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# DETERMINATION OF SYNTHETIC MUSK FRAGRANCES IN FISH SAMPLES USING SPME ARROW – GC – IT – MS/MS

**MASTER'S THESIS**

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# 1. Introduction

The urge of improving quality of life, the rising claim of certain products and the will to reduce the cost of great industrial processes has led to a current situation of excessive chemical compounds production. The lack of regulation because of the continuous appearance of new families of compounds takes society to a scenario of constant emerging pollution cases. Because of all of this, the compounds responsible of this contamination cases are on the spotlight with intention of knowing their effects and have control over them.

In recent years, an elevated number of microorganic contaminants have been found worldwide. Among them, there is a group referred as "emerging organic contaminants" (EOCs). These are natural or synthetic substances, not necessarily recently discovered, that are not generally monitored but are believed to have an undesirable impact in both ecosystems and society [1]. EOCs comprise several groups of pollutants such as pesticides, industrial additives, detergents, pharmaceuticals, "life-style compounds" and personal care products. Some of these compounds present bioaccumulation potential and together with their respective metabolites are identified as persistent organic pollutants (POPs) due to their high stability. This bioaccumulation potential could lead to a degradation of ecosystems and produce side effects in the species where they bioaccumulate as well as in humans due to their toxicology [2,3].

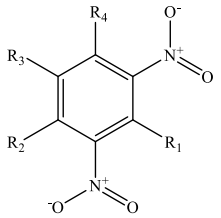
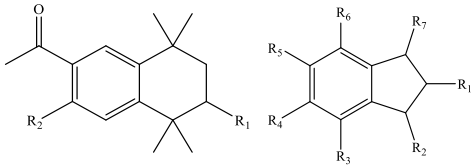
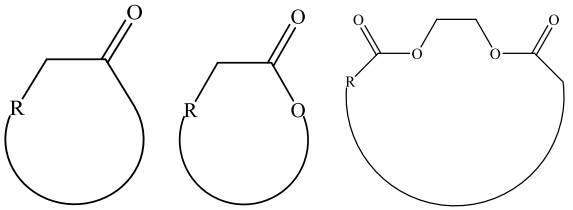
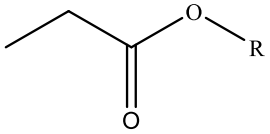
Among EOCs, personal care products (PCPs) are a group with huge concern due to their toxic effects on aquatic biota. They may be absorbed by the body and extracted or washed off after their application heading for domestic effluents. They are produced as the result of the vast use of products such as lotions, toothpaste, cosmetics or food, which may contain disinfectants (triclosan and triclocarban), synthetic musk fragrances (nitro musks and polycyclic musks), UV filters (UV-9, UV-234, UV-320, etc.), preservatives (parabens) and stabilizers (benzotriazoles) as ingredients [4]. These compounds were reported to cause toxicity to aquatic organisms such as algae, daphnia or fishes [5]. The present work focuses on the determination of musk fragrances as they are present in everyday products.

### 1.1. Synthetic musk fragrances

As mentioned before, synthetic musk fragrances pertain to EOCs and include synthetic man-made chemicals produced in large amounts and used mostly in daily products like household products, air fresheners, perfumes, cosmetics and personal care products. These are the responsible of enhanced and persistent scent of the products and were first created to replace natural musks obtained from natural sources in order to reduce the cost of production.

Based on their chemical structure and properties, synthetic musks can be grouped as: nitro musks (NMs), polycyclic musks (PCMs), macrocyclic musks (MCMs) and alicyclic musks (AMs). The basic structures and most relevant compounds of each of the groups are shown in Table 1. Nitro musks were the first synthetic musk fragrances synthetized created by Albert Baur in 1888. They were present in the market till mid 1990s and started disappearing because of their toxicity. Nowadays the use of the most important nitro musks (musk xylene and musk ketone) is restricted by the European Directive 2002/34/EC [6]. In order to replace them, another group of fragrances emerged with the name of polycyclic musks (PCMs). This group comprises acetylated and methylated pyran, tetralin and indane compounds. The characteristic chirality of these compounds is directly related to their odour profile, with galaxolide (HHCB) stereoisomers being the most representative as just two of the four stereoisomers have the distinctive musk odour (4S7R and 4S7S) while the other two are not effective. The vast use of these compounds along with their lipophilic characteristics and poor biodegradability has led to a reduction of their production and use in EU since late 1990s. As an alternative, macrocyclic musks (MCMs) appeared even though they are not widely used due to their high production cost. Moreover, compounds like alicyclic musks (AMs) are supposed to be the fourth generation of musks being more biodegradable and economical [7].

**Table 1.** Musk fragrances classification according to their structure.

| Synthethic musk | Characteristic compounds | Structure | |
|---|---|---|---|
| Nitro musks (NMs) | -Musk xylene (MX)<br>-Musk ketone (MK) |  | $R_1$ - $R_4$ = alkyl, ketone and methoxy groups |
| Polycyclic musks (PCMs) | -Galaxolide (HHCB)<br>-Tonalide (AHTN)<br>-Cashmeran (DPMI)<br>-Celestolide (ADBI) |  | $R_1$ - $R_7$ = alkyl and acetyl groups |
| Macrocyclic musks (MCMs) | -Exaltolide (EXA)<br>-Muscone<br>-Musk NN<br>-Cervolide |  | R = 10 -15 carbons |
| Alycyclic musks (AMs) | -Cyclomusk<br>-Helvetolide<br>-Romandolide |  | R = cycloalkyl |

The widespread use of musk fragrances in everyday life products cause an increase of the concentration of these compounds in household wastewater, what makes household effluents one of the most important contamination sources. These effluents head for the waste water treatment plants (WWTP) where effluent water is treated. Even though these plants are prepared to deal with pathogen agents and organic and inorganic substances, musk fragrances are not completely removed from the water during the process. Studies such as the ones carried out by Homem et al. [8] or Vallecillos et al. [9] show that WWTP effluents still contain concentrations at ng $L^{-1}$ levels of musk fragrances, confirming the low efficacy of these plants regarding fragrances elimination. On the other hand, muds resulting from the process, which also contain low concentrations of eliminated fragrances as stated by Llompart et al. [10], are commonly used as fertilizers in agriculture. Finally, treated WWTP effluent water containing musk fragrances heads for aquatic environment producing its contamination.

Once the musk fragrances reach the aquatic environment they are liable to become part of the aquatic species food chain with the fishes being the most affected. Studies demonstrate the bioaccumulation of musk fragrances in different fish species and mussels. Fussel et al. [11] demonstrated the presence of cashmeran, celestolide, galaxolide and tonalide in fish at low ng $g^{-1}$. Moreover, Vallecillos et al. [12] also demonstrated the presence of musk fragrances in both river and sea fish as well as in mussel.

Therefore, even though cutaneous exposure is the main exposure source to musk fragrances, fish intake becomes an alternative exposure source at a reduced level. Hence, musk fragrances conform a closed cycle as the one represented in Figure 1.

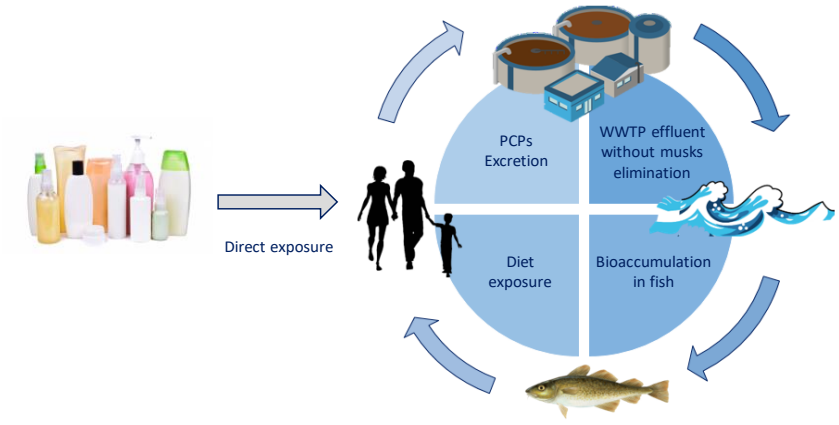



**Figure 1.** Closed cycle of musk fragrances.

Determination of musk fragrances in fish samples as well as population risk assessment has become one of the most outstanding topics on account of the inexistent European regulation on maximum permitted levels. Even though their effects on population have not been confirmed yet, musk fragrances are believed to act as endocrine disruptors [13] aside of the toxicity attached to the compounds. For this reason, the scientific community is focusing on the development of new methods to determine and control musk fragrances in aquatic organisms such as fishes.

Methodologies for the determination of musk fragrances in fish samples differ mainly in the extraction technique used. Up until now, reported methods include soxhlet extraction [14], solid liquid extraction (SLE) [15], solid phase micro extraction (SPME) [16], microwave assisted extraction (MAE) [17], focused-ultrasound solid liquid extraction (FUSLE) [17], pressurized liquid extraction (PLE) [18] and QuEChERs (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure [19]. The main drawbacks of some of those techniques relay in the fact that high amounts of organic solvent are used (Soxhlet) or specific instrumentation is needed (MAE, FUSLE and PLE). However, techniques such as SPME do not require any specific instrumentation apart from the fibres. Nevertheless, SPME could be fully automated with an automatic sampler, what increases the cost of the whole process. New procedures such as QuEChERs are cheaper and use less solvent with same or better results even though higher handling times are required.

Fish sample is a complex matrix as some species have an elevated lipid and protein content which complicates their analysis because of the interferences and the interactions with the analytes. Therefore, matrix clean-up is often needed in order to achieve better sensitivity. Clean-up procedures such as gel permeation chromatography (GPC) [20], solid phase extraction (SPE) [15] or dispersive solid phase extraction (dSPE) [12] demonstrated being able to clean the fish matrix successfully.

As for the separation and detection, gas chromatography coupled to mass spectrometry (GC-MS) results the most suitable technique to determine musk fragrances due to their high volatility. Even though GC-MS is the preferred technique, some authors reported methods were liquid chromatography was successfully applied using tandem mass spectrometry (MS/MS) [21] or fluorescence [22] detectors. Moreover, capillary electrophoresis enabled the separation of certain isomer compounds [23]. Although GC-MS is well stablished, future trends are expected to apply new advanced instruments such as comprehensive two-dimensional gas chromatography (GCxGC) and high resolution

mass spectrometry (HRMS) detectors such as time of flight (TOF) or Orbitrap leading to an increase of sensitivity and/or selectivity [24].

### 1.2. SPME Arrow

Since its development in 1989 by Belardi and Pawliszyn [25], SPME has been one of the most popular and frequently used microextraction techniques as it combines separation of the analytes from the matrix as well as a concentrating step. Even though its high popularity, SPME has some important drawbacks such as limited robustness of the fibre and small sorption phase volumes [26]. The problem with small sorption phase volumes could be solved by using Stir Bar Sorptive Extraction (SBSE), but a specific thermal desorption port is needed when using this technique, thus increasing the equipment cost and losing the full automation. Moreover, commercially available SBSE coatings are limited to PDMS and novel copolymers such as polyacrylate-poly(ethyleneglycol) (PA-PEG) and poly(ethyleneglycol)-modified silicone. With an aim of improving SPME devices and reducing the impact of the main drawbacks, CTC Analytics launched the SPME Arrow in 2015.

The SPME Arrow combines the large sorption phase volumes used in SBSE while maintaining the main advantages of the conventional SPME. The device consists of a steel rod coated with a sorbent material protected by an outer needle. Classical SPME coatings are commercially available for SPME Arrow (Table 2).

**Table 2.** Available SPME Arrow coatings commercialized by CTC Analytics.

| Coating | Diameter | Phase thickness |
|---|---|---|
| Polydimethylsiloxane | 1.1 mm | 100 µm |
| | 1.5 mm | 250 µm |
| Polyacrylate | 1.1 mm | 100 µm |
| Carbon Wide Range / PDMS | 1.1 mm | 120 µm |
| Divinylbenzene / PDMS | 1.1 mm | 120 µm |

As seen in Figure 2 a, the inner steel rod carries the cylindrically shaped sorption phase and connects the upper part with a closed tip, which permits a gentle penetration of the injector and vial septa. As opposed to conventional SPME, which consists of an open capillary, the closed tip allows the device to be completely isolated from the ambient air during transfer processes, thus reducing the risk of adverse influences such as ambient contamination [27]. Figure 2 b shows the differences between a SPME Arrow fibre and a conventional SPME fibre. As previously mentioned, classical SPME have an open tubular tip which may cause coring of injector septa and difficulties during penetration processes.

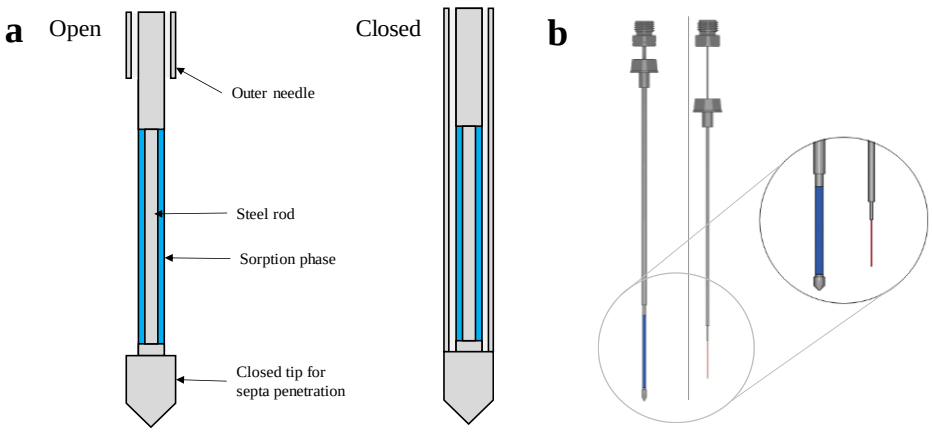



**Figure 2.** SPME Arrow system exposed (**a** left) and covered (**a** right). Comparison of the sorption phase of a SPME Arrow (**b** left) and a conventional SPME fibre (**b** right).

Even though larger volumes of sorption phase are used and the diameter of the fibre is increased, a specific thermal desorption port is not needed as conventional injection ports can be adapted to fit the SPME Arrow, which enables full automation of the process unlike SBSE.

The features commented above suggest that SPME Arrow lead to an enhance of the sensitivity due to the large sorption phases as well as higher robustness of the fibre and hence, the whole technique.

As this novel technique appeared recently, only few methods are reported in the literature demonstrating the suitability of SPME Arrow and its applicability to the analysis of different kind of compounds and samples.

First developed method was reported in 2015 by Helin et al. [28], who determined short chain aliphatic amines in wastewater using SPME Arrows in head-space mode with PDMS/CAR coatings reaching detection limits of low µg $L^{-1}$. This method demonstrated for the first time the effectiveness of the novel technique in terms of enhanced sensitivity and robustness compared to classical SPME fibres.

Likewise, in 2016 Kremser et al. [27] developed an analytical method to determine polycyclic aromatic hydrocarbons (PAHs) immersing the SPME Arrow with a PDMS coating in lab water and wastewater samples, thus lowering even more the detection limits to low ng $L^{-1}$ and emphasizing the fact that SPME Arrow combines effectively the maximal extraction efficiency of SBSE technique while maintaining the classical SPME fibre advantages. A second study carried by Kremser et al. [29] the same year compared static and dynamic head-space sampling techniques such as syringe, loop, SPME, SPME Arrow, trap and ITEX (In-Tube Extraction). Comparing method detection limits (MDLs), relative standard deviations (RSDs) and extraction yields as well as considering cost, footprint, susceptibility to contamination and complexity, Kremser concluded that SPME Arrow and ITEX may be the most efficient choice for many analytical applications.

New coatings are being synthetized for custom SPME Arrows applications, e.g. Lan et al. [30] used a A-ZIF-8 (acidified zeolitic imidazole framework-8) custom SPME Arrow to determine trimethylamine (TMA) and triethylamine (TEA) in wastewater and proved that A-ZIF-8 coating had a potential application as SPME Arrow sorbent due to the extraction of trace levels of amines and its high efficiency and reproducibility. Eventually, Feijó et al. [31] described a method for the determination of biogenic volatile organic compounds (BVOCs) in a boreal forest and compared the efficiency of SPME Arrows with conventional SPME fibres. The study provided useful data on how extraction efficiency of SPME Arrow was 2 times higher than conventional fibres as well as the negative impact of temperature and humidity when PDMS-carbon WR coatings are used, resulting in an extraction amount decrease. Table 3 summarises the studies carried out up to today using SPME Arrow.

**Table 3.** Summary of all the present studies related to SPME Arrow.

| Author and year | Compound | Sample Matrix | Extraction Mode | Arrow coating | Method detection limit (MDL) |
|---|---|---|---|---|---|
| A. Helin (2015) | Dimethylamine (DMA) and trimethylamine (TMA) | Wastewater | Head-space | PDMS/CAR | 0.1 - 10 µg $L^{-1}$ |
| A. Kremser (2016) | Polycyclic aromatic hydrocarbons (PAHs) | Lab Water Wastewater | Immersion | PDMS | 0.1 - 0.8 ng $L^{-1}$ |
| A. Kremser (2016) | Volatile Organic Compounds (VOCs) | Lab Water | Head-space | Carbon WR/PDMS | 0.7 - 4.9 ng $L^{-1}$ |
| H. Lan (2017) | Trimethylamine (TMA) and triethylamine (TEA) | Wastewater Salmon Mushroom | Head-space | A-ZIF-8/PVC (Lab-made) | 1 ng $mL^{-1}$ |
| L.M. Feijó (2018) | Biogenic volatile organic compounds (BVOCs) | Air | On site exposure Static and dynamic sampling | PDMS/DVB Carbon WR/PDMS | 17.7 - 155.2 pg |

To the best of our knowledge, this is the first research project focused on proving the suitability of SPME Arrows for the determination of musk fragrances in fish samples. Moreover, conventional SPME fibres are compared with those studied in order to prove their performance.

## 2. Objectives

The aims of the present study are summarized in the following statements:

1. Development of an analytical method for the determination of musk fragrances based on gas chromatography coupled to ion trap tandem mass spectrometry (GC-IT-MS/MS) using a novel micro extraction technique such as SPME Arrow.

2. Comparison of the effectiveness of using the SPME Arrows compared to the conventional SPME fibres. Analysis of the main advantages and drawbacks of this novel technique.

3. Application of the developed method to commercially available fish samples.

# 3. Experimental Part

## 3.1. Reagents and standards

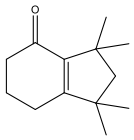
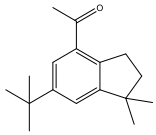
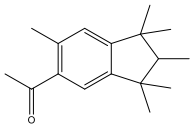
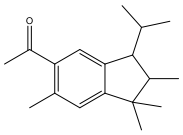
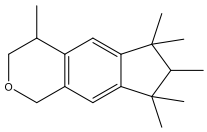
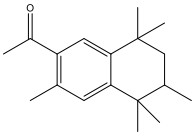
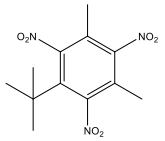
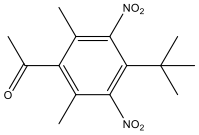
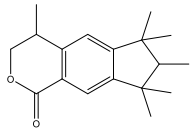
The studied polycyclic musk fragrances (Table 4) were purchased at Promochem Iberia (Barcelona, Spain): 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (cashmeran, DPMI), 4-Acetyl-6-tert-butyl-1,1-dimethylindane (celestolide, ADBI), 6-acetyl-1,1,2,3,3,5-hexamethylindane (phantolide, AHMI), 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (traseolide, ATTI) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (tonalide, AHTN). The polycyclic musk 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran (galaxolide, HHCB) was supplied by Sigma-Aldrich (St. Louis, USA). The nitro musk fragrance 2,4,6-trinitro-1,3-dimethyl-5-tert-butylbenzene (musk xylene, MX) was purchased as a 100 µg $mL^{-1}$ individual solution in acetonitrile from Sigma-Aldrich (St. Louis, USA) and the remaining nitro musk fragrance 4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (musk ketone, MK) was provided by Fluka (Buchs, Switzerland). The compound 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran-1-one (galaxolidone, HHCB-lactone) was supplied by International Flavours & Fragrances Inc. (Barcelona, Spain).

Individual stocks solutions of the synthetic musk fragrances were prepared in acetone at concentrations of 4000 mg $L^{-1}$ for all the compounds except for AHMI which was at 100 mg $L^{-1}$ and for HHCB-lactone which was at 1000 mg $L^{-1}$. A working mixture solution of 1 mg $L^{-1}$ was prepared in ethyl acetate, containing all the compounds except for HHCB-lactone. An individual working solution was prepared in ethyl acetate for HHCB-lactone at the same concentration (1 mg $L^{-1}$). Acetone and ethyl acetate were GC grade with purity >99.9% from J.T. Baker (Deventer, The Netherlands). Helium gas with a purity of 99.999% for chromatographic analysis was purchased at Carburos Metálicos (Tarragona, Spain).

Three SPME Arrows were supplied by CTC Analytics AC (Zwigen, Switzerland). Divinylbenzene/Polydimethylsiloxane (DVB/PDMS) arrow had a 120 µm sorbent film thickness and a film length of 20 mm. The other two: Polydimethylsiloxane (PDMS) and Polyacrylate (PA) arrows both were 100 µm and 20 mm. An additional SPME

conventional fibre of PDMS of 100 µm and 10mm was purchased from Sigma-Aldrich (St. Louis, USA).

**Table 4.** Name, structure, molecular weight and CAS number of the studied compounds.

| Num. | Compound name | Structure | M.W. ($g\ mol^{-1}$) | CAS number |
|---|---|---|---|---|
| 1 | 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (Cashmeran, DPMI) |  | 206.3 | 33704-61-9 |
| 2 | 4-Acetyl-6-tert-butyl-1,1-dimethylindane (Celestolide, ADBI) |  | 244.4 | 13171-00-1 |
| 3 | 6-acetyl-1,1,2,3,3,5-hexamethylindane (Phantolide, AHMI) |  | 244.4 | 64058-43-1 |
| 4 | 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane (Traseolide, ATTI) |  | 258.4 | 68140-48-7 |
| 5 | 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran (Galaxolide, HHCB) |  | 258.4 | 1222-05-5 |
| 6 | 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (Tonalide, AHTN) |  | 258.4 | 1506-02-1 |
| 7 | 2,4,6-trinitro-1,3-dimethyl-5-tert-butylbenzene (Musk xylene, MX) |  | 297.3 | 81-15-2 |
| 8 | 4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (Musk ketone, MK) |  | 294.3 | 81-14-1 |
| 9 | 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran-1-one (HHCB-lactone) |  | 272.4 | * |

### 3.2. Sample collection and pre-treatment

Seafood species such as codfish (*Gadus morhua*), sole (*Solea, solea*) and hake (*Merluccius merluccius*) were purchased at local commercial establishments (local market, supermarket and fish market). Samples were maintained in a refrigerator before their analysis. Fish samples were dissected and the lateral fillets were then homogenised and stored in a freezer. Frozen homogenised samples were lyophilized using the miVac Duo with Speed Trap freeze-drying system from Genevac (Ipswich, United Kingdom) and then ground using a coffee grinder from Moulinex (Alençon, France). In addition, samples were also sieved through a 500 µm mesh in order to homogenise particle diameter.

### 3.3. Analytical method

Fish samples were analysed using a solid-phase micro extraction (SPME) procedure. A portion of 0.25 g of lyophilized fish sample was weighed in a 20 mL head-space SPME glass vial and placed in a tray. Prior to extraction, PDMS SPME Arrow was conditioned at 100 ºC for 15 min in the conditioning port. Once the heat/stir module reached 100ºC, the vial was automatically transported to the module and equilibrated for 1 min. Afterwards, the arrow was exposed for 45 min in the vial head-space. During the extraction, the sample was magnetically stirred at 750 rpm. After 45 min, the desorption of the arrow is conducted in the injection port at 250ºC for 3 min. To prevent carry-over the SPME Arrow was baked for 15 min at 250ºC at the conditioning port after every extraction.

For the conventional SPME fibre procedure a portion of 0.25 g of lyophilized fish sample was weighed in a 20 mL head-space SPME glass vial and placed in a tray. Prior to extraction, PDMS conventional fibre was conditioned at 100 ºC for 15 min in the conditioning port. Once the heat/stir module reached 80ºC, the vial was automatically transported to the module and equilibrated for 1 min. Afterwards, the fibre was exposed for 60 min in the vial head-space. During the extraction, the sample was magnetically stirred at 750 rpm. After 60 min, the desorption of the fibre is conducted in the injection port at 250ºC for 3 min. To prevent carry-over the PDMS fibre was baked for 15 min at 250ºC at the conditioning port after every extraction.

The chromatographic analysis was performed using a Varian 4000 GC/MS/MS with Ion trap system from Varian (Walnut Creek, USA). The system consisted on a 3800 gas chromatograph coupled to a 4000 ion trap mass spectrometer and a 1079 PTV (Programmable Temperature Vaporization) injector. Additionally, the system incorporates a CombiPal auto sampler from CTC Analytics (Zwigen, Switzerland) containing a SPME Arrow holder for automatic extraction, a PAL Heatex Stirrer module and a PAL SPME Arrow conditioning module.

Chromatographic separation was carried out on a ZB-50 analytical column (50% phenyl-50% dimethylpolysiloxane, 30 m x 0.25 mm i.d.; 0.25 µm film thickness) provided by Phenomenex (Torrance, USA). The carrier gas used was helium at a constant flow rate of 1 mL $min^{-1}$. The compounds were separated in 20 min using a temperature program of the oven as follows: 70ºC hold for 3.5 min, raised to 200ºC at 50ºC $min^{-1}$, then to 240ºC at 5ºC $min^{-1}$ and finally to 290ºC at 20ºC $min^{-1}$ and held for 3.4 min. The mass spectrometer operated in electron ionization (EI) mode (70 eV). The trap, manifold and transfer line temperatures were 200ºC, 50ºC and 280ºC, respectively. Tandem mass spectrometry mode (MS/MS) was applied in order to quantify the compounds. Retention times and MS optimized parameters for all the studied compounds are summarized in Table 5.

**Table 5.** Retention times and MS conditions.

| Compound | Retention Time (min) | Precursor Ion (m/z) | Product Ion[a] (m/z) | CID Amplitude (V) | m/z Range | Scan time (s/scans) |
|---|---|---|---|---|---|---|
| DPMI | 7.5 | 191 | 107, 135, **173** | 0.82 | 94 - 201 | 1.08 |
| ADBI | 8.6 | 229 | 131, **173**, 187 | 0.92 | 110 - 239 | 1.08 |
| AHMI | 9.1 | 229 | 131, 145, **187** | 0.93 | 110 - 239 | 1.08 |
| ATTI | 9.7 | 215 | 131, 171, **173** | 0.88 | 104 - 225 | 1.03 |
| HHCB[b] | 9.9 | 243 | 171, **213** | 0.96 | 132 - 253 | 0.53 |
| AHTN[b] | 9.9 | 243 | 145, 159, **187** | 0.96 | 113 - 253 | 0.53 |
| MX | 10.6 | 282 | **265**, 266, 280 | 1.08 | 134 - 292 | 0.34 |
| MK | 12.5 | 279 | **191**, 247, 280 | 1.07 | 132 - 289 | 1.05 |
| HHCB-Lactone | 15.1 | 257 | 183, 201, **239** | 1.00 | 123 - 267 | 1.03 |

[a] Quantification ions (m/z) are shown in bold type.

[b] Compounds were separated using Multiple Reaction Monitoring mode.

# 4. Results and discussion

## 4.1. Chromatographic separation

Compounds were separated using a ZB-50 analytical column (50% phenyl-50% dimethylpolysiloxane, 30 m x 0.25 mm i.d.; 0.25 µm film thickness) which is a high polarity column. Temperature program of the oven was adapted from Trabalón et al. [32]. All the compounds were chromatographically separated except for HHCB and AHTN which had the same retention time. Acquisition mode of the MS was set as multiple reaction monitoring (MRM), which permitted the separation of these two compounds based on their MS spectra and selective ions. Figure 3 shows the extracted ion chromatogram (XIC) chromatogram of the quantifier ion of each of the compounds.

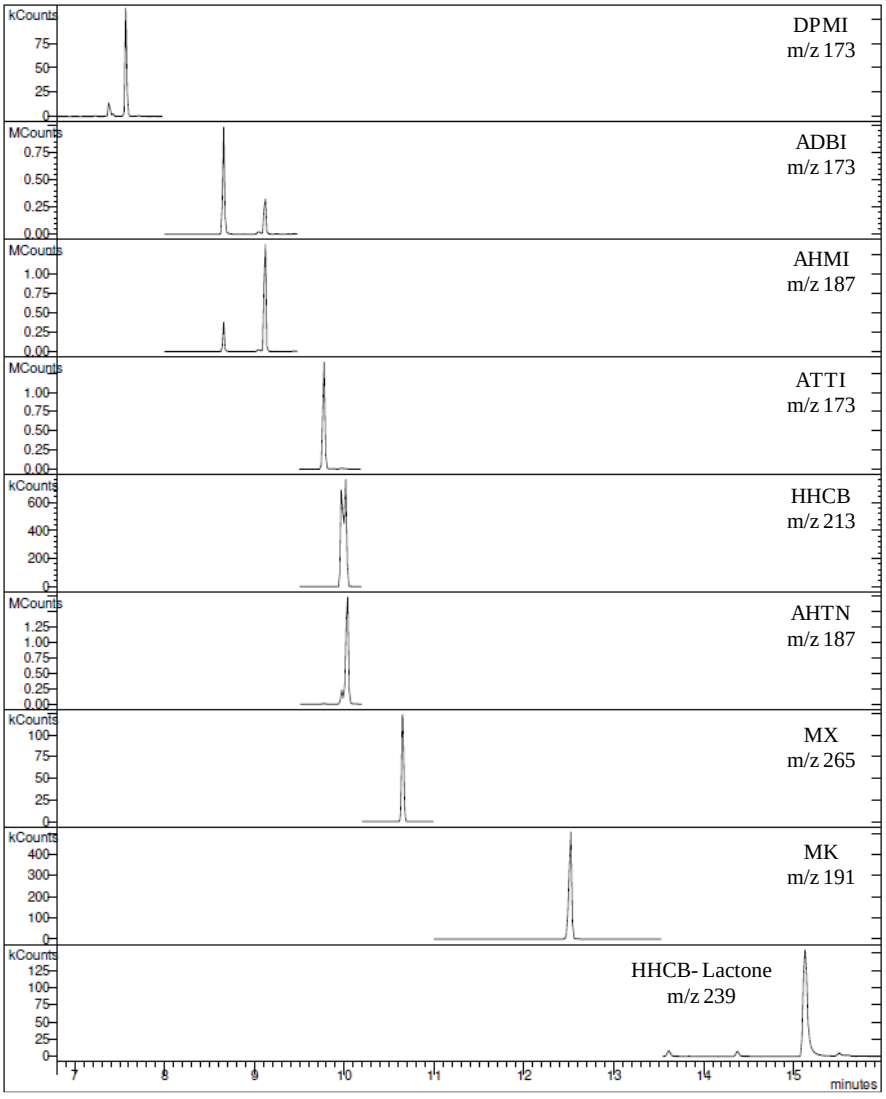



**Figure 3**. Extracted ion chromatogram (XIC) of the quantifier ions of the 9 musk fragrances at a concentration level of 1 mg $L^{-1}$.

Mass spectrometry related parameters such as precursor and product ions, collision induced dissociation (CID) amplitude and mass/charge (m/z) range were optimized with the optimal results being the ones summarized in Table 5 (section 3.3.).

### 4.2. SPME Arrow optimization

In order to set up the optimal extraction conditions different extraction experiments were performed using SPME Arrow in head-space mode (HS). The experiments were carried using 0.25 g of cod (*Gadus morhua*) spiked at a concentration of 1 mg $kg^{-1}$ (dry weight, d.w.) of all the musk fragrances except for HHCB-lactone as it is a degradation product of HHCB. The presence of both HHCB and HHCB-lactone could make results difficult to understand if both of them are spiked together. Therefore, HHCB-lactone extraction was not optimized and was included in the method later. Cod fish was chosen for the extraction optimization as it has a reduced lipid content, which causes to have less interferences in the analysis. Analysis of blank samples were performed in order to subtract the blank signal of the compounds present in the fish.

The parameters that can be optimised for SPME Arrow are the same as for conventional SPME: type of coating, the extraction time and temperature and other parameters such as water addition or desorption temperature and time. The different parameters studied and the optimal values obtained are discussed and described in detail in the following sections.

#### 4.2.1. Coating selection

Three different types of SPME Arrow sorbents were compared in the extraction of musk fragrances from the fish samples. Two absorbent type coatings (polyacrylate, PA and polydimethylsiloxane, PDMS) and an adsorbent type (polydimethylsiloxane-divinylbenzene, PDMS-DVB) were tested. Extraction and desorption conditions were the same for all the SPME Arrows. Samples were extracted at 80 ºC for 45 min while stirred at 750 rpm and desorbed at 250ºC for 3 min. Peak area values were considered to select which type of sorbent was the best (Figure 4).

Results show that PDMS and PDMS/DVB coatings were the best for the extraction of musk fragrances while PA was fairly below. This agrees with the fact that PDMS and

PDMS/DVB coatings are non-polar phases aimed to extract compounds with low polarity and mid-high volatility. On the other hand, PA coatings have a moderate polarity and are preferred for mid-high polar compounds. Since musk fragrances are volatile and nonpolar compounds, PDMS and PDMS/DVB could be the most suitable coatings. As PDMS had slightly better results than PDMS-DVB it was chosen as the preferred coating for the SPME Arrow extraction.

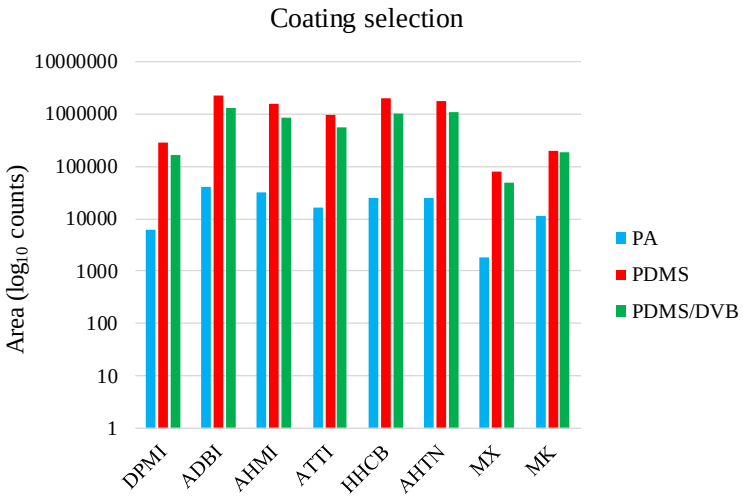



**Figure 4.** Comparison of different SPME Arrow coatings.

### 4.2.2. Extraction temperature

Increasing the extraction temperature may affect the extraction efficiency. Thus, extraction temperatures ranging from 60 to 120 ºC were tested. All experiments were carried out with the same extraction time (45 min) and desorption conditions (250ºC for 3 min). The results are represented in Figure 5.

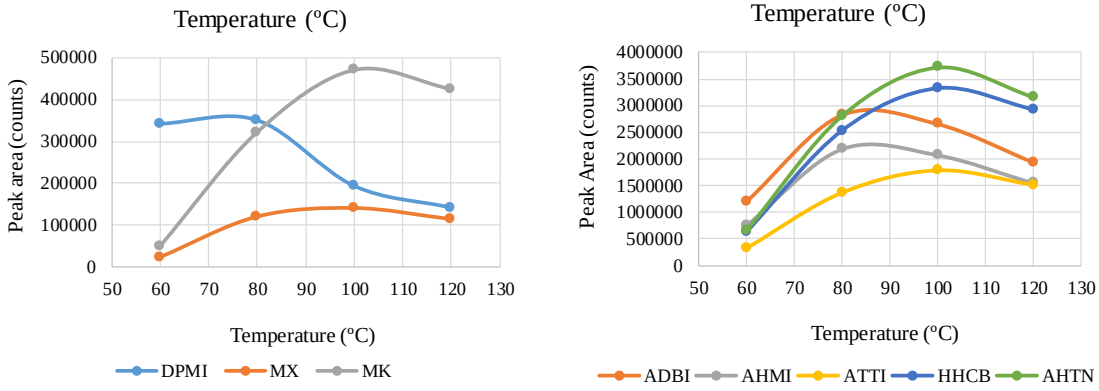



**Figure 5.** The effect of temperature in extraction efficiency.

Increasing the temperature affects SPME collection as it increases the partitioning of compounds into the head-space of the vial. However, at the same time the partitioning into the sorbent may decrease. As observed in the figure, all the compounds were affected by the temperature changes, mostly enhancing the extraction efficiency while increasing the temperature. For DPMI, ADBI and AHMI the opposite effect occurs. These three compounds are the most volatile of the musk fragrances studied, which could be the reason to the decrease of the efficiency with temperature.

As stated before, volatile compounds are more sensitive to decrease the partitioning into the sorbent as the temperature increases. This explains why DPMI, being the most volatile compound, is the most affected compound in terms of losing extraction efficiency.

Even though these three compounds have their maximum extraction efficiency at 80 ºC, the rest of the compounds show better results when heated at 100ºC. Therefore, the selected temperature for the extraction was 100ºC.

### 4.2.3. Extraction time

After selecting the optimal temperature for the extraction (100ºC) experiments with different extraction times ranged between 20 and 80 min were performed to construct the extraction time profiles. The desorption conditions were kept as the ones used for the temperature optimization, 250ºC for 3 min. The extraction time profiles obtained are represented in Figure 6.

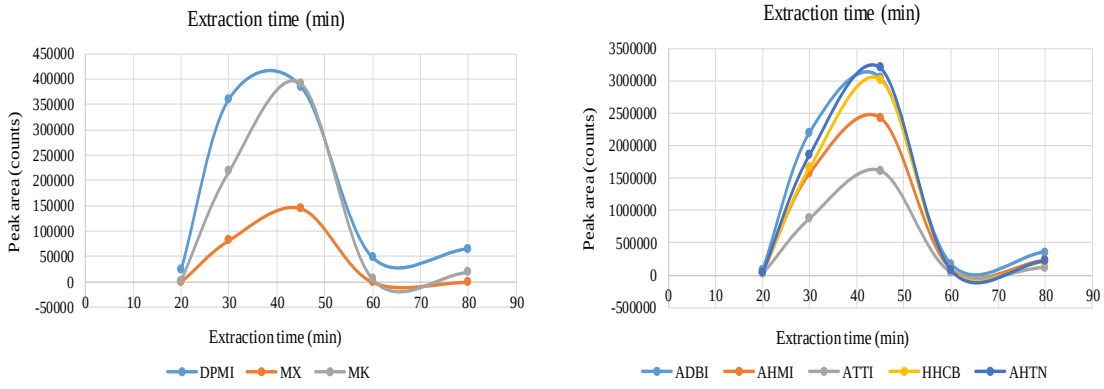



**Figure 6.** Extraction time profile for the studied compounds.

All the compounds reached their maximum extraction efficiency at 45 min except for the most volatile compound, DPMI, which optimal extraction time was between 30 and 40 min. The results show that no equilibrium is reached once the highest amount is extracted since the amounts decrease significantly with time. Reported methods in the literature do not explain similar effects when using PDMS SPME Arrow. Further experimental research must be done in order to explain this trend. Consequently, extraction time was set at 45 min.

### 4.2.4. Water addition

The addition of certain volumes of water was tested to check if extraction efficiency was enhanced. In this case, water volumes of 0.5 and 1 mL of ultrapure water were added to the SPME vial. Experiments were performed with the same extraction conditions: 100ºC for 45 min while stirred at 750 rpm and desorbed at 250ºC for 3 min. Results were compared with the ones obtained without the addition of water and are represented in figure 7.

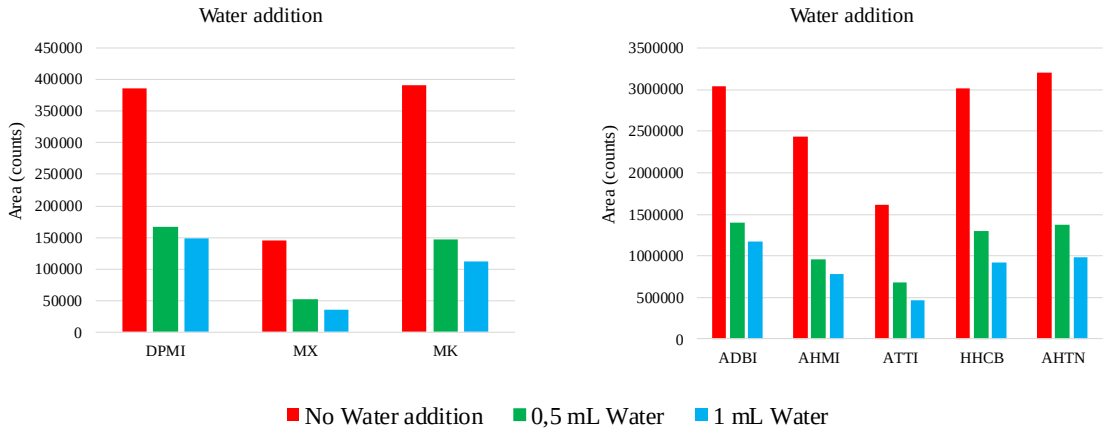



**Figure 7.** Effect of the addition of water.

Results show that the addition of water reduces the extraction efficiency. As the volume of water added to the vial increases, the extraction efficiency decreases. This can be related to the humidity formed during the extraction. The sealed vial along with the high temperatures used for the extraction cause water to be vaporized, which increases humidity in the head-space of the vial. This water vapor could be condensed and trapped onto the sorbent, thus reducing the extraction efficiency of the SPME Arrow. Authors such as Helin et al. [28] and Feijó et al. [31] also reported a reduction of extraction

efficiency when increasing the humidity while using SPME Arrows. Therefore, no water was added to the vial for the extraction.

Desorption conditions were tested but no improvement was seen when changing the temperature or increasing the desorption time. Thus, initial conditions (250ºC for 3 min) were selected and no carryover was observed.

### 4.3. Conventional SPME fibre optimization

In order to compare the differences of using SPME Arrow instead of a conventional SPME fibre, a conventional SPME fibre was tested. The same coating sorbent used for the SPME Arrow was selected to compare how the increase of sorbent volume affected the extraction efficiency A 100 µm PDMS conventional fibre was optimized for the extraction of musk fragrances from fish samples. Thus, temperature and time of the extraction procedure were optimized. The experiments were performed in head-space mode and with the same sample conditions as for SPME Arrow optimization.

#### 4.3.1. Extraction temperature

The initial extraction conditions for testing the conventional fibre were the ones obtained for the SPME Arrow. Then, further experiments were carried out in order to find the optimal temperature of the extraction. Extraction temperatures ranging from 40 to 120 ºC were tested. All experiments were carried out with the same extraction time (45 min) and desorption conditions (250ºC for 3 min). The results are represented in Figure 8.

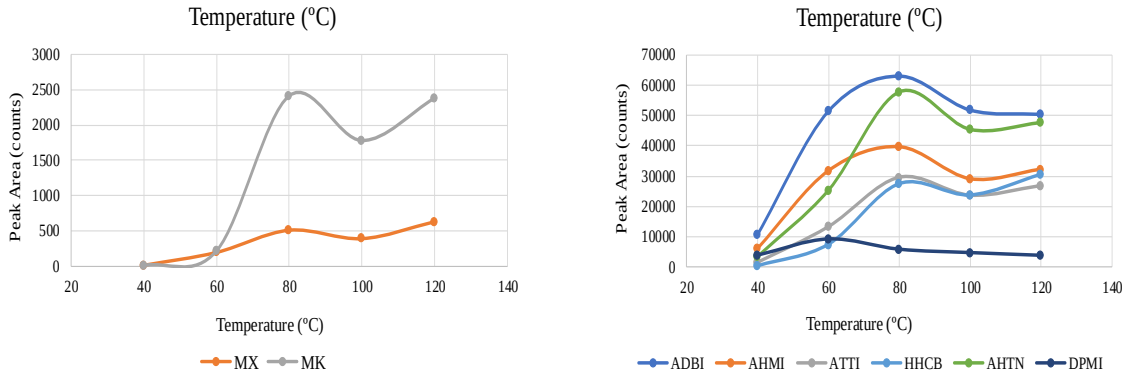



**Figure 8.** Effect of the temperature when using a conventional SPME fibre.

The results show that except for DPMI, the most volatile compound, the optimal extraction temperature was 80 ºC. The fact that DPMI showed a lower optimal temperature, 60 ºC, could be explained for the same reasons stated in the discussion of temperature extraction for SPME Arrow.

### 4.3.2. Extraction time

Once the optimal temperature was selected, experiments with different extraction times ranged between 10 and 80 min were performed to construct the extraction time profiles of the compounds for the conventional fibre. Time ranges tested were lower than the SPME Arrow ones as conventional fibres takes shorter times to reach equilibrium [31]. The time profiles obtained can be seen in Figure 9.

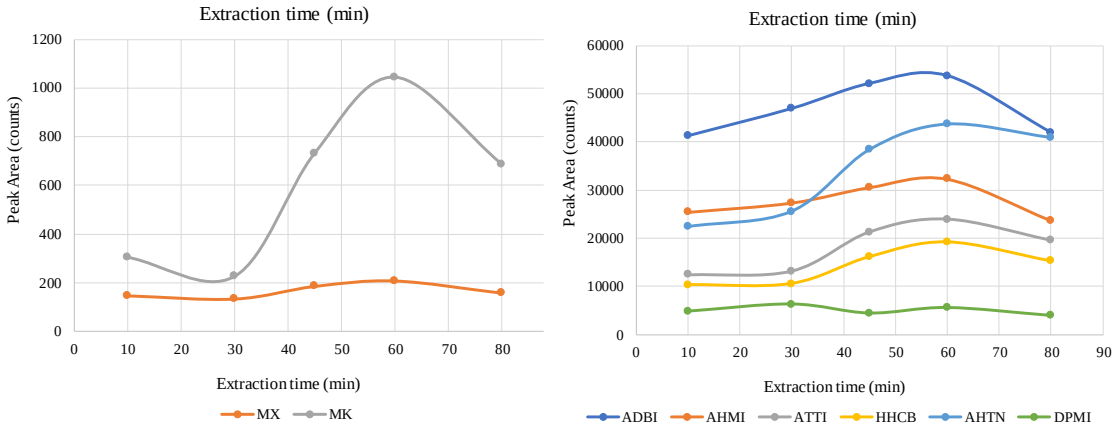



**Figure 9.** Extraction time profile when using a conventional SPME fibre.

The extraction time profiles obtained for the compounds show that the highest extraction efficiency is reached at 60 min, when the equilibrium is reached for most of the compounds. DPMI reached the equilibrium before all the other compounds at 30 min, due to its high volatility. Longer extraction times than 60 min show a slightly decrease of extracted amounts of compounds, which is the same that happened with SPME Arrow. Therefore, 60 min was chosen as optimal extraction time.

### 4.4. SPME Arrow and conventional SPME fibre comparison

Samples of cod spiked at the same concentration (1 mg $kg^{-1}$) were analysed by both techniques at their optimal conditions:

- SPME Arrow: extracted with a PDMS coating at 100ºC for 45 min and desorbed at 250ºC for 3 min.
- Conventional SPME fibre: extracted with a PDMS coating at 80ºC for 60 min and desorbed at 250ºC for 3 min.

The results obtained are represented in Figure 10.

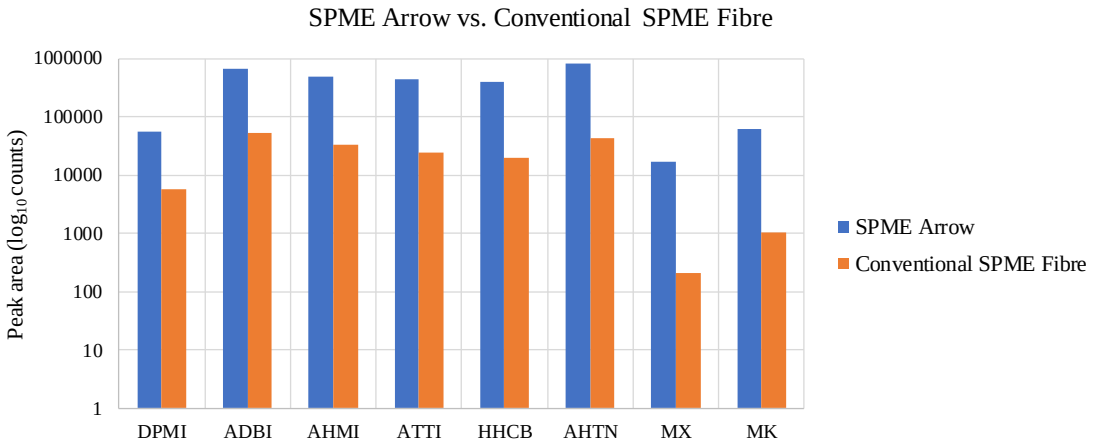



**Figure 10.** Comparison of the signal obtained when analysing a cod sample spiked at 1 mg $kg^{-1}$ using SPME Arrow and a conventional SPME fibre.

As seen in the figure, SPME Arrow had extraction efficiencies up to 10 times higher for most of the compounds when compared with conventional SPME fibres. Therefore, sensitivity of the method was enhanced, thus lowering the detection limits when using SPME Arrow instead of conventional fibre. Even though higher extraction efficiencies comprise better results in terms of method limits, the larger sorption volumes had an accentuated extraction efficiency loss at higher extraction times when compared with conventional fibres (as seen in extraction time profiles for both techniques). This problem could be solved by not surpassing the highest extraction efficiency time. These experiments demonstrated the advantages of SPME Arrow in front of the conventional fibres.

### 4.5. Method quality parameters

The analytical performance of the SPME Arrow method was investigated under optimal conditions by establishing the linear ranges, method detection limits (MDLs), method quantification limits (MQLs), repeatability (intra-day) and reproducibility (inter-day).

Matrix matched calibration was selected due to extraction technique used (SPME) and compounds interaction with matrix. The linear range was evaluated by constructing matrix match calibration curves by spiking cod samples at different concentrations ranging from 2.5 to 500 ng $g^{-1}$ (d.w.). Non-spiked samples were analysed to subtract the signal of the compounds present in the samples. All the compounds showed two linear ranges at low ng $g^{-1}$ (ranging between 2.5 – 75 ng $g^{-1}$) and high ng $g^{-1}$ (ranging between 75 – 500 ng $g^{-1}$) except for DPMI, ADBI and HHCB-lactone, which only had one. All the compounds achieved good linearity with $R^2 > 0.992$.

Method detection limits (MDLs) were estimated as the concentration that gave a signal average of three times the standard deviation of the compound in the blank samples. For the compounds that were not present in the blank sample, the MDL corresponded to the concentration that provided a signal/noise ratio equal to 3. Hence, MDLs ranged between 0.5 ng $g^{-1}$ (d.w.) and 2.5 ng $g^{-1}$ (d.w.) for cod samples. Method quantification limits (MQLs) were fixed as the lowest points of the calibration curves and ranged from 2.5 ng $g^{-1}$ (d.w.) to 5 ng $g^{-1}$ (d.w.). There are no reported methods for the determination of musk fragrances in fish using SPME Arrow nor conventional SPME. Thus, the MDLs obtained for this method cannot be compared with other data obtained with the same extraction technique. However, authors like Vallecilos el at. [12] or Trabalón et al.[32] developed methods using pressurized liquid extraction (PLE) and QuEChERS followed by IT-MS/MS with MDLs ranging between 0.25 - 5 ng $g^{-1}$ (d.w.) and 1 – 5 ng $g^{-1}$ (d.w.), respectively. Therefore, the developed method achieves similar or even lower MDLs using a technique that requires less sample handling.

Repeatability and reproducibility, expressed as relative standard deviation (%RSD), were calculated by spiking (n=5) cod samples at 10 and 100 ng $g^{-1}$ (d.w.) for all the compounds, with values below 23 % which were similar to those obtained by the methods mentioned above. The results of the method validation are summarized in Table 8.

**Table 8.** Validation parameters of the method.

| Compound | MDL (ng $g^{-1}$) | MQL (ng $g^{-1}$) | Linear range (ng $g^{-1}$) | $r^2$ | Repeatibility[a] | | Reproducibility[a] | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 10 ng $g^{-1}$ | 100 ng $g^{-1}$ | 10 ng $g^{-1}$ | 100 ng $g^{-1}$ |
| DPMI | 0.5 | 5 | 5 - 250 | 0.9920 | 4 | 8 | 11 | 14 |
| ADBI | 0.5 | 2.5 | 2.5 - 75<br>75-500 | 0.9996<br>0.9990 | 4 | 5 | 12 | 10 |
| AHMI | 0.5 | 5 | 5 - 250 | 0.9998 | 5 | 4 | 10 | 14 |
| ATTI | 1 | 5 | 5-75<br>75-500 | 0.9977<br>0.9993 | 5 | 4 | 13 | 18 |
| HHCB | 1 | 5 | 5 - 75<br>75-500 | 0.9997<br>0.9950 | 15 | 4 | 16 | 11 |
| AHTN | 0.5 | 2.5 | 2.5 - 75<br>75-500 | 0.9995<br>0.9990 | 2 | 2 | 9 | 20 |
| MX | 2.5 | 5 | 5-50<br>50-500 | 0.9989<br>0.9991 | 15 | 6 | 23 | 20 |
| MK | 0.5 | 2.5 | 2.5 - 25<br>25-500 | 0.9992<br>0.9991 | 6 | 3 | 12 | 10 |
| HHCB-Lactone | 1 | 2.5 | 2.5 - 100<br>100-500 | 0.9999<br>0.9996 | 8 | 11 | 9 | 6 |

[a] Expressed as Relative Standard Deviation (%RSD); n=5.

### 4.6. Application of the method

The determination of the nine musk fragrances in fish samples was performed using the optimised and validated method. Three different fish species were analysed: cod (*Gadus morhua*), sole (*Solea solea*) and hake (*Merluccius merluccius*). All the samples pertained to fish species with low lipid content. The concentrations obtained from the analysis of triplicates using the developed method are summarized in Table 9.

**Table 9.** Concentration of 9 musk fragrances (ng $g^{-1}$ dry weight) in three different fish species.

| Compound | COD (*Gadus morhua)* | SOLE (*Solea solea)* | HAKE (*Merluccius merluccius)* |
|---|---|---|---|
| DPMI | n.d. | <MQL | n.d. |
| ADBI | n.d. | n.d. | n.d. |
| AHMI | n.d. | n.d. | n.d. |
| ATTI | <MQL | <MQL | n.d. |
| HHCB | 17.5 | 6.5 | 12.6 |
| AHTN | 5.1 | 2.9 | 3.1 |
| MX | n.d. | n.d. | n.d. |
| MK | n.d. | n.d. | n.d. |
| HHCB-lactone | n.d. | <MQL | n.d. |

MQL: method quantification limit; n.d.: not detected.

HHCB and AHTN were found in all the samples at concentrations ranging from 6.5 to 17.5 ng $g^{-1}$ (d.w.) and 2.9 to 5.1 ng $g^{-1}$ (d.w.), respectively. Their appearance agrees with the fact that these are the most used polycyclic musk fragrances worldwide. However, AHTN lower concentrations than HHCB may be explained by the recent European directive 2008/42/EC, 2008 [33] that regulated its use in cosmetics. HHCB-lactone was only detected in sole and its concentration was found below the method quantification limit. The concentration ranges for cod and hake agrees with the ones reported by Cunha et al. [34] for the same species: 15.3 to 18.5 ng $g^{-1}$ (d.w.) and 6.3 to 7.6 ng $g^{-1}$ (d.w.) for HHCB and AHTN in cod samples and 14.7 to 29.3 ng $g^{-1}$ (d.w.) and 5.7 to 6.6 ng $g^{-1}$ (d.w.) for hake samples. As for the other polycyclic musks, DPMI was also found in sole, while ATTI was found in both sole and cod.

Results show that cod had the greatest concentration of musk fragrances followed by hake and sole. These results are in agreement with those reported by Trabalón et al. [32] where the overall concentration of musk fragrances had the same pattern as the obtained in this study. However, concentrations reported for cod samples were higher, and hake was the specie were the most compounds were detected.

Finally, none of the nitro musk fragrances (MX and MK) were detected in any of the samples analysed. This was expected as the use of these compounds in cosmetics was regulated or prohibited by the European directives 2002/34/EC, 2002 [6] and 1223/2009/EC, 2009 [35].

## 5. Conclusions

A novel SPME Arrow extraction technique was testedfor the determination of synthetic musk fragrances in fish samples. The developed method allowed the determination of the fragrances with MDLs ranging between 0.5 ng $g^{-1}$ (d.w.) and 2.5 ng $g^{-1}$ (d.w.) and MQLs ranged from 2.5 ng $g^{-1}$ (d.w.) to 5 ng $g^{-1}$ (d.w.) with only a lyophilization of the sample step as sample handling.

SPME Arrow demonstrated being able of extracting the compounds from the fish samples with an efficiency up to 10 times higher than conventional SPME fibres, providing better sensitivity and robustness.

Trace levels of these compounds were determined in different commercial fish samples with concentration levels of low ng $g^{-1}$ of HHCB and AHTN.

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