-*tert*-butyllindane (ADBI, celestolide), 6-acetyl-1,1,2,3,3,5-hexamethylindane (AHMI, phantolide), 5-acetyl- 1,1,2, 6-tetramethyl-3-isopropylindane (ATII, traseolide), 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-*(g)*-2-benzopyran (HHCB, galaxolide) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN, tonalide). The nitro musk fragrances 2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX, musk xylene) and 1,1,3,3,5-pentamethyl-4,6-dinitroindane (MM, musk moskene) were purchased as 100 μg mL^{-1} individual solutions in acetonitrile from Sigma–Aldrich (Steinheim, Germany) and Riedel de Haën (Seelze, Germany), respectively. The standard 4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (MK, musk ketone) was provided by Fluka (Buchs, Switzerland). International Flavors & Fragances Inc. (Barcelona, Spain) supplied 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-*[g]*-2-benzopyran-1-one (HHCB-lactone, galaxolidone) while the internal standard ^{2}H15-musk xylene (^{2}H15-MX) came as a 100 μg mL^{-1} solution in acetone from Symta (Madrid, Spain). Suppl. Table 1 shows the main characteristics (formula name, molecular structure, CAS number, molar mass and boiling point) of the target compounds [1,34,35].

Individual standard solutions of the synthetic musk fragrances were prepared in acetone at concentrations of 4000 μg mL^{-1} for polycyclic musks and 1000 μg mL^{-1} for musk ketone and HHCB-lactone. A working mixture solution of 100 μg mL^{-1} was prepared in ethyl acetate except for MX, MM and ^{2}H15-MX which were supplied directly at a concentration of 100 μg mL^{-1} in acetonitrile and used as received. Acetone and ethyl acetate were GC grade with purity > 99.9% from Prolabo (VWR, Llinars del Vallès, Barcelona, Spain).

The extraction solvents dichloromethane, methanol, and hexane were GC grade (of > 99.9% purity) from Prolabo, while acetonitrile was HPLC grade from Prolabo. Ultrapure water was obtained using an ultrapure water purification system from Veolia Water (Sant Cugat del Vallés, Barcelona, Spain). Helium gas with a purity of 99.999% was used for the chromatographic analysis (Carburos Metálicos, Tarragona, Spain).

2.2. Sampling and sample pre-treatment

Red mulltet (*Mullus surmuletus*) and mussels (*Mytilus galloprovincialis)* were selected for method development, optimisation and validation. The method was successfully applied to determine musk fragrances in gilt head bream (*Sparus aurata*), turbot (*Psetta maxima*), red mullet (*Mullus surmuletus*) and mussels (*Mytilus galloprovincialis*), which had been purchased locally (Tarragona market) and mostly caught or collected in the Mediterranean Sea between May and December 2013. Perch (*Perca fluviatilis*), sheatfish (*Silurus glanis*) and carp (*Cyprinus carpio*) samples, which had been caught in the wild (between May and November 2013) and collected from the Ebro River, were also analysed.

After collection, the samples were immediately preserved in a refrigerated box. Lateral fillets were then dissected from the fish, homogenised and stored in a freezer until analysis. Frozen samples were lyophilised using the freeze-drying system (Labconco, Kansas City, MO, USA), crushed using a mortar and pestle and sieved through a 125 μm screen to homogenise the diameter of the particles.

2.3. Sample extraction

2.3.1. Quick, easy, cheap, effective, rugged and safe (QuEChERS)

A total of 0.5 g (d.w.) of freeze-dried sample was weighed into 50 mL centrifuge tubes from Scharlab (Barcelona, Spain), 10 mL of ultrapure water was added to the tube, and the tube was shaken vigorously for 1 min. Then, 10 mL of acetonitrile was added, followed by an extraction salt packet (Scharlab) for the European Committee for Standardization (CEN) extraction method [36], which contains 4 g of magnesium sulphate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate. The mixture was then vortexed (3 min) and centrifuged for 5 min at 7000 rpm (Hettich Universal 32 R, Tuttlingen, Germany). The supernatant (acetonitrile layer) was removed and transferred to a 15 mL centrifuge tube containing 1 g of florisil (Sigma-Aldrich) for the dSPE clean-up. The tube was vortexed for 3 min and centrifuged again at 7000 rpm for 5 min and the supernatant was evaporated under a gentle stream of nitrogen to a final volume of ≈ 1 mL. The internal standard (IS, 50 ng g^{-1}) was added to the extract before it was reconstituted to 2 mL with ethyl acetate. Extracts were filtered with a 0.22 μm PTFE syringe filter and analysed by GC-IT-MS/MS.

2.3.2. Pressurised liquid extraction (PLE)

Extraction of fish and mussel samples was carried out using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA; USA). Stainless steel extraction cells and glass collecting vials of 11 mL and 20 mL volume respectively were used. A cellulose filter was placed at the bottom of the 11 mL stainless steel extraction cell. It was then filled with 1 g of florisil (in-cell clean-up sorbent) previously conditioned at 400 °C overnight, 0.5 g (d.w.) of freeze-dried sample mixed with 1 g of diatomaceous earth (conditioned at 400 °C for 8 h), and 1 g of diatomaceous earth. This was finally compacted and closed before extraction. The extraction was carried out with one cycle of dichloromethane at 60 °C and 1500 psi for 5 min. The preheating time was 5 min, flush volume was 100% of cell volume and purge time was 90 s. The sample extract was evaporated with a rotary evaporator (R-114, Büchi, Switzerland) set at 30 °C, the IS (50 ng g^{-1}) was added to the residue (≈ 1 mL) before it was reconstituted to 2 mL with ethyl acetate and filtered with a 0.22 μm PTFE syringe filter, and finally analysed by GC-IT-MS/MS system.

2.4. Gas chromatography-ion trap-tandem mass spectrometry

The GC–IT–MS/MS analyses were performed using a Varian ion trap GC–MS system (Varian, Walnut Creek, CA, USA), equipped with a 3800 gas chromatograph, a 4000 ion trap mass detector, a 1079 programmable vaporising temperature injector and a CombiPal autosampler (CTC Analytics, Zwigen, Switzerland). The mass spectrometer was operated in the electron ionisation (EI) mode (70 eV) and the system was controlled by Varian MS Workstation v.6.9 software. A fused silica capillary column (3 m × 0.25 mm i.d.) from Micron Phenomenex (Torrance, California, USA) was used as a guard column. The chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl/dimethylpolysiloxane, 30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Micron Phenomenex. The oven temperature was programmed as follows: 70 °C hold for 3.5 min, raised at 50 °C min^{-1} to 200 °C, then 5 °C min^{-1} to 240 °C and finally 20 °C min^{-1} to 290 °C (hold 3.4 min). The carrier gas employed was helium with a purity of 99.999% at a constant column flow of 1 mL min^{-1}. During the injection of the 10 μL, the 1079 injector operated in large volume injection (LVI) mode and a 2 mm i.d. insert liner packed with glass wool (Varian) was used. During injection in split mode at a rate of 50 mL min^{-1} the 1079 injector temperature was set at 70 °C. The ethyl acetate was purged out with a vent flow of 100 mL min^{-1} within 0.5 min (vent time). The splitless mode was then programmed for 2.5 min while the temperature was increased at 100 °C min^{-1} to 300 °C for 5 min. Transfer line, manifold and trap temperatures were 280 °C, 50 °C and 200 °C respectively. For quantitative analysis of the target compounds, the tandem mass spectrometry (MS/MS) mode was applied. Retention times as well as optimal MS parameters of the target compounds are summarised at Suppl. Table 2.

3. Results and discussion

3.1. Large volume injection GC-IT-MS/MS optimisation

A mixed solution of 10 μg mL^{-1} of the target musk fragrances and 1 μg mL^{-1} of ^{2}H15-MX as IS was prepared in ethyl acetate and 10 μL of this solution was directly injected into the GC–IT-MS, using electron impact ionisation in full scan mode. All the compounds were identified by their molecular ion and afterwards the chromatographic separation was optimised by testing several oven temperature programs. All compounds were separated in just 16 min using the chromatographic conditions described in Section 2.4. In order to achieve maximum sensitivity/selectivity of the compounds, the MS/MS method was carried out by selecting appropriate precursor/product ions and IT–MS/MS parameters based on a previous paper [37]. In Suppl. Table 2 are also summarised, the parent ion, optimum amplitude excitation voltage, CID storage level, product ions (quantifiers and qualifiers), the m/z range of ions analysed by EI-MS/MS and scan time of each target compound. Each compound was acquired separately in one segment, except HHCB and AHTN and ^{2}H15-MX, MX and MM; because of this, the scan time of these compounds was shorter than the others.

3.2. QuEChERS optimisation

QuEChERS extraction involves two extraction steps, the first of which, a salting-out liquid-liquid extraction to extract the analytes of interest from the matrix while the second, a dSPE for the clean-up of the sample. To achieve efficient extraction of the target compounds from a fish or mussel sample using the QuEChERS system, certain variables such as the salts were adopted from the original method (AOAC) [33]. Other significant parameters that affect extraction and clean-up performance, i.e. the ratio between the sample mass and the volume of solvent, type of extraction solvent and dSPE sorbents, were optimised.

Lyophilised fish (red mullet) and mussel samples were spiked at a concentration of 1 μg g^{-1} (d.w.) for each compound to ensure that peak areas of the analytes present in the samples (< 10% of peak areas from spiked samples) do not affect the accurate quantification of analytes during the optimisation of the QuEChERS variables. To calculate the QuEChERS recoveries, internal standard calibration curves were constructed by using fish and mussel samples spiked after the extraction. Then, samples spiked previously to the extraction were analysed and calculated concentrations by those calibration curves and theoretical concentrations were compared. Thus, recoveries do not take into account the differences caused by the matrix effect, only the extraction yield [38].

The influence of the sample mass/solvent volume ratio was studied by mixing different sample amounts (0.25, 0.50, 0.75 and 1.00 g (d.w.)) with 10 mL of ultrapure water followed by the addition of 10 mL of acetonitrile and the QuEChERS extraction salts. The other parameters are described in Section 2.3.1. The best QuEChERS recoveries (between 54–97% for fish samples and between 47–85% for mussel samples) were obtained working with 0.50 g (d.w.) of sample amount. Some agglomerates were formed for higher sample amounts, indicating that the amount of $MgSO_4$ used was not enough to remove all the water. This situation negatively affected the extraction method, obtaining QuEChERS recoveries 10% and 20% lower for fish and mussel samples, respectively. Furthermore, the use of large sample quantities can lead to a higher co-extraction of matrix interferents.

For the best extraction of musk fragrances and to guarantee a minimal co-extraction of matrix interferences, the selection of an appropriate solvent is a crucial step in this phase of the optimisation process. Four different solvents were tested: dichloromethane, acetonitrile, ethyl acetate and hexane. Acetonitrile is the common solvent used in QuEChERS methodology and the other solvents were chosen based on literature [25,39,40].

The acetonitrile showed the best extraction performance with QuEChERS recoveries between 54% and 97% for fish and 47% and 85% for mussels. No significant differences were obtained working with dichloromethane, ethyl acetate or hexane, with QuEChERS recoveries in any case lower than those obtained with acetonitrile. Therefore acetonitrile was chosen as the extraction solvent because it was the only one capable of fully dispersing the matrix and increasing the surface contact area between the sample and the extraction solvent, resulting in higher QuEChERS recoveries.

One of the major drawbacks in the analysis of biological samples is the high matrix effect observed, especially when MS is used, which involves ion suppression or enhancement of the signal. Consequently to achieve better quantification limits of the target analytes, a dSPE was tested to clean-up the sample. In this clean-up step, a commercially available dSPE tube containing primary and secondary amine exchange sorbent (PSA) and octadecyl-silica (C18) was used. The PSA sorbent is used to remove sugars, fatty acids, organic acids, lipids and certain pigments, while the C18 sorbent is used to remove long chain fatty acid compounds and other non-polar interferences [41]. Home-made dSPE tubes containing florisil (1 g), silica (1 g) and alumina (1 g) as clean-up sorbents were also tested due to their ability in the removal of lipids, oils and waste from PLE extracts [42,43].

The matrix effect (ME, %) was calculated with Eq. 1:

$$ME(\%) = \frac{(C_{sample} - C_{standard})}{C_{standard}} \times 100 \quad (1)$$

where C_{sample} is the concentration determined by spiking a fish or mussel extract after QuEChERS and using an internal standard calibration curve obtained by direct injection of the standards. $C_{standard}$ is the theoretical concentration. Moreover, apparent recoveries (R_{app}), which include QuEChERS recovery and matrix effect, were calculated by analysing a spiked fish or mussel sample and using the same calibration curve as before. Working without a clean-up step a high ME was observed for all of the target analytes with R_{app} between 6% and 90% independently of the kind of sample analysed. It is worth noting that ME values between were −95% and −85% for MM, MK and HHCB-lactone. However, when a dSPE was applied different behaviour of the target analytes was observed. For polycyclic musks, florisil was the best dSPE sorbent with ME between −28% and 16% and between −52% and 31% for fish and mussel samples, respectively and R_{app} ranging between 59 and 110% (see Fig. 1). Nitro musks and HHCB-lactone showed the highest R_{app} (36–66%) and lowest ME, between −58% and −15% for fish samples and −62% and −28% for mussel samples, working with a mixture of PSA and C_{18} sorbents as dSPE sorbent. Therefore florisil was chosen as the dSPE sorbent as a compromise.

To summarise, working with QuEChERS as extraction technique, optimum results for both fish and mussel samples were achieved when 10 mL of ultrapure water was mixed with 0.5 g (d.w.) of sample. Then 10 mL of acetonitrile followed by 4 g of magnesium sulphate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate were added. A dSPE with 1 g of florisil as a clean-up step was performed. Table 1 summarises the R_{app} and ME found under optimal conditions working with fish and mussel matrices.

3.3. PLE optimisation

To achieve efficient extraction of the target compounds from a fish or mussel sample using a PLE system, several operational parameters as extraction solvent, temperature, time and number of cycles must be optimised. In addition, an in-cell clean-up sorbent was tested in order to reduce ME. Other parameters such as pressure, flush volume and purge time can also be optimised, but it is well known that these parameters have no significant effect on extraction efficiency.

Lyophilised fish and mussel samples (0.5 g (d.w.)) spiked at 1 μg g^{-1} (d.w.) were mixed with 1 g (d. w.) of diatomaceous earth and they were placed into a stainless-steel cell. The initial experimental conditions were set according to previous literature [21,23]: 80 °C, 2 cycles, 5 min static time, 120 s of purge time, 1500 psi and 100% flush volume.

First the extraction solvent was optimised. Hot water, which was successfully applied by our group for the determination of nitrosamines and aliphatic amines in sewage sludge [44,45], was chosen as the initial extraction solvent instead of organic solvents because makes the extraction more environmentally friendly. However, the high lipid percentage of the mussels caused a solid mass to form inside the extraction cell that made it impossible to extract the target compounds present in mussels by PLE using water as the extraction solvent. When working with fish samples, PLE recoveries were below 30% for all the target compounds. Therefore it was decided to work with organic solvents previously used as extraction solvents to extract musk fragrances or other personal care products from sludge samples [21,46] and biota samples [23,25]. Of the organic solvents studied – methanol (polarity index=5.1), ethyl acetate (polarity index=4.4), dichloromethane (polarity index=3.1) and hexane (polarity index=0.0), as can be seen in Fig. 2 – dichloromethane was the most efficient solvent for extracting the target analytes from fish and mussels, with PLE recoveries between 57%–86% and 51%–91%, respectively. A nonpolar solvent (polarity ≤ dichloromethane) as hexane did not provide good PLE recovery values; below 50% for fish samples and up to 38% for mussel samples. Among relatively polar solvents (polarity ≥ dichloromethane), methanol was also capable of extracting musk fragrances from fish or mussels samples with PLE recoveries between 49%–82% for fish and 44%–90% for mussels), respectively. However, due to the lipophilic properties of musk fragrances, they can be retained in the fatty precipitates that appear in the PLE extracts. While, as can be seen in Fig. 2 the target compounds were not effectively extracted with ethyl acetate as extraction solvent with PLE recovery values lower than 48% and 31% for fish and mussels, respectively. As a compromise between analytes recoveries and co-extracted matrix components, dichloromethane was therefore been selected as extraction solvent.

The extraction temperature was then studied by comparing PLE recoveries obtained at 60 °C, 80 °C and 100 °C. The other extraction conditions were the same as described above. A temperature of 60 °C provided the best PLE recoveries for the entire target analytes (between 67 and 95% for fish and 64 and 101% for mussel) independently of the matrices analysed. PLE recoveries decreased when the temperature was increased to 80 °C and were below 40% for most of the compounds when the temperature was 100 °C. This is probably due to the presence of high amounts of fatty precipitates in the PLE extract increasing with the temperature, which makes a filtration step with a PTFE filters (0.45 μm) previous to filtration with PTFE filters of 0.20 μm before GC–MS mandatory.

Static time and number of cycles were also studied in order to enhance the efficiency of PLE extraction. Static times of 5, 10 and 15 min were studied. The best results were obtained working with 5 min. Static times of 10 and 15 min did not result in a significant increase in extraction efficiency. Regarding the number of cycles, 1, 2 and 3 cycles with a static extraction time of 5 min were tested. Two extraction cycles did not improve PLE recoveries significantly for the vast majority of the target compounds, and so any increase in the number of cycles was discarded. Therefore, one cycle and a static time of 5 min were chosen as the optimal parameters for PLE extraction.

Due to the optimisation of the PLE parameters, the presence of fatty precipitates in the PLE extract was considerably reduced until turbidity. However, in order to minimise the ME and avoid a clean-up step previous to GC-IT-MS/MS, an in-cell clean-up sorbent was tested. So 1 g of in-cell clean-up sorbent was placed on the cellulose filter at the bottom of the extraction cell to retain the interfering substances when the PLE was carried out under optimum conditions. Three sorbents were tested to work as in-cell clean-up sorbent – florisil, alumina and silica - all conditioned at 400 °C overnight. R_{app} as well as ME, calculated as has been described in Section 3.2, were taken into account to select the optimal in-cell

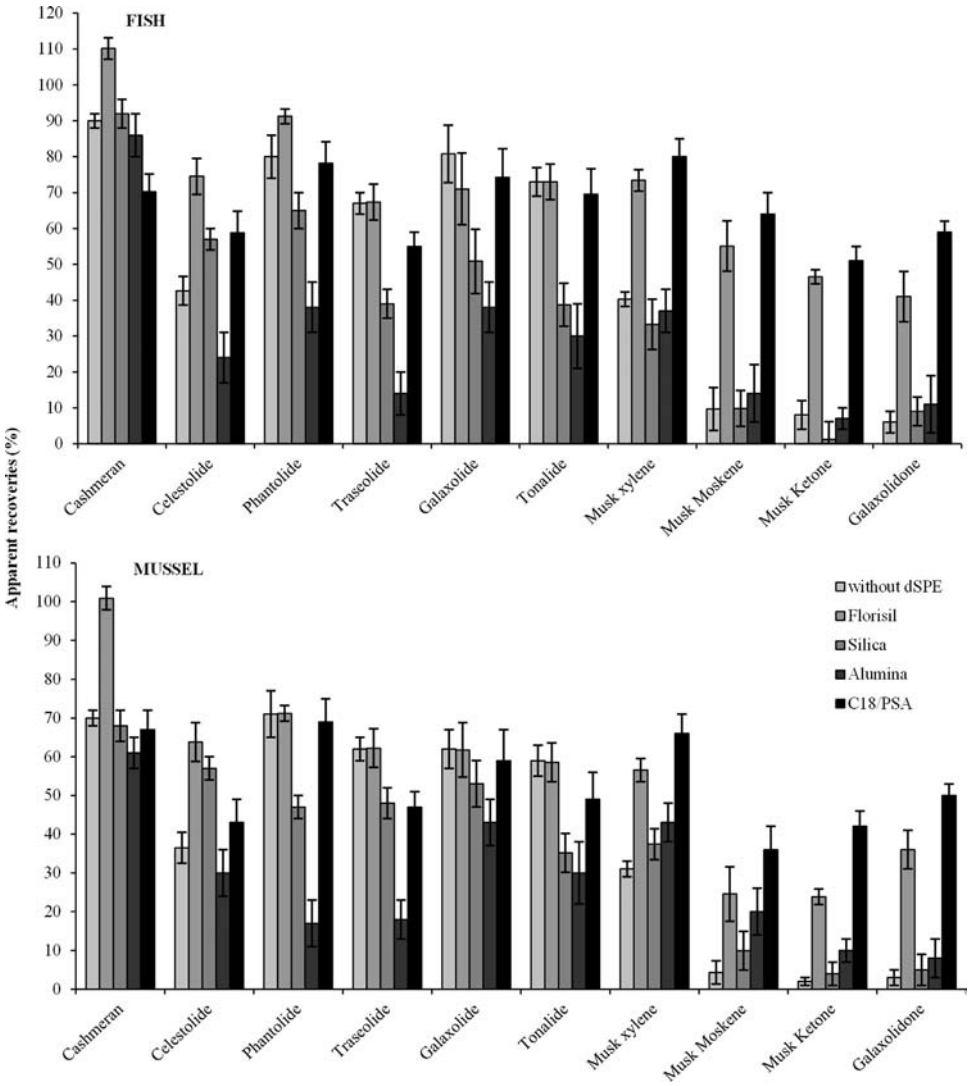

Fig. 1. Optimisation of the dSPE clean-up sorbent under QuEChERS optimal conditions: 0.5 g sample amount, 10 mL acetonitrile as extraction solvent and QuEChERS containing 4 g of magnesium sulphate, 1 g of sodium chloride, 0.5 g of sodium citrate dibasic sesquihydrate and 1 g of sodium citrate dyhidrate.

Table 1
Apparent recoveries (R_{app}, %) and matrix effect (ME, %) obtained under PLE and QuEChERS optimal conditions for both fish and mussel samples ($n=3$, 1 μg g^{-1}).

Compounds	FISH				MUSSEL			
	PLE		QuEChERS		PLE		QuEChERS	
	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)	R_{app} (%)	ME (%)
Cashmeran	109	8	110	16	79	19	110	31
Celestolide	94	−19	75	−28	88	−20	64	−32
Phantolide	91	10	91	−15	80	−13	71	−21
Traseolide	95	9	67	−10	87	−11	62	−23
Galaxolide	95	16	71	−17	91	−53	62	−52
Tonalide	96	5	73	−21	86	−41	59	−46
Musk xylene	67	−29	73	−20	67	−39	57	−34
Musk moskene	64	−33	55	−54	57	−41	25	−65
Musk ketone	69	−47	46	−56	54	−58	24	−67
Galaxolidone	61	−49	41	−62	45	−58	36	−70

clean-up sorbent. Results showed that florisil was the only sorbent that provided lower ME, ranging between −49% to 16% for fish samples and between −58% to19% for mussel samples and an enhancement of R_{app} for all of the target analytes until the values placed in Table 1. Silica seemed not to affect the response of analytes and working with alumina a decrease of between 10–20% of R_{app} of nitro musks and also HHCB-lactone was observed, probably because the analytes were adsorbed by alumina.

The best $R_{app,}$ which were summarised in Table 1, were achieved under the following conditions: 60 °C, 1 cycles 5 min static time, 120 s purge time, 1500 psi, 0.5 g (d.w.) sample, 100% flush volume and 1 g florisil as in-cell clean-up sorbent.

3.4. Method validation

As both extraction methodologies are suitable for the extraction of the target analytes present in fish and mussel samples, both methods were validated. Linear range, method detection limits (MDLs), method quantification limits (MQLs), intra-day and inter-day repeatability (expressed as % Relative Standard Deviation) were the validation parameters evaluated. Although ME had been significantly reduced by the optimisation of the clean-up step, it was decided to use a matrix-matched calibration curve for the quantification of analytes in order to obtain more accurate results. In addition, as large volume injection mode was used (10 μL), the IS ^{2}H15-MX was used to improve the repeatability.

The fish (*Mullus surmuletus*) and mussel (*Mytilus galloprovincialis*) samples used to validate the method were analysed ($n=5$)

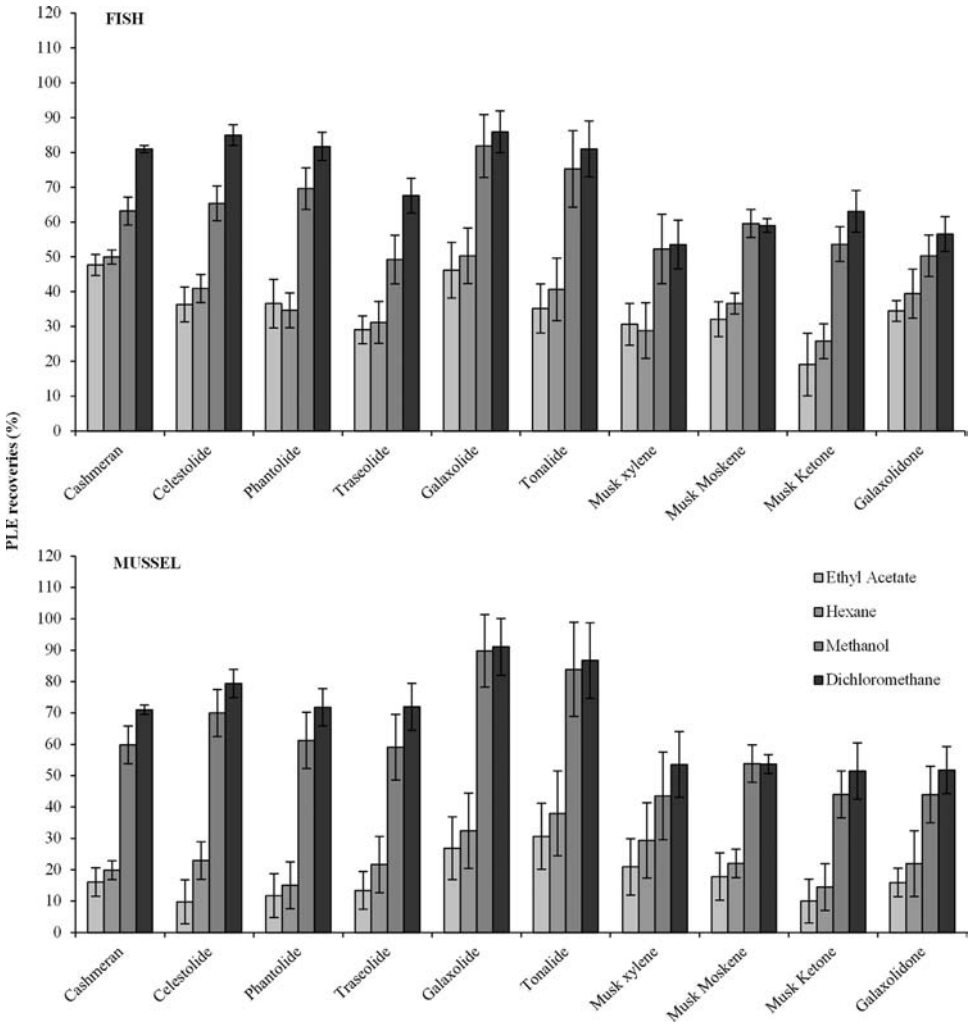

Fig. 2. Optimitsation of the PLE extraction solvent under the following initial experimental conditions: 80 °C, 2 cycles, 5 min static time, 120 s purge time, 1500 psi, 0.5 g (d.w.) sample amount and 100% flush volume.

to determine if any target analyte was present, and the results revealed peaks of HHCB and AHTN in the chromatogram. The average peak area of each compound detected was subtracted from the corresponding peak areas of each spiked sample.

Linear range, MQLs and MDLs were obtained experimentally by spiking fish and mussel samples at different levels (IS=50 ng g^{-1}) prior to the extraction procedure by PLE or QuEChERS (Tables 2 and 3). The linear range started at the MQL (defined as the lowest calibration point) and went up to 100 ng g^{-1} (d.w.) or 250 ng g^{-1} (d.w.) depending on the target analyte, with good linearity for all of the target compounds ($r^2 > 0.994$) provided by the presence of the IS. The MDLs were calculated by the S/N of 3 for the compounds that did not appear in the fish and mussel samples. MDLs for HHCB and AHTN were estimated as the concentration that gave a signal average of plus three times the standard deviation of the signal obtained for blank samples. Thus MQLs and MDLs were between 1 ng g^{-1} and 20 ng g^{-1} and 0.5 ng g^{-1} and 10 ng g^{-1}, respectively, independently of the matrix analysed. As can be seen in Tables 2 and 3, slightly better MQLs and MDLs were obtained working with PLE as extraction technique for both fish and mussel samples. In addition, the developed methods provided better MDLs than those reported in the literature by Subedi et al. [25] and Mottaleb et al. [26]. Considering that MDLs representing the lowest concentration of each analyte that may be reported in a defined matrix with 99% confidence that the concentration is non zero [47], Subedi et al. [25] reported MDLs between 1.6 and 38 ng g^{-1}working with PLE followed by GPC and GC-IT-MS/MS. While Mottaleb et al. [26] obtained MDLs between 12–397 ng g^{-1} working with LLE followed by GPC and GC-IT-MS/MS as separation and detection technique.

Intra-day and inter-day repeatability were obtained with five replicates of a fish sample and a mussel sample spiked at 50 ng g^{-1} (d.w.). The presence of the IS improved the method repeatabilities obtaining intra-day repeatability values always less than 10% for fish samples and 14% for mussel samples, and no significant differences were observed between work with PLE or QuEChERS as the extraction technique. Inter-day repeatability was always less than 16% or 19% (%RSD, $n=5$) for fish and mussel samples respectively.

PLE and QuEChERS extraction procedures were compared in terms of validation parameters, analysis time and ME when applied to fish and mussel. Although both extraction techniques are suitable for the extraction of musk fragrances from fish and mussel tissues, the results showed that PLE was the extraction procedure that provided the lowest ME. As can be seen in Figs. 3a and b the chromatograms obtained by PLE showed lower base lines, well-defined peaks for all of the target musk fragrances and an absence of interfering peaks. As a result slightly better validation parameters were obtained for both fish and mussel samples when the PLE based method was used. That together with the absence of significant differences in terms of extraction time meant that PLE was chosen to determine the target musks present in the different kinds of fish samples. However, QuEChERS could

Table 2
Method validation parameters obtained working with fish (*Mullus surmuletus*) samples and PLE or QuEChERS as extraction technique.

	MDLs (ng g^{-1})		Linear range[a] (ng g^{-1})		Intra-day Repeatability[b] (%)		Inter-day Repeatability[b] (%)	
	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS
Cashmeran	0.5	0.5	2.5–250	2.5–100	4	6	11	9
Celestolide	0.25	0.5	1–100	2.5–250	5	4	7	8
Phantolide	0.25	0.25	2.5–250	2.5–250	3	3	6	6
Traseolide	0.5	1	2.5–250	5–250	5	2	9	5
Galaxolide	0.25	0.25	1–100	1–100	4	5	8	7
Tonalide	0.25	0.25	1–100	1–100	3	5	6	7
Musk xylene	5	10	10–250	20–250	7	6	8	16
Musk moskene	5	10	10–250	20–250	9	7	11	13
Musk ketone	5	10	10–250	20–250	8	7	8	11
Galaxolidone	0.25	0.5	2.5–100	5–250	10	4	12	7

[a] MQLs (ng g^{-1}) were fixed as the lowest calibration point.
[b] RSD (%), $n=5$, 50 ng g^{-1}.

Table 3
Method validation parameters obtained working with mussel (*Mytilus galloprovincialis*) samples and PLE or QuEChERS as extraction technique.

Compounds	MDLs (ng g^{-1})		Linear range[a] (ng g^{-1})		Intra-day Repeatability[b] (%)		Inter-day Repeatability[b] (%)	
	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS	PLE	QuEChERS
Cashmeran	0.5	1	2.5–250	5–250	6	6	9	9
Celestolide	1	2.5	5–250	5–250	4	7	9	8
Phantolide	2.5	2.5	5–250	5–250	2	3	4	16
Traseolide	1	2.5	5–250	5–250	7	5	8	18
Galaxolide	0.5	0.5	2.5–100	2.5–250	7	7	9	9
Tonalide	0.5	0.5	2.5–100	2.5–250	7	6	9	13
Musk xylene	5	7.5	10–250	20–250	9	4	12	9
Musk moskene	5	7.5	10–250	20–250	12	14	12	19
Musk ketone	5	7.5	10–250	20–250	10	5	14	10
Galaxolidone	2.5	5	7.5–250	10–250	2	14	16	16

[a] MQLs (ng g^{-1}) were fixed as the lowest calibration point.
[b] RSD (%), $n=5$, 50 ng g^{-1}.

be used to determine musk fragrances in fish and mussel samples if PLE is not available.

3.5. Method application

The PLE GC-IT-MS/MS method was applied to determine musk fragrances in fish samples of red mullet (*Mullus surmuletus*), gilt head bream (*Sparus aurata*), turbot (*Psetta maxima*) and mussels (*Mytilus galloprovincialis*) from Tarragona market, and also in perch (Perca fluviatilis), sheatfish (Silurus glanis) and carp (*Cyprius carpio*) samples from the River Ebro (Section 2.2). As it has been already described, two matrix-matched calibration curves were used, one for fish samples and the other for mussel samples, for the quantification of analytes in order to obtain more accurate results.

Table 4 presents the results of the average concentrations of musk fragrances found in each sample ($n=8$) analysed. HHCB and AHTN, which were usually determined in wastewater [17–19] or river waters [23–25] at concentrations ranging from μg L^{-1} to mg L^{-1}, were present in all the samples analysed at concentrations ranging between 2.97 ng g^{-1}–18.04 ng g^{-1} and 1.17 ng g^{-1}–8.42 ng g^{-1} for HHCB and AHTN, respectively. Perch and sheatfish were the fish samples with the highest concentrations of HHCB (18.04 ng g^{-1}) and AHTN (8.42 ng g^{-1}), respectively. DPMI was found in all the samples analysed (12.83 ng g^{-1}–33.53 ng g^{-1}), except red mullet and mussels. ADBI was determined in red mullet, turbot and carp samples at concentrations between 1.56 ng g^{-1} and 8.26 ng g^{-1}. The presence of AHMI was demonstrated only in the turbot and carp samples, with an average concentration of 2.61 ng g^{-1} and 12.51 ng g^{-1}, respectively. None of the samples contained detectable traces of MX, MM or MK. HHCB-lactone was found only in the perch (15.99 ng g^{-1}) and sheatfish (17.94 ng g^{-1}) samples from the Ebro River. Fig. 3c shows the PLE GC-IT-MS/MS chromatogram of a turbot sample in which the presence of DPMI, ADBI, AHMI, HHCB and AHTN is shown.

Previous research [23–26,28,48] that has focused on determining musk fragrances in fish samples (lateral fillets) from river or sea waters confirm the findings of this study, that is, that the most abundant polycyclic musks are HHCB and AHTN, although other polycyclic musks such as DPMI, ADBI, AHMI and ATII can also be present in fish samples in minor concentrations. However, the nitro musks (MX, MM and MK) show significant differences in concentration depending on the location of the fish samples, and if the study was carried out before nitro musk fragrances became subject to regulation [25,28,48].

4. Conclusions

Two extraction methodologies, QuEChERS and PLE, were compared for determining ten musk fragrances in fish and mussel samples. A dSPE clean-up with florisil or an in-cell clean-up with florisil was applied to de-fat the sample extract and reduce the matrix effect in QuEChERS and PLE, respectively. Despite the reduction of ME observed by applying those strategies, matrix-matched calibration curves were applied to ensure more accurate results.

The methods were validated for both extraction procedures, with slightly better results being obtained working with PLE plus in-cell

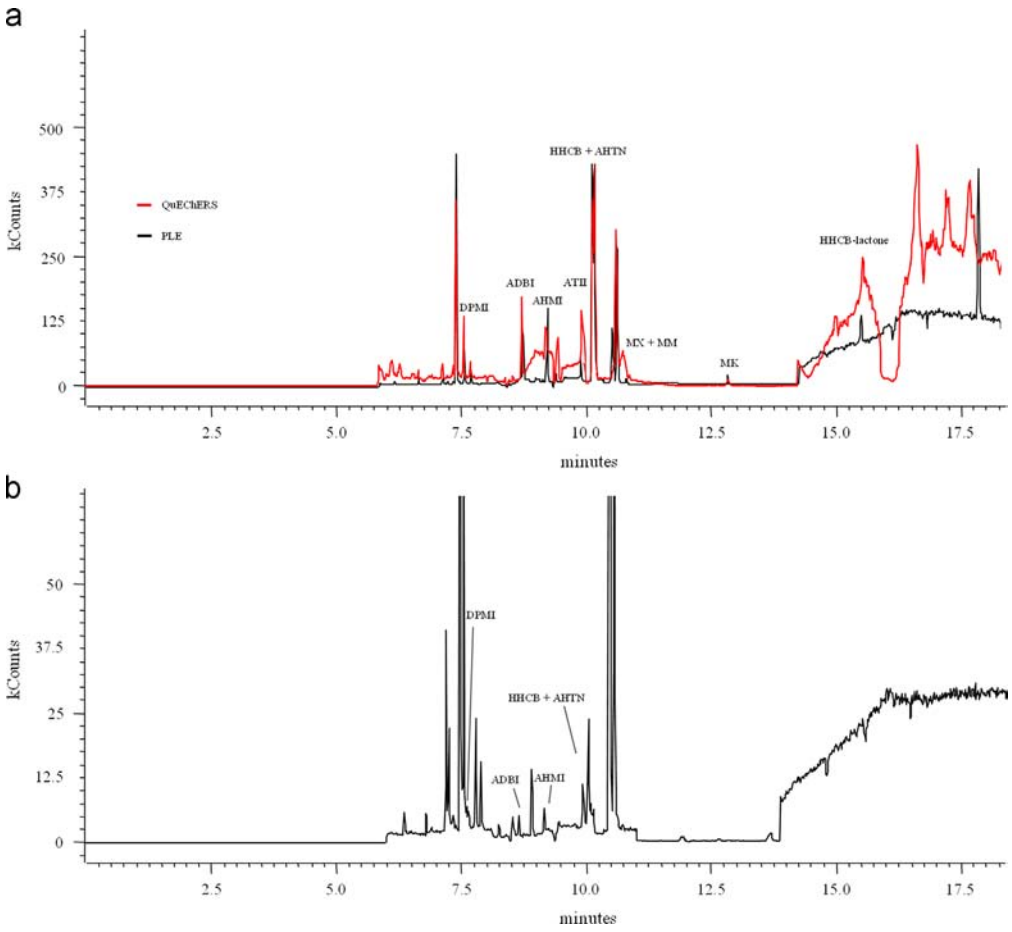

Fig. 3. Chromatograms obtained by LVI-GC-IT-MS/MS: a) mussel sample spiked at 50 ng g^{-1} and extracted by QuEChERS (red line) and PLE (black line), respectively. b) non-spiked turbot sample extracted by PLE and analysed by LVI-GC-IT-MS/M. DPMI (Cashmeran), ADBI (Celestolide), AHMI (Phantolide), ATII (Traseolide), HHCB (Galaxolide), AHTN (Tonalide), MX (Musk xylene), MM (Musk moskene), MK (Musk ketone), HHCB-lactone (Galaxolidone).

Table 4
Musks concentrations (ng g^{-1} (d.w.)) determined in samples analysed.

Compounds	Tarragona market				Ebro river		
	Red mullet *(Mullus surmuletus)*	Gilt head bream *(Sparus aurata)*	Turbot *(Psetta maxima)*	Mussel *(Mytilus galloprovincialis)*	Perch *(Perca fluviatilis)*	Sheatfish *(Silurus glanis)*	Carp *(Cyprinus carpio)*
Cashmeran	n.d.	12.83	15.69	n.d.	13.36	33.53	14.06
Celestolide	6.25	n.d.	8.26	n.d.	n.d.	n.d.	1.56
Phantolide	n.d.	n.d.	2.61	n.d.	n.d.	n.d.	12.51
Traseolide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Galaxolide	2.97	6.12	9.67	8.94	18.04	16.23	12.68
Tonalide	1.17	3.61	5.19	5.65	7.53	8.42	1.38
Musk xylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Musk moskene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Musk ketone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Galaxolidone	n.d.	n.d.	n.d.	n.d.	15.99	17.94	n.d.

n.d.: not detected

clean-up as the extraction technique, with MDLs ranging between 0.5 ng g^{-1} and 10 ng g^{-1} depending on the target analyte, intra-day repeatabilities lower than 14% for all the compounds analysed, and inter-day repeatabilities between 4% and 16%.

The applicability of the PLE/GC-IT-MS/MS method has been demonstrated by analysing different kinds of fish such as red mullet, gilt head bream, turbot and mussels, from the local market in Tarragona and perch, sheatfish and carp taken from the Ebro River. The results showed that HHCB and AHTN were present in all the samples analysed, with perch and sheatfish being the samples that present the highest concentrations of HHCB (18.04 ng g^{-1}) and AHTN (8.42 ng g^{-1}), respectively.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.010.

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