Solid-phase microextraction method for the determination of volatile compounds associated to oxidation of fish muscle

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Abstract

A procedure for the determination of volatile compounds derived from lipid oxidation of fish muscle samples is presented. Analytes are concentrated on a solid-phase microextraction fiber employed in headspace mode (HS-SPME), and selectively determined using gas chromatography in combination with mass spectrometry (GC–MS). The influence of several parameters on the efficiency of microextraction such as type of fiber, volume of sample, time, temperature, salting out effect and stirring was systematically investigated. A saline extraction of fish muscle followed by incubation on a CAR-PDMS fiber during 30 minutes at 60 ºC gave the most effective and accurate extraction of the analytes. Quantification of them was performed by MS in selected ion monitoring mode (SIM) and by the internal standard method. Satisfactory linearity, repeatability and quantification limits were achieved under these conditions. The method was applied for determining the volatile compounds associated to oxidation of Atlantic Horse Mackerel (*Trauchurus trauchurus*) minced muscle and excellent correlations were obtained with chemical indexes for monitoring lipid oxidation as peroxide value and thiobarbituric acid reactive substances. This combined technique is fast, simple, sensitive, inexpensive and useful to monitor target compounds associated to fish rancidity as 1-penten-3-ol, 2,3-pentanedione or 1-octen-3-ol.

Keywords: SPME, fish, volatiles, lipid oxidation, gas chromatography/mass spectrometry

1. Introduction

Fatty fish is an important and nutritional seafood particularly owing to the high concentration of polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (20:5ω-3) (EPA) and docosahexanoic acid (22:6ω-3) (DHA) [1]. Degradation of PUFAs by auto or enzymatic oxidation during storage and processing of fatty fish, easily leads to the formation of volatiles associated with rancidity [2]. For this, lipid oxidation has long been recognized as a leading cause of quality deterioration in fish muscle foods and is often the decisive factor in determining their shelf-life [3]. Most effort has been devoted to chemical methods aimed to measure oxidation. Peroxide value (PV) and the 2-thiobarbituric acid reactive substances assay (TBARS) are common measurements of lipid oxidation; however, because peroxides are decomposing to secondary products relatively quickly, and TBARS is not specific for malonaldehyde, the measurement of volatile compounds has become a well accepted indicator of lipid oxidation [4].

Several volatiles have been associated to the characteristic odors and flavors of oxidized fish, described as rancid, painty, fishy and cod-liver like [2]. Oxidation of unsaturated fatty acids in fish was related to the formation of *E*-2-pentenal, *E*-2-hexenal, *Z*-4-heptenal, (*E,E*)-2,4-heptadienal and 2,4,7-decatrienal [1]. Other volatiles formed during oxidation of fish lipids are 1-penten-3-ol, 1-octen-3-ol, 1,5-octadien-3-one and 2,6 nonadienal, some of them having high odor impact [5, 6]. Fish volatiles have been conventionally analyzed by gas chromatographic (GC) techniques. GC analyses for volatiles correlate with flavor scores by sensory analyses and detect low levels of oxidation in oils and foods [1]. Simultaneous steam distillation with solvent extraction has been employed for determining volatiles in fish muscle [7, 8] but is time- and solvent-consuming, which may result in the loss or degradation of some of the volatile compounds [9]. Analysis of volatiles in fish and seafood has been widely performed by several headspace techniques [10-15]. Both, dynamic head space and purge-and-trap (DHS techniques) coupled with gas chromatography have been extensively used for the analysis of aroma compounds in fish muscle and provided better sensitivity and efficacy than static head space. However, DHS techniques are more complex and expensive than other sample introduction systems and are not convenient for routine analysis [4].

Solid-phase microextraction (SPME) is an alternative extraction technique developed by Pawliszyn and co-workers in the early 90's that combines sampling and sample preparation in one step [16]. It is a solventless and economical method for sample preparation before gas chromatography and provides several advantages over other well established techniques for analyzing volatiles in foods in terms of sensitivity, selectivity and suitability for routine analysis [17]. This technique, used in the head space mode (HS-SPME), is particularly suitable for the analysis of volatiles and has been used for the analysis of flavor and freshness in several foodstuffs included seafood. The method has been applied to determine the concentration of aliphatic amines [18], volatiles of yellowfish tuna [19], differences in volatiles of raw and smoked fish species [20], the volatile composition of fish stored under controlled atmospheres and its relationship with the flavor [21] or the oxidative stability of microencapsulated fish oils [22].

SPME based techniques require careful optimization and selection of several parameters having significant influence in the analyses. Variables such as the type of fiber which determines the specificity of the extraction, the sample amount, the time and temperature of extraction, the salting-out effect or the desorption time of the fiber in the injector affect the preconcentration efficiency. In a recent paper, an optimization and detailed study of these parameters have been performed in order to propose a HS-SPME method for the analysis of oxidation products formed in fish oil enriched foods [23].

In this paper, a method based on HS-SPME coupled to GC-MS is proposed for the analysis of volatile compounds formed from lipid oxidation of fish muscle. The suitability of different fiber coatings has been determined. Then, the influence of the main factors affecting the microextraction has been extensively studied. The method has been carefully validated and was applied to determinate the lipid oxidation occurred in Atlantic Horse Mackerel minced muscle during chilled storage. The results obtained for volatiles were correlated with PV and TBARS indexes.

2. Experimental

2.1. Reagents

Acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, 2-ethylfuran, 2-pentylfuran, *E*-2-pentenal, *E*-2-hexenal, *E*-2-heptenal, *Z*-4-heptenal, 1-penten-3-one, 1-penten-3-ol, 1-octen-3-ol, 2,3-pentanedione, (*E,E*)-2,4-heptadienal and 3-methyl-3-buten-1-ol (internal standard) was obtained from Sigma-Aldrich (Steinheim, Germany). All chemicals and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

2.2. Materials

Four different coating fibers for HS-SPME were tested: 75µm Carboxen/polydimethylsiloxane coating (CAR-PDMS), 65µm polydimethylsiloxane/divinylbenzene coating (PDMS-DVB), 2 cm-50/30 µm Carboxen/polydimethylsiloxane/divinylbenzene (CAR-PDMS-DVB) and 65µm Carbowax/divinylbenzene coating fibers. These fibers were selected according to the different polarities and molecular weights of the studied analytes and since they were tested for determination of volatile compounds in several food matrixes [24-26]. They were obtained from Supelco (Bellefonte, PA, USA).

Oxidized Atlantic Horse Mackerel (*Trauchurus trauchurus*) muscle was used for method optimization. The oxidation was carried in our laboratory by holding it at -20 ºC during 7 months. A PV of 18.7 mequivO_2/kg lipid was achieved after this storage period. For the chilled experiments, fresh Atlantic Horse Mackerel, caught the night before of the study, was supplied by a local market (peroxide value of 0.45 mequivO_2/kg lipid) and was composed by 2.3% of lipid. The lipid composition is indicated in Table 1.

2.3. HS-SPME-GC method.

The oxidized Atlantic Horse Mackerel muscle was used for studying the different parameters affecting the SPME.

2.3.1. Extraction of volatile compounds.

For such purpose, 3 grams of oxidized minced muscle were homogenized for 2 minutes with a volume of 8 mL of ultrapure water or 8 mL of ultrapure water saturated in NaCl. The mixture was centrifuged (10 minutes, 3500 rpm) and a volume of supernatant, depending on the experiment, was successively analyzed.

2.3.2. Fiber selection

The different fibers were exposed to the head space of 1 mL of the saline extract obtained from the oxidized fish homogenate during 15 minutes at 60 ºC. The volatiles were desorbed in the GC injection port for 10 minutes at 300 ºC for CAR-PDMS, at 250 ºC for PDMS-DVB fiber, at 270 ºC for CAR-PDMS-DVB fiber and at 220 ºC for CW-DVB fiber according to technical recommendations. The absence of artifacts due to compounds remained into the fiber after desorption was also checked.

2.3.3. Experimental conditions for CAR-PDMS fiber.

The influence of salting out and stirring on the yield of the volatile extraction from fish muscle was evaluated after the extraction of 3 grams of oxidized minced fish muscle according with the conditions described above. A volume of 6 mL was then extracted by CAR-PDMS fiber during 15 minutes to 60 ºC with and without stirring.

A factorial design was performed to evaluate other parameters affecting the HS-SPME extraction efficiency as temperature and time of extraction and sample amount. The experiments were performed with saline extracts from oxidized fish muscle and with stirring. A two-level factorial design (2^3) was selected. This design was used to obtain the surface responses, fitting the data to a mathematical model, and to know what factors are statistically significant evaluating the effects of each factor and the interactions between factors. Two centerpoints were added and all the experiments were randomly performed. Corresponding experimental conditions studied are shown

in Table 2. The interval of sample amount was established between 0.5 and 6 mL. The upper limits of temperature and time (60 ºC and 30 minutes) were established in order to keep the composition of the fish muscle extracts since are very susceptible to oxidation. In addition, 30 minutes of extraction time allows automate the analyses. Data analysis was performed by means of the statistical package Statgraphics Plus for Windows V. 5.1.

The optimization of sample volume was performed using 20 ml vials with the oxidised fish muscle saline extracts. Different volumes (1, 3, 5 and 6 mL), depending of the experiment, were placed and the analyses were performed extracting the volatiles with a CAR-PDMS fiber to 60 ºC during 30 minutes and with stirring.

2.3.4. GC-MS analysis conditions

GC–MS analysis was performed in a Thermo Finnigan ThermoQuest (San Jose, CA, USA) gas chromatograph equipped with a split/splitless injector and coupled with a Trace quadrupole mass detector. Compounds were separated on a 30 m x 0.32 mm, 1 μm film thickness, fused silica DB-1701 (Folsom, CA, USA) capillary column. The GC oven temperature program was: 35 ºC for 3 minutes, followed by an increase of 3 ºC/minute to 70 ºC; then an increase of 10 ºC/minute to 200 ºC and finally an increase of 20 ºC/minute to a final temperature of 260 ºC hold for 5 minutes. Helium was employed as carrier gas, with a constant flow of 1.5 mL/min. Injector was operated in the split mode and its temperature was set at 220, 260, 270 or 300 °C (depending on the type of fiber coating to be desorbed). Transfer line temperature was maintained at 265 °C. The quadrupole mass spectrometer was operated in the electron impact mode (EI) and the source temperature was set at 200 °C. Initially, full scan mode data were acquired to determine appropriate masses for the later acquisition in selected ion monitoring mode (SIM) under the following conditions: mass range: 10-200 amu and scan rate: 0.220 s/scan. All the analyses were performed setting ionization energy at 70 eV, filament emission current at 150 μA and the electron multiplier voltage at 500 V.

2.3.5. Qualitative and quantitative analyses

Identification of components was based on computer matching with the reference mass spectra of the Wiley 6, Mainlib and Replib libraries and standards. Semiquantitative determination of volatiles was performed by the method of internal standard using 3-methyl-3-buten-1-ol. Quantitative determination was carried out by using an internal calibration curve that was built using stock solutions of the compounds in ultrapure water saturated in salt and analyzing them by the optimized HS-SPME method. Quantification limits were calculated to a signal to noise (S/N) ratio of 10. Repeatability was evaluated by analyzing 6 replicates of oxidized fish muscle. The stability of the samples was evaluated by comparing the results obtained after analysis of the same oxidized fish muscle sample under storage during 6 months to -80 ºC.

2.4. Chilled storage of Atlantic Horse Mackerel minced muscle

8 kg of fresh Atlantic Horse Mackerel *(Trachurus trachurus),* 20-24 different fish, were debonned, eviscerated and the white muscle was separated and minced. Streptomycine sulfate (200 ppm) was added for inhibiting microbial growth. Portions of 8 g were placed into 50-ml Erlenmeyer flasks and were kept refrigerated at 4 ºC on ice during 5 days. Triplicate samples were taken at different sampling times for performing the different analyses. Oxidation was calculated as the time (in days) required for a sudden change in the rate of the oxidation by the method of tangents to the two parts of the kinetic curve [27].

2.4.1. Peroxide value

Peroxide value of fish muscle was determined by the ferric thiocyanate method [28] and was expressed as mmol oxygen/kg lipid.

2.4.2. TBARS index

The thiobarbituric acid reactive substances index (TBARS) (mg malonaldehyde/kg muscle) was determined according to Vyncke [29].

2.4.3. Volatile analysis

Volatile compounds were analyzed by means of the optimized HS-SPME method described as follows: volatiles were extracted by homogenizing 3 g of fish muscle with 8 mL of saline ultrapure water solution and 6 mL of supernatant were placed in a 20 mL vial fitted with a silicone septum. The CAR-PDMS fiber was exposed to the headspace of extract by incubating to 60 ºC during 30 minutes under magnetic stirring. The fiber was immediately desorbed in the gas chromatograph injector to 300 ºC during 10 minutes.

2.4.4. Statistical analysis

Analyses were performed in triplicate. The data were compared by one-way analysis of variance (ANOVA) [30], and the means were compared by a least squares difference method. Linear regressions were employed for the calibration graphs by Pearson coefficients. Significance was declared at $p < 0.05$. Statistical analyses were performed with the statistical package Statgraphics Plus for Windows V. 5.1.

3. Results and discussion

3.1 HS-SPME-GC-MS methodology

A set of preliminary experiments were conducted to perform a good chromatographic separation of the volatiles associated to fish rancidity. Volatiles present in 3 g of oxidized Atlantic Horse Mackerel muscle were extracted with 8 mL of ultrapure water and then, 1 mL of the supernatant was incubated in a CAR-PDMS 75 µm fiber (60 ºC during 15 min). Fiber was desorbed in the injector of the gas chromatograph and volatile compounds were analyzed in full scan mode (Fig. 1). As a result, 79 compounds were identified (Table 3) and 16 of them selected (Table 4) as representatives of lipid oxidation in fish muscle according to previous studies [1, 14, 26, 31-33]. The different mass spectra of the target compounds were carefully studied for selecting the correct ions for injection in SIM mode (Table 4), in order to improve the signal to noise ratio. The optimization of the SPME methodology and the subsequent quantification in the storage experiment were performed in SIM mode. Fiber coating has shown to determine qualitative and quantitative differences in fish volatile profiles obtained by SPME technique [34]. In order to avoid possible interferences, the memory effect of the different fibers was studied. A desorption of the fibers into the injector of the chromatograph during 2 minutes was performed and after that, the fibers were reinserted and blank analyses were run. Any of the selected compounds was observed in the blank chromatogram for any fiber. A desorption time of 10 min was selected in order to automatize the GC analysis and to assure the total clean of the fiber. The increment between 2 and 10 minutes didn't influence the resolution of the peaks.

The four coating fibers studied in this work gave different results in terms of sensitivity. Figure 2 shows the analysis corresponding to volatiles extracted from oxidized Atlantic Horse Mackerel muscle using the different fibers. The results clearly showed that CAR-PDMS fiber enabled the detection of a wider range of compounds and produced higher signal intensities than CAR-PDMS-DVB, PDMS-DVB and CW-DVB fibers, especially for the smallest molecular weight analytes. Therefore, it was selected as the fiber for the HS-SPME method here proposed.

This fiber has been also used for determining volatile spoilage indicators in several fish species [21, 33]. Guillen M. D. et al. [34] found that CAR-PDMS (100 µm film thickness) was the most suitable fiber for the retention of the most volatile compounds in smoked fish. These authors [34] also tested the utility of polyacrilate (85 µm film thickness) fiber which was suitable for determining the head space composition in a broader volatility range and PDMS (100 µm film thickness) fiber which showed minor retention of the target compounds. CAR-PDMS (75 µm film thickness) was also chosen by Duflos G. et al. [35] for analyzing the freshness of Whiting since it combined the best signal to noise ratio with maximum extraction of compounds. In a recent paper,

CAR-PDMS (75 μm film thickness) has also demonstrated to provide the highest sensitivity and reproducibility in the analysis of volatiles associated to oxidation of fish oil enriched food emulsions [23]. In situ derivatization of volatiles on CAR-PDMS fiber coating surface has been recently proposed for quantifying the formation of formaldehyde in fish muscle [36]. This last technique allows to reach higher selectivity and sensitivity, but the methodology only determinates specific compounds.

As regards to the effect of the salting out effect and stirring, the highest sensitivity for almost all the target compounds was achieved by the extraction in ultrapure water saturated in NaCl and with stirring. The addition of salt increases the ionic strength of the water sample by lowering the solubility of analytes in the aqueous phase and stirring enhance the extraction efficiency in non-equilibrium situations increasing the sensitivity [37].

A selection of the microextracting conditions of CAR-PDMS was then performed by a factorial design to get information about the significance of the experimental parameters. Table 2 shows the corresponding experimental design matrix. Response was evaluated in terms of peak area for all compounds. As an example of the behavior of the target volatiles associated to fish lipid oxidation, Fig. 3 shows the Pareto chart for *E*-2-Hexenal. Results showed that all selected variables produced significant effects and that no significant interactions between factors were apparent. Sample amount, extraction temperature and extraction time were statistically significant factors and, therefore, the peak area of *E*-2-Hexenal increased when the three factors increased (positive effect).

Factorial experimental design only explains what factors are significant but can not optimize the response because only evaluates 2 levels per factor. An extraction time of 30 min at 60 ºC procured the best extraction of volatiles associated to oxidation. These values were the maximum in the experimental design and factors as time and temperature of incubation are positively correlated with the efficiency of the extraction [37]. In addition, these conditions are not able to provoke oxidation of the samples. Therefore, temperature and time were fixed in 60 ºC and 30 min respectively.

The fiber efficiency in SPME is not always directly proportional to sample amount [37]. In the present study, the volume of the saline fish muscle extract was optimized by evaluating the responses obtained after the extraction of 1, 3, 5 and 6 mL. The highest signals (peak areas) were obtained extracting the maximum volume evaluated according to Górecki et al. [38], that established that the head space volume in the vial should be minimized to increase the extraction efficiency. Consequently, 6 mL was finally selected as the amount of sample.

The SIM chromatogram obtained after the analysis of an oxidized Atlantic Horse Mackerel muscle sample using the optimized conditions achieved during this study is shown in Figure 4.

3.2. Validation of the method

The method employed in the optimized conditions was validated for 16 of the 76 compounds detected (Table 5). Its linearity was evaluated by using samples of ultrapure water saturated in NaCl spiked with increasing concentrations of the analytes ranged between 0.2 to 500 ng/ml. A satisfactory linearity (correlation coefficients from 0.985 to 0.999) was obtained for all compounds. As for repeatability, relative standard deviations of peak areas between 0.6 and 13.9% were achieved (n=6). The results of the stability test indicated that during the storage period to -80 ºC the volatile composition remained stable since relative standard deviations between 0.6 and 12.2% were achieved for almost all the target compounds. 2-ethylfuran (30.3%), *E*-2-pentenal (20.2%) and *E*-2-hexenal (28.3%) were the only compounds that didn't show satisfactory stability.

Quantification limits of the method, defined for a signal to noise ratio (S/N) of 10, ranged from 0.03 to 0.34 ng per gram of fish muscle depending of compound. A potential shortcoming of SPME based methods is that the extraction yield can be matrix dependent. In such a case, quantification should be performed using the time consuming standard addition method. Possible matrix effects of the HS-SPME method here proposed were investigated by evaluating the recoveries of each volatile. For such purpose, extracts of samples of Atlantic Horse Mackerel muscle were spiked with 100 ng/g of the selected compounds. The response corresponding to each compound was corrected

with that obtained for non-spiked aliquots of the same extract. Spiked and non-spiked samples were processed in triplicate. Obtained results suggest that the efficiency of the process is scarcely affected by the matrix for the most of compounds evaluated (Table 5). Few compounds, like 1-penten-3-one, *E*-2-hexenal or (*E,E*)-2,4-heptadienal showed a very poor recoveries, therefore, standard addition method should be use for their quantification.

3.3. Chilled storage of Atlantic Horse Mackerel minced muscle

The proposed methodology was employed to assess the oxidative deterioration in terms of volatile formation during the storage of Atlantic Horse Mackerel minced muscle at 4 ºC. Propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage. Other target volatiles formed in significant concentrations and closely related to lipid oxidation were 2-ethylfuran, *Z*-4-heptenal and 3,5-octadien-2-one.

Propanal is a product from 16-hydroperoxide formed by autoxidation of methyl linolenate and from 15-hydroperoxide formed by photosensitized oxidation of methyl linolenate [1]. 1-penten-3-ol is formed by the action of 15-lipoxygenase on EPA (20:5 n-3) [13, 31]. Hexanal can be produced via linoleic acid 13-hydroperoxide and, in addition, a degradation of preformed volatiles as 2,4-decadienal or 2-octenal has been considered responsible for the abundant occurrence of this compound in different lipid systems [32, 39]. 1-octen-3-ol is an important contributor to off-flavors due to its low odor score and it has been reported to be formed from oxidation of arachidonic acid by 12-lipoxygenase [2]. 2-ethylfuran can be produced via 12-hydroperoxide of linolenate (18:3 n-3), via 14-hydroperoxide EPA (20:5 n-3) or via 16-hydroperoxide DHA (22:6 n-3) [40]. *Z*-4-heptenal is produced via 2,6-nonadienal that is produced by the action of 12-lipoxygenase on EPA [14, 26]. An autoxidation of EPA to (*E,Z*)-2,4-heptadienal and (*E,Z*)-3,5-octadien-2-one has been also proposed [31, 41].

During the storage of fish muscle, a strong increase in the volatile formation was achieved after the second day of storage in agreement with a first detection of rancid off-flavors. This increment was especially important for 1-penten-3-ol and 2,3-pentanedione. The formation of these compounds showed induction periods of 1.9 and 1.9 days respectively (Fig. 5) and the levels achieved by the second and third days were: 56.0 ± 9.8 and 168.2 ± 7.6 ng/g for 1-penten-3-ol (increment of 200.2%) and 96.0 ± 7.8 and 234.2 ± 8.2 ng/g for 2,3-pentanedione (increment of 144.0%). Formation of 1-octen-3-ol showed an induction period of 2.1 days and the levels achieved during the days 2 and 3 were 61.7 ± 0.8 and 65.8 ± 0.3 ng/g respectively (Fig. 5). This little increment (6.6%) was enough for correlating this analysis with the detection of rancid off-flavors since 1-octen-3-ol is a potent odorant of the unpleasant rancid flavor with a very low sensorial threshold value [1].

Analyses of volatile compounds were correlated with PV and TBARS since these indexes showed induction periods of 2 and 1.9 days respectively (Fig. 5). 1-penten-3-ol, 2,3-pentanedione and 1-octen-3-ol were the compounds showing the higher correlations (Fig. 5). Pearson coefficients obtained between the formation of 1-penten-3-ol and PV and TBARS indexes were 0.9832 and 0.9970 respectively. Similar correlations were obtained for 2,3-pentanedione (R^2= 0.9834 and R^2= 0.9986) and for 1-octen-3-ol (R^2= 0.9593 and R^2= 0.9767). According to these results, these volatiles were chosen the best markers of lipid oxidation.

4. Conclusions

The developed analytical method, simple and inexpensive, enables the simultaneous determination of volatile compounds associated to oxidation of fish muscle. The procedure exhibited several advantages over more conventional methods including the use of smaller amounts of sample (only 3 grams of fish muscle were necessary), minimal sample handling, low cost, time consuming or suitability for routine analysis. Type of fiber, salting out effect, stirring, exposure temperature, exposure time and sample volume were parameters influencing SPME carefully studied and optimized. Validation showed satisfactory results in terms of linearity, sensitivity, repeatability,

accuracy and stability of the samples. Analysis of volatile compounds could be successfully applied to indicate the oxidative deterioration in fish muscle since Pearson coefficients higher than 0.97 were achieved with PV and TBARS indexes. Because to the high levels of 1-penten-3-ol, 2,3-pentanedione and 1-octen-3-ol formed during the storage and the high correlations with the chemical indexes for assessing the extent of oxidation, they were preferred as potential markers to evaluate the lipid oxidation in fish muscle.

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Figure Captions

Figure 1: Full scan chromatogram obtained after analysis of oxidized Atlantic Horse Mackerel muscle. Peaks are identified as in Table 4.

Figure 2: Evaluation of extraction efficiencies from different fiber coatings.

Figure 3: Standardized Pareto chart obtained for *E*-2-hexenal. Vertical line indicates the statistical significance bound for the different effects.

Figure 4: SIM chromatogram obtained from oxidized Atlantic Horse Mackerel muscle in the optimized conditions.

Figure 5: Time course of lipid oxidation of Atlantic Horse Mackerel muscle measured by headspace volatiles, and PV and TBARS indexes.

413 Figure 1

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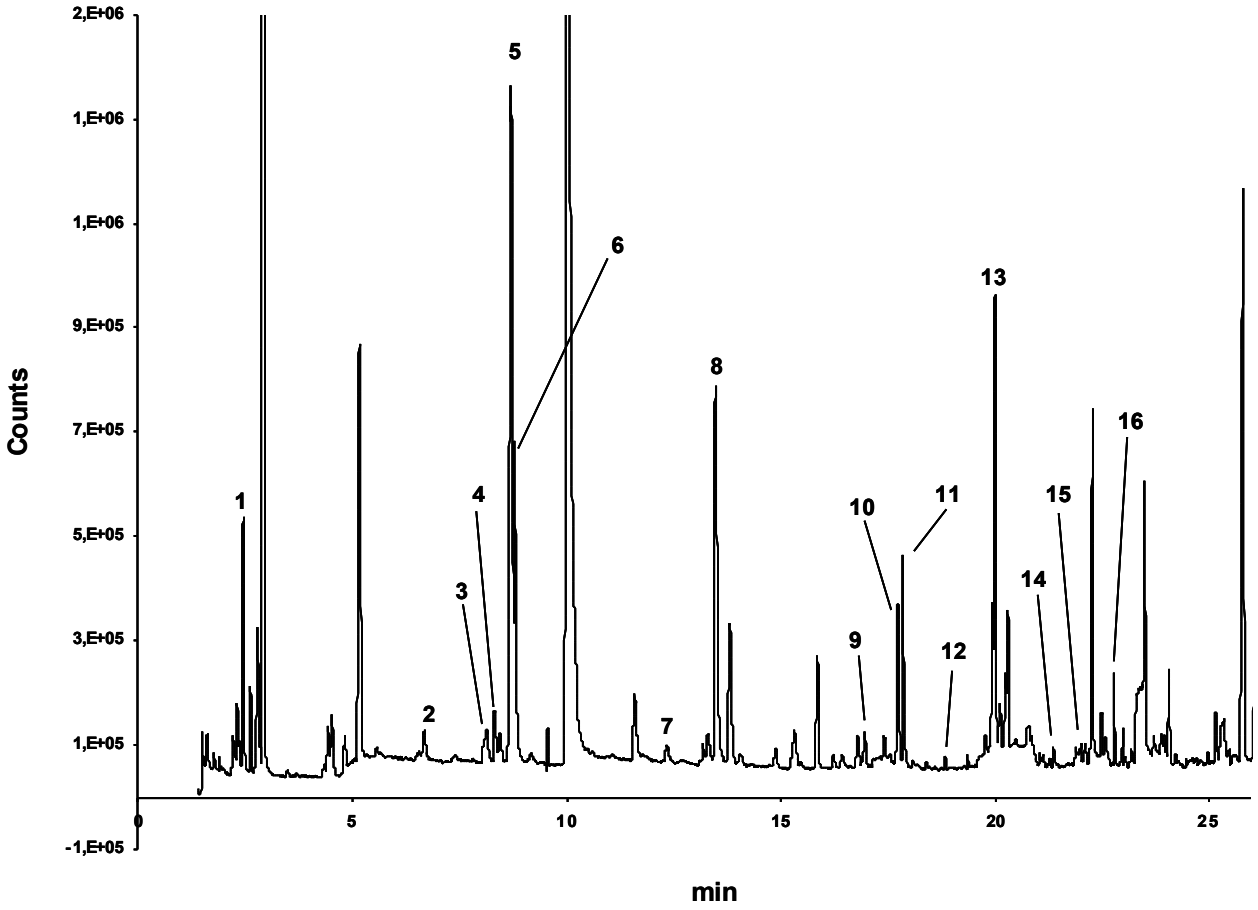

417 Figure 2

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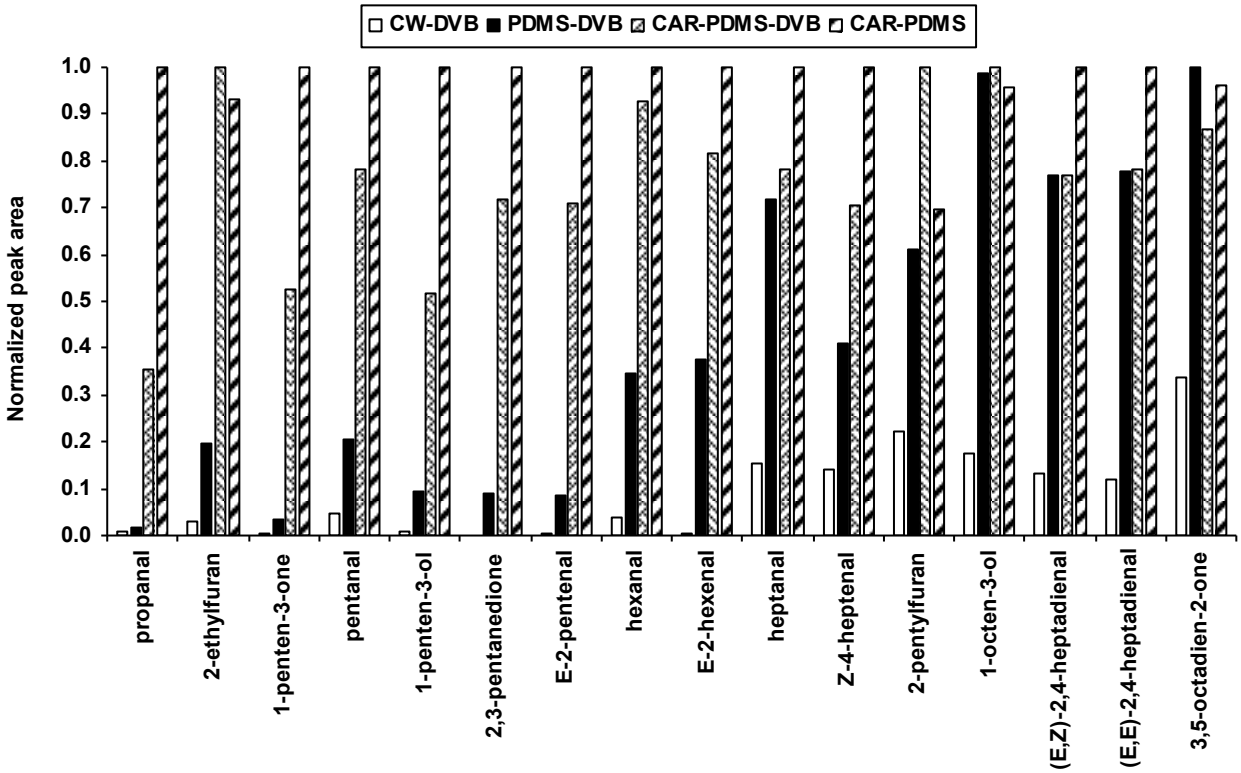

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426 Figure 3

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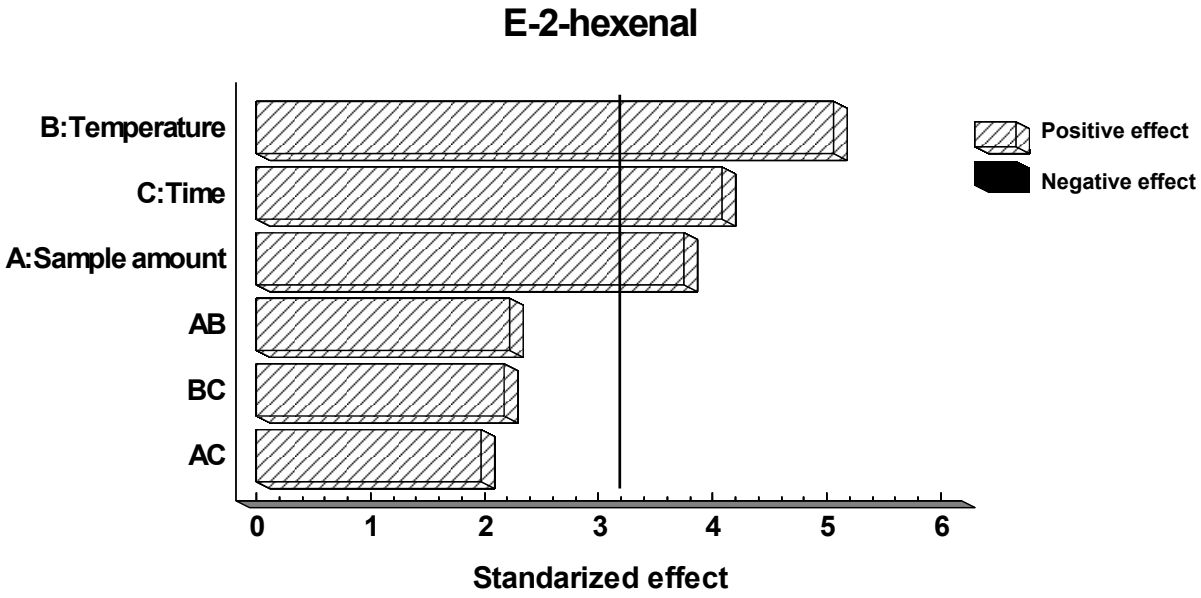

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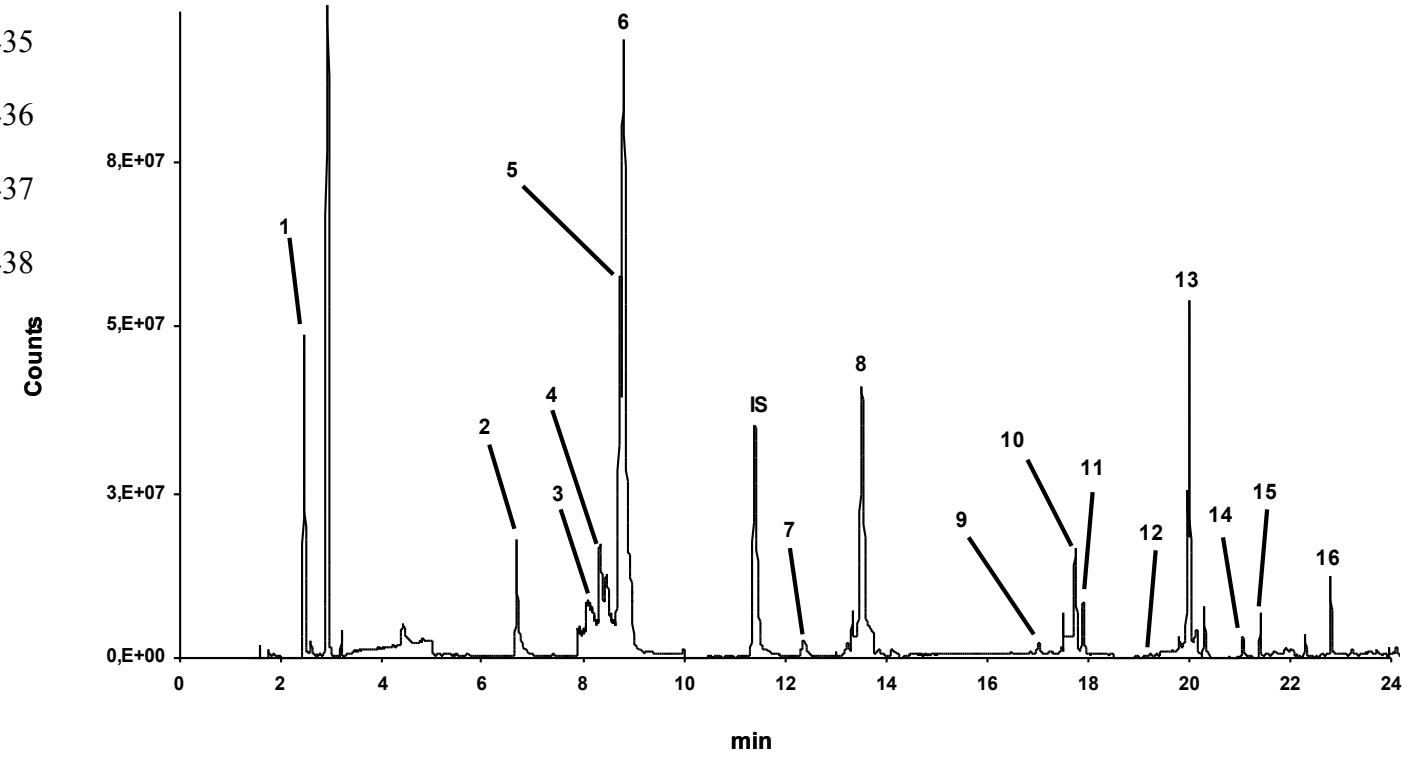
Figure 4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5,E+07
3,E+07
0,E+00
0
2
4
6
8
10
12
14
16
18
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min
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IS
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439 Figure 5

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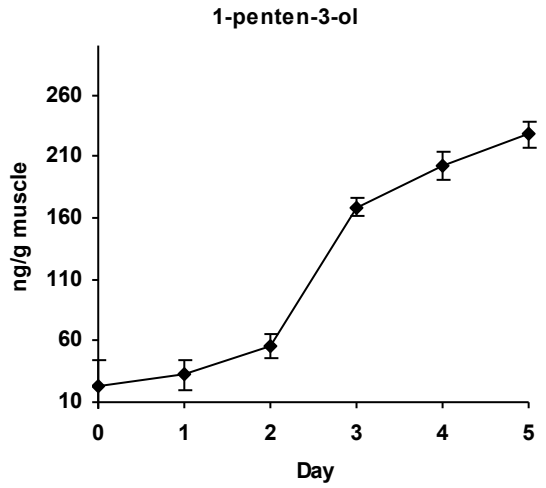

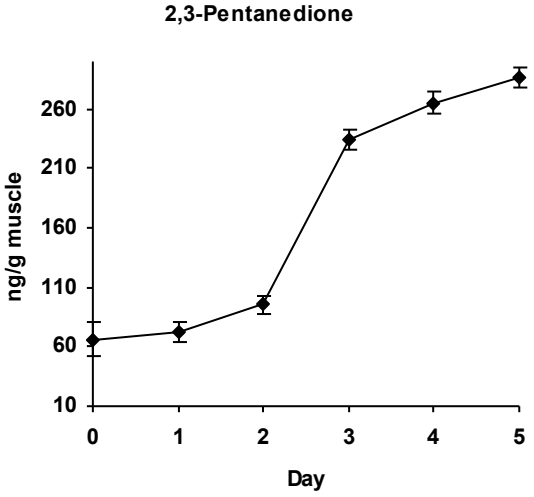

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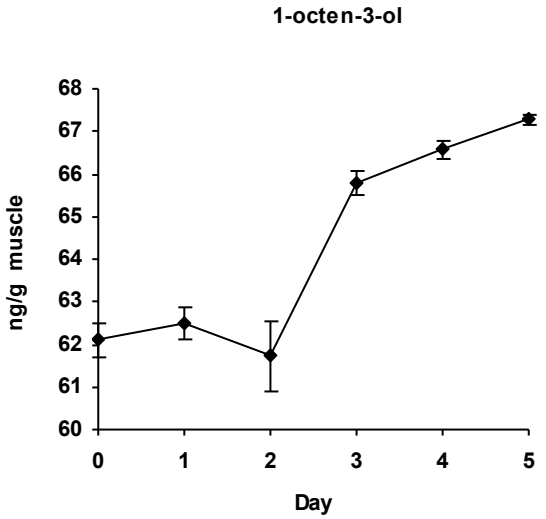

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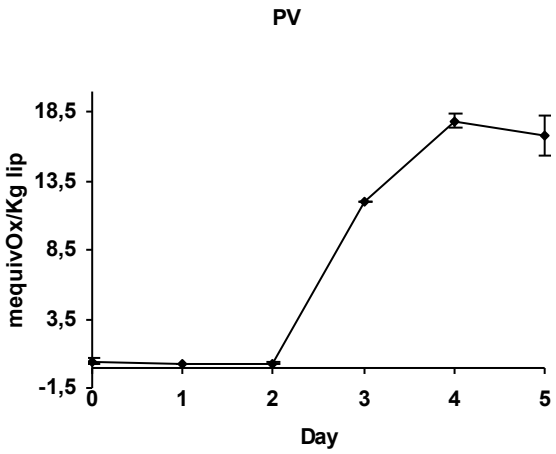

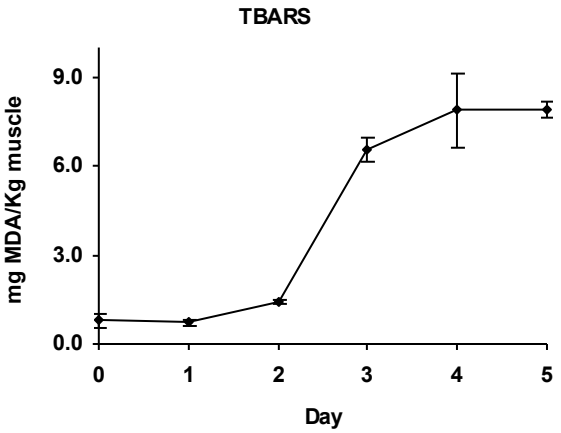

443

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445 Table 1: Fatty Acids (w/w % of Total Fatty Acids) of Atlantic Horse Mackerel muscle used for the

446 chilled experiments.

447

Fatty acid	*% of total fatty acids*	*Fatty acid*	*% of total fatty acids*
14:0	**5.3**	**20:1n-9**	**4.5**
15:0	**0.5**	**18:4n-3**	**1.4**
16:0	**19.6**	**20:2**	**0.3**
16:1n-7	**3.9**	**20:4 n- 6**	**1.1**
17:0	**0.6**	**22:1n-11**	**6.5**
18:0	**4.3**	**22:1n-9**	**0.6**
18:1n-9	**10.5**	**20:4n-3**	**0.8**
18:1n-7	**2.3**	**20:5n-3**	**8.4**
19:0	**11.9**	**24:1n-9**	**1.4**
18:2n-6	**1.1**	**22:5n-3**	**2.2**
18:3n-3	**1.1**	**22:6n-3**	**23.5**

448

449

450 Table 2: Experimental matrix and responses obtained in the factorial screening design.

451

Experiment	*Amount of sample (mL)*	*Temperature (°C)*	*Time (min)*	*Peak area[a] (counts)*
1	**3.5**	**50**	**20**	**1 153 157**
2	**1**	**60**	**30**	**1 067 379**
3	**1**	**60**	**10**	**509 695**
4	**6**	**40**	**10**	**295 690**
5	**3.5**	**50**	**20**	**1 022 004**
6	**1**	**40**	**10**	**114 483**
7	**6**	**40**	**30**	**777 308**
8	**6**	**60**	**30**	**2 856 975**
9	**6**	**60**	**10**	**1 011 315**
10	**1**	**40**	**30**	**370 766**

452 **[a] Peak area values corresponding to *E*-2-hexenal**.

453

454

455

456 Table 3: Volatile compounds identified in an oxidized Atlantic Horse Mackerel muscle sample.

457

Compound	Identification [a]
Alcohols	
Ethanol	**MS**
1-Propanol	**MS**
2-Methyl-1-propanol	**MS**
1-Penten-3-ol	**MS, STD**
1-Pentanol	**MS**
2-Penten-1-ol	**MS**
2-Hepten-1-ol	**MS**
3-Pentanol	**MS**
3-Hexen-1-ol	**MS**
1-Hexanol	**MS**
4-Heptanol	**MS**
1-Octen-3-ol	**MS, STD**
1-Octanol	**MS**
2-Octen-1-ol	**MS**
2-Nonen-1-ol	**MS**
Phenol	**MS**
Ethyllinalool	**MS**
Aldehydes	
Acetaldehyde	**MS, STD**
Propanal	**MS, STD**
2-Methylpropanal	**MS**
Butanal	**MS, STD**
Pentanal	**MS, STD**
E-2-Pentenal	**MS, STD**
Hexanal	**MS, STD**
E-2-Hexenal	**MS, STD**
Heptanal	**MS, STD**
Z-4-Heptenal	**MS, STD**
E-2-Heptenal	**MS, STD**
Benzaldehyde	**MS**
Octanal	**MS, STD**
(E,Z)-2,4-Heptadienal	**MS**
(E,E)-2,4-Heptadienal	**MS, STD**
Nonanal	**MS, STD**
3,7-Dimethyl-6-octenal	**MS**
Decanal	**MS**
Amines	
Trimethylamine	**MS**
Aromatics	
Toluene	**MS**
Ethylbenzene	**MS**
Xilene (not identified)	**MS**
Styrene	**MS**
Propylbenzene	**MS**
Trimethylbenzene	**MS**
Esters	
Ethylacetate	**MS, STD**
Furans	
2-Methylfuran	**MS**
2-Ethylfuran	**MS, STD**
2-Pentylfuran	**MS**
Noncyclic hydrocarbons	
Pentane	**MS**
3-Methyl-1-butene	**MS**
2-Pentene	**MS**
2-Methyl-1,3-pentadiene	**MS**
Hexane	**MS**
Octane	**MS**
1-Octadecene	**MS**
Pentadecane	**MS**
Eicosane	**MS**
5-Eicosene	**MS**
5-Nonadecene	**MS**
Nonadecane	**MS**
Octadecane	**MS**
Ketones	
Acetone	**MS, STD**
2,3-Butanedione	**MS**
2-Pentanone	**MS**
1-Penten-3-one	**MS, STD**
3-Pentanone	**MS**
2,3-Pentanedione	**MS, STD**
3-Hidroxy-2-butanone	**MS**
3,3-Dimethyl-2-butanone	**MS**
2-Hydroxi-3-pentanone	**MS**
6-Methyl-2-heptanone	**MS**
2,3-Octanedione	**MS**
6-Octen-2-one	**MS**
2-Nonanone	**MS**
Acetophenone	**MS**
3,5-Octadien-2-one	**MS**
4-Methyl-ciclohexanone	**MS**
Chlorinated compounds	
Dichloromethane	**MS, STD**
Chloroform	**MS, STD**
Sulfur compounds	
Carbon disulphide	**MS**

458 [a] Compounds were identified by comparison with reference substances on the basis of the following criteria: MS
459 obtained from Mainlib, Wiley 6 and Replib libraries (MS) and retention time and spectra of authentic reference
460 compounds (STD).
461

462 Table 4: Selected compounds for optimization of HS-SPME method and selected mass in the SIM
463 chromatograms.
464

Number	Compound name	Retention time (min)	SIM Mass
1	Propanal	2.51	29+57+58
2	2-Ethylfuran	6.75	53+81+96
3	1-Penten-3-one	8.30	27+55+84
4	Pentanal	8.40	29+41+44+57+58
5	1-Penten-3-ol	8.83	57+86
6	2,3-Pentanedione	8.85	43+100
7	*E*-2-Pentenal	12.34	55+83+84
8	Hexanal	13.46	44+56+72+82
9	*E*-2-Hexenal	17.04	41+55+69+83+98
10	Heptanal	17.78	44+55+70
11	*Z*-4-Heptenal	17.92	41+55+68
12	2-Pentylfuran	18.94	81+138
13	1-Octen-3-ol	20.04	52+72
14	(*E*,*E*)-2,4-Heptadienal	21.05	81+110
15	(*E*,*Z*)-2,4-Heptadienal	21.43	81+110
16	3,5-Octadien-2-one	22.80	81+95+124
IS	3-Methyl-3-buten-1-ol (IS)	11.46	43+55+69

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469 Table 5: Linearity, recovery, detection limits and precision of the optimized HS-SPME method.

470

Compound	Correlation[a]	Recovery[a] (%)	LOQs[a] (ng/g fish muscle)	RSD (%) (n=6)
Propanal	**0.999**	**77.5 ± 6.6**	**0.34**	**13.9**
2-Ethylfuran	**0.995**	**115.8 ± 12.2**	**0.04**	**10.5**
1-Penten-3-one	**0.996**	**31.6 ± 1.7**	**0.05**	**5.4**
Pentanal	**0.999**	**109.7 ± 7.7**	**0.05**	**7.0**
1-Penten-3-ol	**0.999**	**105.9 ± 7.3**	**0.13**	**6.9**
2,3-Pentanedione	**0.999**	**102.8 ± 11.2**	**0.10**	**10.9**
***E*-2-Pentenal**	**0.998**	**116.9 ± 15.3**	**0.15**	**13.1**
Hexanal	**0.991**	**103.2 ± 11.3**	**0.06**	**11.0**
***E*-2-Hexenal**	**0.996**	**66.0 ± 1.3**	**0.09**	**2.0**
Heptanal	**0.992**	**109.1 ± 5.7**	**0.04**	**5.3**
***Z*-4-Heptenal**	**0.986**	**93.5 ± 5.0**	**0.04**	**5.4**
2-Pentylfuran	**0.987**	**93.6 ± 5.1**	**0.06**	**4.8**
1-Octen-3-ol	**0.987**	**103.3 ± 4.6**	**0.03**	**4.5**
***(E,E)*-2,4-Heptadienal**	**0.987**	**59.3 ± 4.8**	**0.10**	**4.8**
(*E,Z*)-2,4-Heptadienal	**—**	**—**	**—**	**0.6**
3,5-Octadien-2-one	**—**	**—**	**—**	**8.7**

471 [a] Values of correlation, recovery and LOQs for (*E,E*)-2,4-heptadienal and 3,5-octadien-3-one not
472 showed because no availability of standards.

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