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

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11

12 **Abstract**

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

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

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31

32 **1. Introduction**

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

44 Several volatiles have been associated to the characteristic odors and flavors of oxidized fish,
45 described as rancid, painty, fishy and cod-liver like [2]. Oxidation of unsaturated fatty acids in fish
46 was related to the formation of *E*-2-pentenal, *E*-2-hexenal, *Z*-4-heptenal, (*E,E*)-2,4-heptadienal and
47 2,4,7-decatrinal [1]. Other volatiles formed during oxidation of fish lipids are 1-penten-3-ol, 1-
48 octen-3-ol, 1,5-octadien-3-one and 2,6 nonadienal, some of them having high odor impact [5, 6].
49 Fish volatiles have been conventionally analyzed by gas chromatographic (GC) techniques. GC
50 analyses for volatiles correlate with flavor scores by sensory analyses and detect low levels of
51 oxidation in oils and foods [1]. Simultaneous steam distillation with solvent extraction has been
52 employed for determining volatiles in fish muscle [7, 8] but is time- and solvent-consuming, which
53 may result in the loss or degradation of some of the volatile compounds [9]. Analysis of volatiles in
54 fish and seafood has been widely performed by several headspace techniques [10-15]. Both,
55 dynamic head space and purge-and-trap (DHS techniques) coupled with gas chromatography have
56 been extensively used for the analysis of aroma compounds in fish muscle and provided better
57 sensitivity and efficacy than static head space. However, DHS techniques are more complex and
58 expensive than other sample introduction systems and are not convenient for routine analysis [4].

59 Solid-phase microextraction (SPME) is an alternative extraction technique developed by Pawliszyn
60 and co-workers in the early 90's that combines sampling and sample preparation in one step [16]. It
61 is a solventless and economical method for sample preparation before gas chromatography and
62 provides several advantages over other well established techniques for analyzing volatiles in foods
63 in terms of sensitivity, selectivity and suitability for routine analysis [17]. This technique, used in
64 the head space mode (HS-SPME), is particularly suitable for the analysis of volatiles and has been
65 used for the analysis of flavor and freshness in several foodstuffs included seafood. The method has
66 been applied to determine the concentration of aliphatic amines [18], volatiles of yellowfish tuna
67 [19], differences in volatiles of raw and smoked fish species [20], the volatile composition of fish
68 stored under controlled atmospheres and its relationship with the flavor [21] or the oxidative
69 stability of microencapsulated fish oils [22].

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

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

83 2. Experimental

84 2.1. Reagents

85 Acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, 2-ethylfuran, 2-
86 pentylfuran, *E*-2-pentenal, *E*-2-hexenal, *E*-2-heptenal, *Z*-4-heptenal, 1-penten-3-one, 1-penten-3-ol,
87 1-octen-3-ol, 2,3-pentanedione, (*E,E*)-2,4-heptadienal and 3-methyl-3-buten-1-ol (internal standard)
88 was obtained from Sigma-Aldrich (Steinheim, Germany). All chemicals and solvents used were
89 either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

90

91 2.2. Materials

92 Four different coating fibers for HS-SPME were tested: 75µm Carboxen/polydimethylsiloxane
93 coating (CAR-PDMS), 65µm polydimethylsiloxane/divinylbenzene coating (PDMS-DVB), 2 cm-
94 50/30 µm Carboxen/polydimethylsiloxane/divinylbenzene (CAR-PDMS-DVB) and 65µm
95 Carbowax/divinylbenzene coating fibers. These fibers were selected according to the different
96 polarities and molecular weights of the studied analytes and since they were tested for
97 determination of volatile compounds in several food matrixes [24-26]. They were obtained from
98 Supelco (Bellefonte, PA, USA).

99 Oxidized Atlantic Horse Mackerel (*Trauchurus trauchurus*) muscle was used for method
100 optimization. The oxidation was carried in our laboratory by holding it at -20 °C during 7 months. A
101 PV of 18.7 mequivO₂/kg lipid was achieved after this storage period. For the chilled experiments,
102 fresh Atlantic Horse Mackerel, caught the night before of the study, was supplied by a local market
103 (peroxide value of 0.45 mequivO₂/kg lipid) and was composed by 2.3% of lipid. The lipid
104 composition is indicated in Table 1.

105 2.3. HS-SPME-GC method.

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

108

109 2.3.1. Extraction of volatile compounds.

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

114

115 2.3.2. Fiber selection

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

122

123 2.3.3. Experimental conditions for CAR-PDMS fiber.

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

128 A factorial design was performed to evaluate other parameters affecting the HS-SPME extraction
129 efficiency as temperature and time of extraction and sample amount. The experiments were
130 performed with saline extracts from oxidized fish muscle and with stirring. A two-level factorial
131 design (2³) was selected. This design was used to obtain the surface responses, fitting the data to a
132 mathematical model, and to know what factors are statistically significant evaluating the effects of
133 each factor and the interactions between factors. Two centerpoints were added and all the
134 experiments were randomly performed. Corresponding experimental conditions studied are shown

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

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

144

145 2.3.4. GC-MS analysis conditions

146 GC-MS analysis was performed in a Thermo Finnigan ThermoQuest (San Jose, CA, USA) gas
147 chromatograph equipped with a split/splitless injector and coupled with a Trace quadrupole mass
148 detector. Compounds were separated on a 30 m x 0.32 mm, 1 µm film thickness, fused silica DB-
149 1701 (Folsom, CA, USA) capillary column. The GC oven temperature program was: 35 °C for 3
150 minutes, followed by an increase of 3 °C/minute to 70 °C; then an increase of 10 °C/minute to 200
151 °C and finally an increase of 20 °C/minute to a final temperature of 260 °C hold for 5 minutes.
152 Helium was employed as carrier gas, with a constant flow of 1.5 mL/min. Injector was operated in
153 the split mode and its temperature was set at 220, 260, 270 or 300 °C (depending on the type of
154 fiber coating to be desorbed). Transfer line temperature was maintained at 265 °C. The quadrupole
155 mass spectrometer was operated in the electron impact mode (EI) and the source temperature was
156 set at 200 °C. Initially, full scan mode data were acquired to determine appropriate masses for the
157 later acquisition in selected ion monitoring mode (SIM) under the following conditions: mass range:
158 10-200 amu and scan rate: 0.220 s/scan. All the analyses were performed setting ionization energy
159 at 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V.

160

161 2.3.5. Qualitative and quantitative analyses

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

171

172 2.4. Chilled storage of Atlantic Horse Mackerel minced muscle

173 8 kg of fresh Atlantic Horse Mackerel (*Trachurus trachurus*), 20-24 different fish, were deboned,
174 eviscerated and the white muscle was separated and minced. Streptomycine sulfate (200 ppm) was
175 added for inhibiting microbial growth. Portions of 8 g were placed into 50-ml Erlenmeyer flasks
176 and were kept refrigerated at 4 °C on ice during 5 days. Triplicate samples were taken at different
177 sampling times for performing the different analyses. Oxidation was calculated as the time (in days)
178 required for a sudden change in the rate of the oxidation by the method of tangents to the two parts
179 of the kinetic curve [27].

180

181 2.4.1. Peroxide value

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

184

185 2.4.2. TBARS index

186 The thiobarbituric acid reactive substances index (TBARS) (mg malonaldehyde/kg muscle) was
187 determined according to Vyncke [29].
188

189 2.4.3. Volatile analysis

190 Volatile compounds were analyzed by means of the optimized HS-SPME method described as
191 follows: volatiles were extracted by homogenizing 3 g of fish muscle with 8 mL of saline ultrapure
192 water solution and 6 mL of supernatant were placed in a 20 mL vial fitted with a silicone septum.
193 The CAR-PDMS fiber was exposed to the headspace of extract by incubating to 60 °C during 30
194 minutes under magnetic stirring. The fiber was immediately desorbed in the gas chromatograph
195 injector to 300 °C during 10 minutes.
196

197 2.4.4. Statistical analysis

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

203 3. Results and discussion

204 3.1 HS-SPME-GC-MS methodology

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

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

229 This fiber has been also used for determining volatile spoilage indicators in several fish species [21,
230 33]. Guillen M. D. et al. [34] found that CAR-PDMS (100 μm film thickness) was the most
231 suitable fiber for the retention of the most volatile compounds in smoked fish. These authors [34]
232 also tested the utility of polyacrilate (85 μm film thickness) fiber which was suitable for
233 determining the head space composition in a broader volatility range and PDMS (100 μm film
234 thickness) fiber which showed minor retention of the target compounds. CAR-PDMS (75 μm film
235 thickness) was also chosen by Duflos G. et al. [35] for analyzing the freshness of Whiting since it
236 combined the best signal to noise ratio with maximum extraction of compounds. In a recent paper,

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

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

248 A selection of the microextracting conditions of CAR-PDMS was then performed by a factorial
249 design to get information about the significance of the experimental parameters. Table 2 shows the
250 corresponding experimental design matrix. Response was evaluated in terms of peak area for all
251 compounds. As an example of the behavior of the target volatiles associated to fish lipid oxidation,
252 Fig. 3 shows the Pareto chart for *E*-2-Hexenal. Results showed that all selected variables produced
253 significant effects and that no significant interactions between factors were apparent. Sample
254 amount, extraction temperature and extraction time were statistically significant factors and,
255 therefore, the peak area of *E*-2-Hexenal increased when the three factors increased (positive effect).
256 Factorial experimental design only explains what factors are significant but can not optimize the
257 response because only evaluates 2 levels per factor. An extraction time of 30 min at 60 °C procured
258 the best extraction of volatiles associated to oxidation. These values were the maximum in the
259 experimental design and factors as time and temperature of incubation are positively correlated with
260 the efficiency of the extraction [37]. In addition, these conditions are not able to provoke oxidation
261 of the samples. Therefore, temperature and time were fixed in 60 °C and 30 min respectively.

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

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

270

271 3.2. Validation of the method

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

281 Quantification limits of the method, defined for a signal to noise ratio (S/N) of 10, ranged from 0.03
282 to 0.34 ng per gram of fish muscle depending of compound. A potential shortcoming of SPME
283 based methods is that the extraction yield can be matrix dependent. In such a case, quantification
284 should be performed using the time consuming standard addition method. Possible matrix effects of
285 the HS-SPME method here proposed were investigated by evaluating the recoveries of each
286 volatile. For such purpose, extracts of samples of Atlantic Horse Mackerel muscle were spiked with
287 100 ng/g of the selected compounds. The response corresponding to each compound was corrected

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

293 294 3.3. Chilled storage of Atlantic Horse Mackerel minced muscle

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

300 Propanal is a product from 16-hydroperoxide formed by autoxidation of methyl linolenate and from
301 15-hydroperoxide formed by photosensitized oxidation of methyl linolenate [1]. 1-penten-3-ol is
302 formed by the action of 15-lipoxygenase on EPA (20:5 n-3) [13, 31]. Hexanal can be produced via
303 linoleic acid 13-hydroperoxide and, in addition, a degradation of preformed volatiles as 2,4-
304 decadienal or 2-octenal has been considered responsible for the abundant occurrence of this
305 compound in different lipid systems [32, 39]. 1-octen-3-ol is an important contributor to off-flavors
306 due to its low odor score and it has been reported to be formed from oxidation of arachidonic acid
307 by 12-lipoxygenase [2]. 2-ethylfuran can be produced via 12-hydroperoxide of linolenate (18:3 n-
308 3), via 14-hydroperoxide EPA (20:5 n-3) or via 16-hydroperoxide DHA (22:6 n-3) [40]. *Z*-4-
309 heptenal is produced via 2,6-nonadienal that is produced by the action of 12-lipoxygenase on EPA
310 [14, 26]. An autoxidation of EPA to (*E,Z*)-2,4-heptadienal and (*E,Z*)-3,5-octadien-2-one has been
311 also proposed [31, 41].

312 During the storage of fish muscle, a strong increase in the volatile formation was achieved after the
313 second day of storage in agreement with a first detection of rancid off-flavors. This increment was
314 especially important for 1-penten-3-ol and 2,3-pentanedione. The formation of these compounds
315 showed induction periods of 1.9 and 1.9 days respectively (Fig. 5) and the levels achieved by the
316 second and third days were: 56.0 ± 9.8 and 168.2 ± 7.6 ng/g for 1-penten-3-ol (increment of
317 200.2%) and 96.0 ± 7.8 and 234.2 ± 8.2 ng/g for 2,3-pentanedione (increment of 144.0%).
318 Formation of 1-octen-3-ol showed an induction period of 2.1 days and the levels achieved during
319 the days 2 and 3 were 61.7 ± 0.8 and 65.8 ± 0.3 ng/g respectively (Fig. 5). This little increment
320 (6.6%) was enough for correlating this analysis with the detection of rancid off-flavors since 1-
321 octen-3-ol is a potent odorant of the unpleasant rancid flavor with a very low sensorial threshold
322 value [1].

323 Analyses of volatile compounds were correlated with PV and TBARS since these indexes showed
324 induction periods of 2 and 1.9 days respectively (Fig. 5). 1-penten-3-ol, 2,3-pentanedione and 1-
325 octen-3-ol were the compounds showing the higher correlations (Fig. 5). Pearson coefficients
326 obtained between the formation of 1-penten-3-ol and PV and TBARS indexes were 0.9832 and
327 0.9970 respectively. Similar correlations were obtained for 2,3-pentanedione ($R^2= 0.9834$ and $R^2=$
328 0.9986) and for 1-octen-3-ol ($R^2= 0.9593$ and $R^2= 0.9767$). According to these results, these
329 volatiles were chosen the best markers of lipid oxidation.

330

331 4. Conclusions

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

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

345

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- 396

397 **Figure Captions**

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

400
401 Figure 2: Evaluation of extraction efficiencies from different fiber coatings.

402
403 Figure 3: Standardized Pareto chart obtained for *E-2-hexenal*. Vertical line indicates the statistical
404 significance bound for the different effects.

405
406 Figure 4: SIM chromatogram obtained from oxidized Atlantic Horse Mackerel muscle in the
407 optimized conditions.

408
409 Figure 5: Time course of lipid oxidation of Atlantic Horse Mackerel muscle measured by headspace
410 volatiles, and PV and TBARS indexes.

411

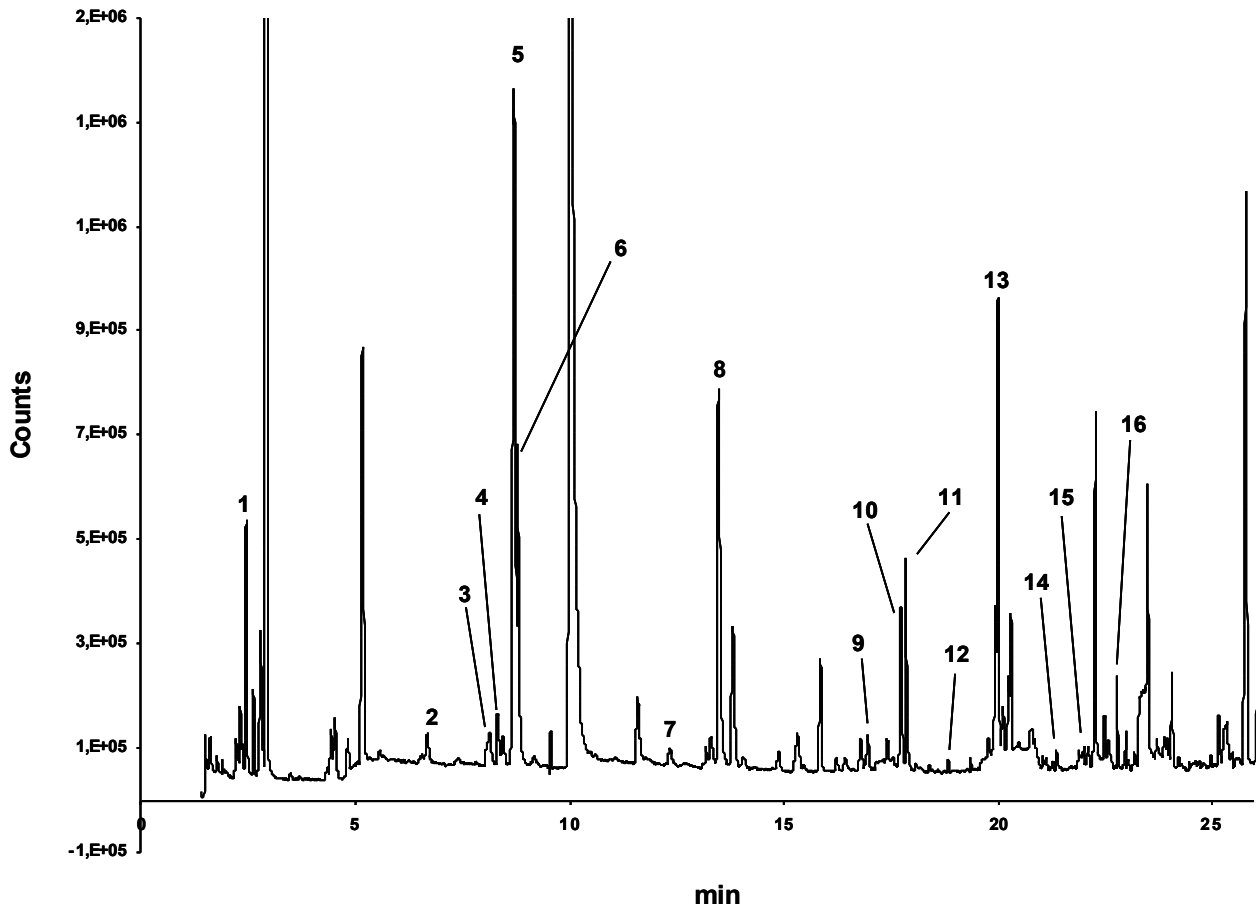
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413 Figure 1

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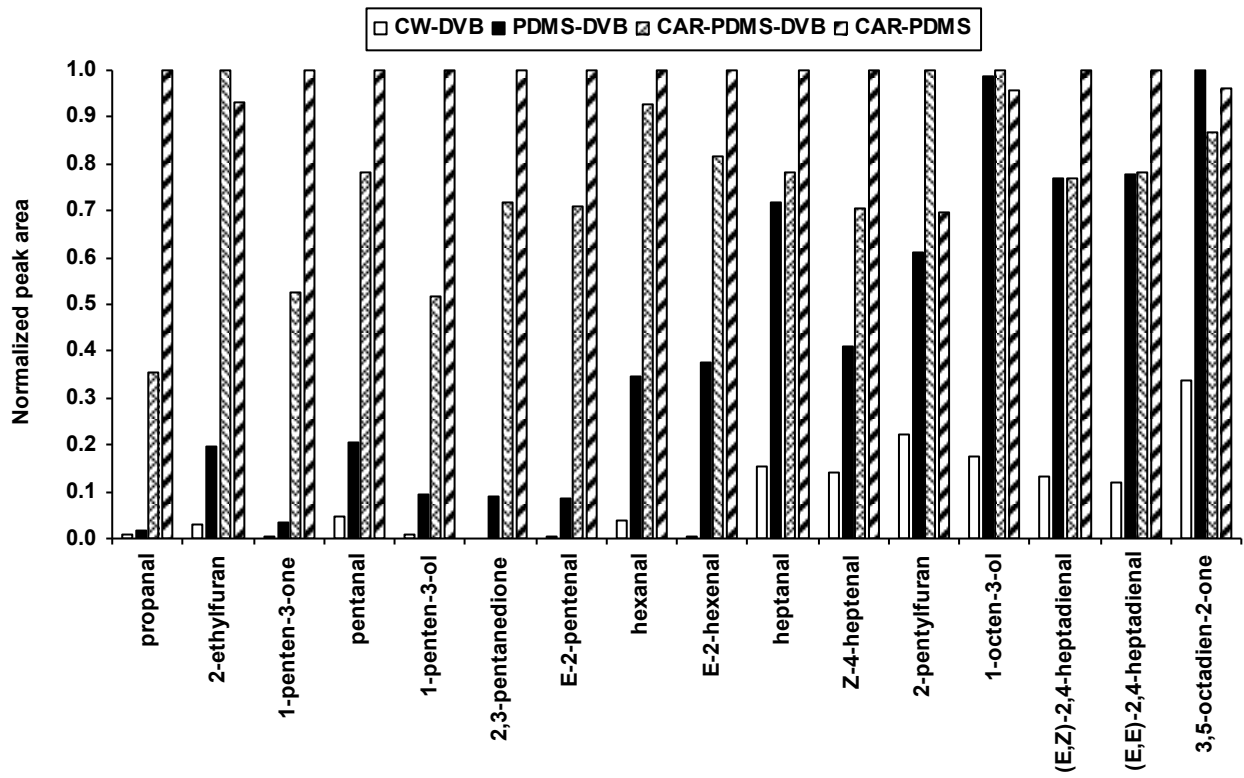
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417 Figure 2

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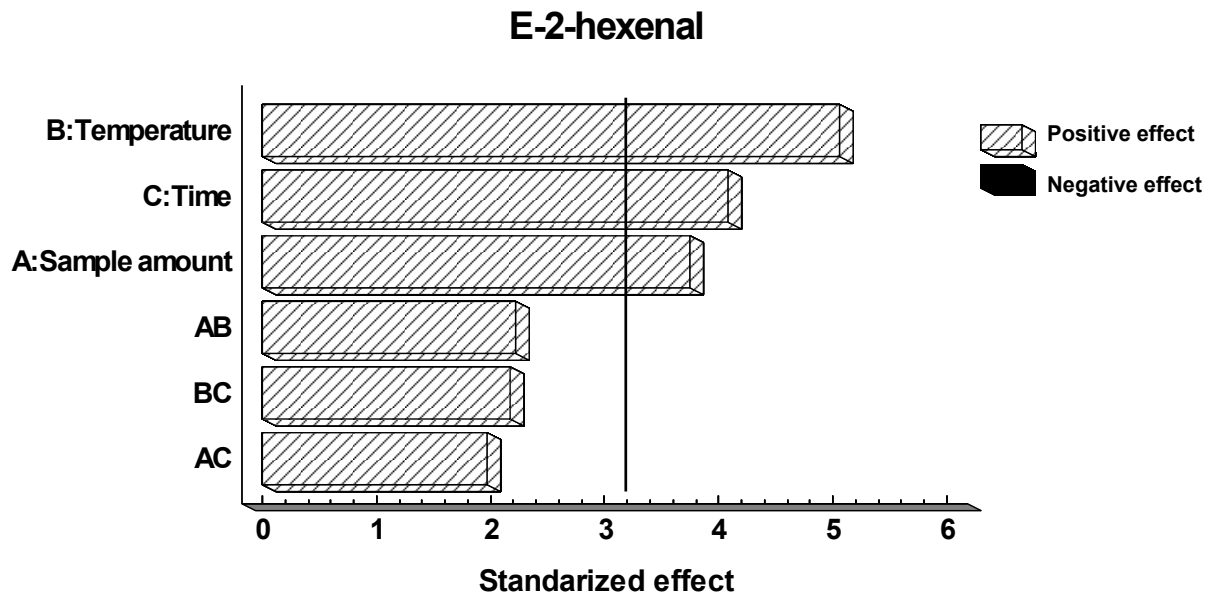
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426 Figure 3

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433 Figure 4

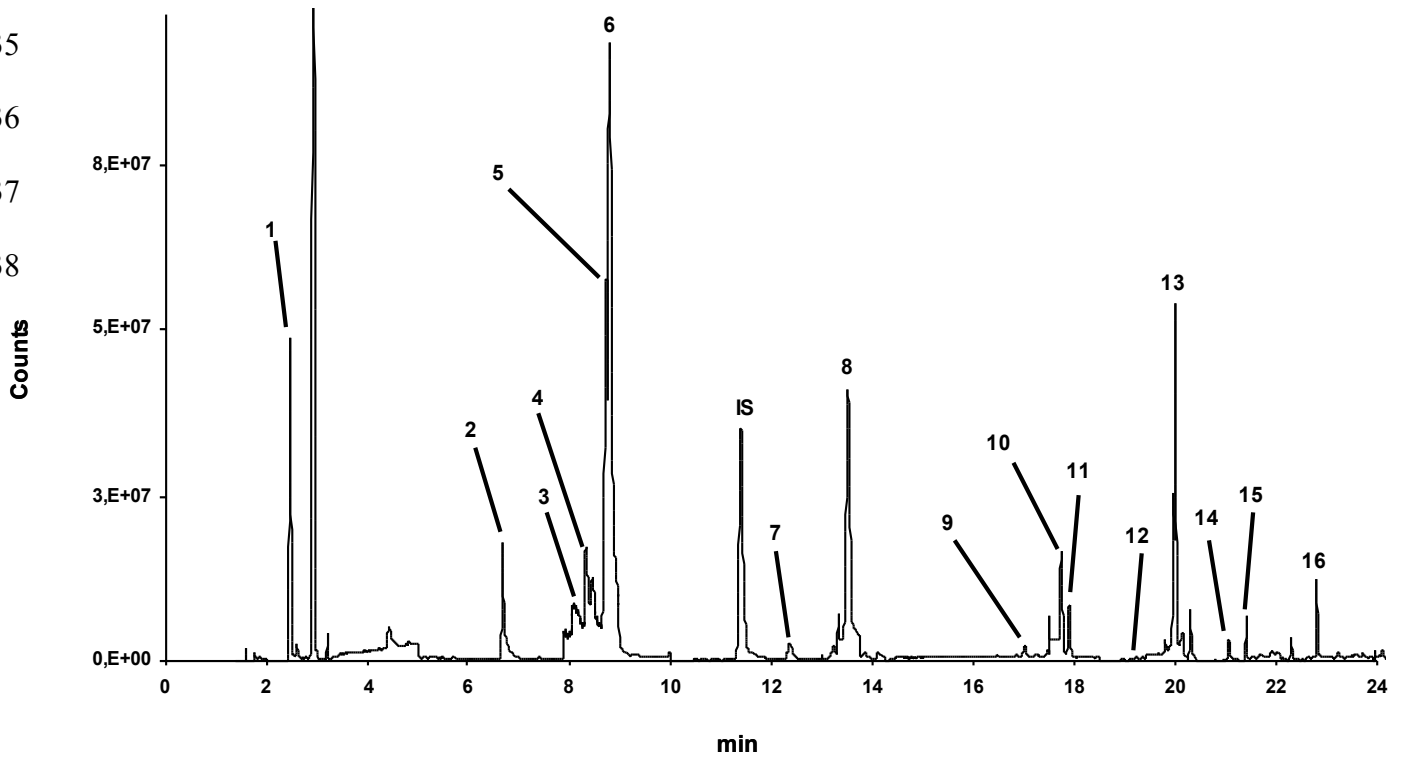
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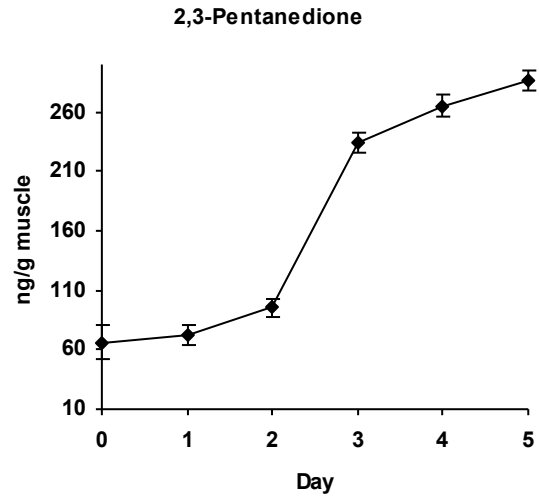
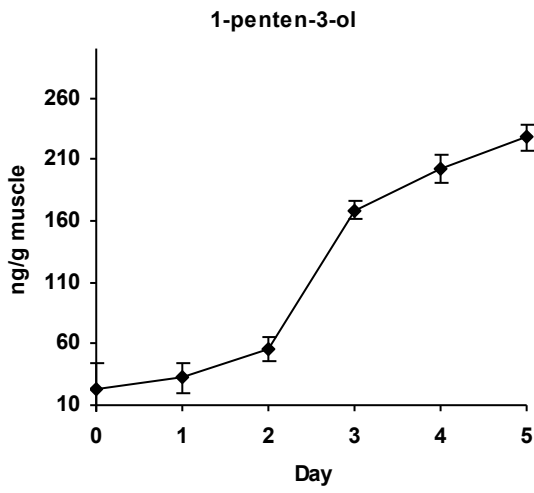
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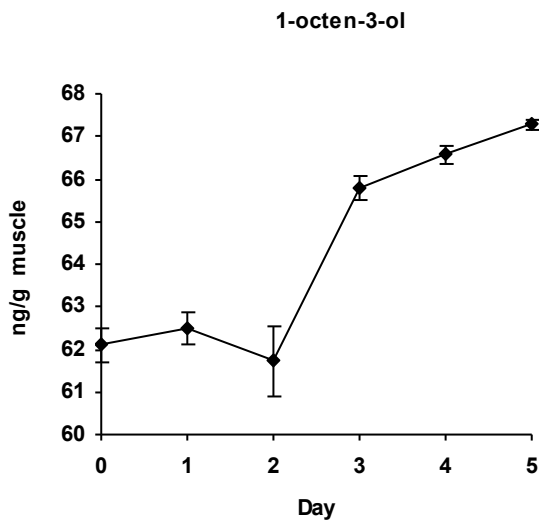


439 Figure 5

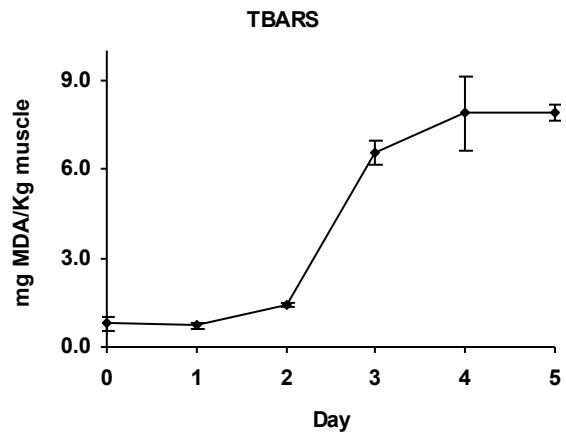
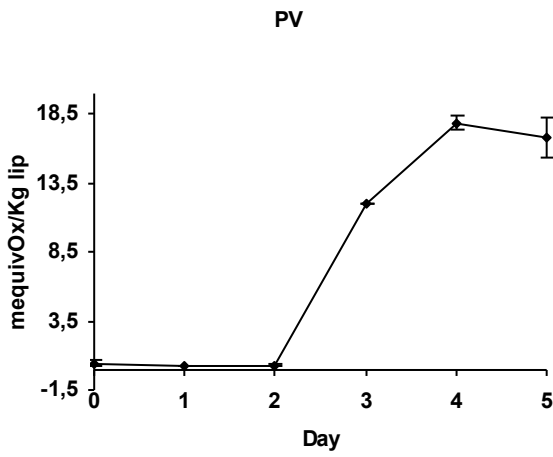
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445 Table 1: Fatty Acids (w/w % of Total Fatty Acids) of Atlantic Horse Mackerel muscle used for the
446 chilled experiments.

447

<i>Fatty acid</i>	<i>% of total fatty acids</i>	<i>Fatty acid</i>	<i>% of total fatty acids</i>
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

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

451

<i>Experiment</i>	<i>Amount of sample (mL)</i>	<i>Temperature (°C)</i>	<i>Time (min)</i>	<i>Peak area^a (counts)</i>
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.

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

457

Compound	Identification ^a	Compound	Identification ^a
<u>Alcohols</u>		Propylbenzene	MS
Ethanol	MS	Trimethylbenzene	MS
1-Propanol	MS	<u>Esters</u>	
2-Methyl-1-propanol	MS	Ethylacetate	MS, STD
1-Penten-3-ol	MS, STD	<u>Furans</u>	
1-Pentanol	MS	2-Methylfuran	MS
2-Penten-1-ol	MS	2-Ethylfuran	MS, STD
2-Hepten-1-ol	MS	2-Pentylfuran	MS
3-Pentanol	MS	<u>Noncyclic hydrocarbons</u>	
3-Hexen-1-ol	MS	Pentane	MS
1-Hexanol	MS	3-Methyl-1-butene	MS
4-Heptanol	MS	2-Pentene	MS
1-Octen-3-ol	MS, STD	2-Methyl-1,3-pentadiene	MS
1-Octanol	MS	Hexane	MS
2-Octen-1-ol	MS	Octane	MS
2-Nonen-1-ol	MS	1-Octadecene	MS
Phenol	MS	Pentadecane	MS
Ethylalcohol	MS	Eicosane	MS
<u>Aldehydes</u>		5-Eicosene	MS
Acetaldehyde	MS, STD	5-Nonadecene	MS
Propanal	MS, STD	Nonadecane	MS
2-Methylpropanal	MS	Octadecane	MS
Butanal	MS, STD	<u>Ketones</u>	
Pentanal	MS, STD	Acetone	MS, STD
E-2-Pentenal	MS, STD	2,3-Butanedione	MS
Hexanal	MS, STD	2-Pentanone	MS
E-2-Hexenal	MS, STD	1-Penten-3-one	MS, STD
Heptanal	MS, STD	3-Pentanone	MS
Z-4-Heptenal	MS, STD	2,3-Pentanedione	MS, STD
E-2-Heptenal	MS, STD	3-Hydroxy-2-butanone	MS
Benzaldehyde	MS	3,3-Dimethyl-2-butanone	MS
Octanal	MS, STD	2-Hydroxy-3-pentanone	MS
(E,Z)-2,4-Heptadienal	MS	6-Methyl-2-heptanone	MS
(E,E)-2,4-Heptadienal	MS, STD	2,3-Octanedione	MS
Nonanal	MS, STD	6-Octen-2-one	MS
3,7-Dimethyl-6-octenal	MS	2-Nonanone	MS
Decanal	MS	Acetophenone	MS
<u>Amines</u>		3,5-Octadien-2-one	MS
Trimethylamine	MS	4-Methyl-ciclohexanone	MS
<u>Aromatics</u>		<u>Chlorinated compounds</u>	
Toluene	MS	Dichloromethane	MS, STD
Ethylbenzene	MS	Chloroform	MS, STD
Xilene (not identified)	MS	<u>Sulfur compounds</u>	
Styrene	MS	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	<i>E</i> -2-Pentenal	12.34	55+83+84
8	Hexanal	13.46	44+56+72+82
9	<i>E</i> -2-Hexenal	17.04	41+55+69+83+98
10	Heptanal	17.78	44+55+70
11	<i>Z</i> -4-Heptenal	17.92	41+55+68
12	2-Pentylfuran	18.94	81+138
13	1-Octen-3-ol	20.04	52+72
14	(<i>E,E</i>)-2,4-Heptadienal	21.05	81+110
15	(<i>E,Z</i>)-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.

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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
<i>E</i> -2-Pentenal	0.998	116.9 ± 15.3	0.15	13.1
Hexanal	0.991	103.2 ± 11.3	0.06	11.0
<i>E</i> -2-Hexenal	0.996	66.0 ± 1.3	0.09	2.0
Heptanal	0.992	109.1 ± 5.7	0.04	5.3
<i>Z</i> -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
<i>(E,E)</i> -2,4-Heptadienal	0.987	59.3 ± 4.8	0.10	4.8
<i>(E,Z)</i> -2,4-Heptadienal	—	—	—	0.6
3,5-Octadien-2-one	—	—	—	8.7

471

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

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