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## Development of an SPME-GC-MS method for the specific quantification of dimethylamine and trimethylamine: use of a new ratio for the freshness monitoring of cod fillets

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1 **Development of an SPME-GC-MS method for the specific quantification of dimethylamine and**  
2 **trimethylamine: use of a new ratio for the freshness monitoring of cod filets.**

3 Running title: **Quantification of TMA & DMA by SPME-GC-MS for cod freshness monitoring**

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18

## ABBREVIATIONS

19 CAR: Carboxen ®; DMA: Dimethylamine; DMA-N: Dimethylamine expressed as nitrogen equivalent;  
20 DTR: DMA-N/TMA-N ratio; DVB: Divinylbenzene; FWHM: Full-width at half-maximum; GC: Gas  
21 chromatography; LRI: Linear retention indices; MS: Mass spectrometry; PDMS: Polydimethylsiloxane;  
22 PVB-N: Partial volatile basic nitrogen; QIM: Quality index method; SIM: Single ion monitoring;  
23 SPME: Solid phase microextraction; TCA: Trichloroacetic-acid; TF: Tailing factor; TIC: Total ion  
24 chromatogram; TMA: Trimethylamine; TMA-N: Trimethylamine expressed as nitrogen equivalent;  
25 TVB-N: Total volatile basic nitrogen.

26

## ABSTRACT

27 BACKGROUND: Fish is a highly perishable food, so it is important to be able to estimate its freshness  
28 to ensure optimum quality for consumers. The present study describes the development of an SPME-  
29 GC-MS technique capable of quantifying both trimethylamine (TMA) and dimethylamine (DMA),  
30 components of what has been defined as partial volatile basic nitrogen (PVB-N). This method was used  
31 together with other reference methods to monitor the storage of cod fillets (*Gadus morhua*) conserved  
32 under melting ice.

33 RESULTS: Careful optimisation enabled definition of the best parameters for extracting and separating  
34 targeted amines and an internal standard. The study of cod spoilage by sensory analysis and TVB-N  
35 assay led to the conclusion that the shelf-life of cod fillet was between 6 and 7 days. Throughout the  
36 study, TMA and DMA were specifically quantified by SPME-GC-MS; the first was found to be highly  
37 correlated with the values returned by steam distillation assays. Neither TMA-N nor DMA-N were able  
38 to successfully characterize the decrease in early freshness, unlike dimethylamine/trimethylamine ratio  
39 (DTR), whose evolution is closely related to the results of sensory analysis until the stage where fillets  
40 need to be rejected.

41 CONCLUSION: DTR was proposed as a reliable indicator for the early decrease of freshness until fish  
42 rejection.

43 **Keywords:** SPME-GC-MS, TMA, DMA, TVB-N, PVB-N, QIM, fish freshness, storage assessment

## INTRODUCTION

44

45 Fish is increasingly consumed around the world despite disparities recorded between continents. <sup>1</sup> It is a  
46 very convenient food, with good nutritional properties but is nevertheless highly perishable, with a short  
47 shelf-life ranging from a few days to around twenty days in cold storage, depending on fish species and  
48 packaging. <sup>2,3</sup> Freshness estimation is a key issue concerning this food matrix, as in fish there is a close  
49 link between freshness and overall quality. <sup>4</sup> Consumers are more and more concerned about the quality  
50 of what they eat, with specific attentions to price and freshness. <sup>5</sup> Accurate indicators and tools charac-  
51 terizing the decrease in freshness are therefore required. To date, numerous techniques have been devel-  
52 oped including sensory, <sup>6,7</sup> physical, <sup>8,9</sup> biochemical <sup>10-13</sup> and microbial <sup>14,15</sup> methods, but sensory analy-  
53 sis remains the most widely used technique for freshness estimation by the industry. <sup>16</sup> Unfortunately,  
54 these techniques have certain drawbacks. Concerning sensory analysis, the need for a constantly trained,  
55 objective and available sensory panel can be problematic. <sup>17</sup> Physical methods usually require whole fish  
56 or fillets with skin, which makes them less efficient with processed fish. The main problem encountered  
57 with chemical analysis is their lack of reliability during the early stages of freshness decay. <sup>3</sup> Finally,  
58 microbial methods require very long processing periods and these methods are highly variable regarding  
59 numerous parameters. <sup>18</sup>

60 After fish death and rigor mortis resolution, several processes occur, leading to freshness decrease and  
61 fish spoilage. Among these processes, endogenous enzyme activities, nucleotide degradation, lipid oxi-  
62 dation or development of specific spoilage organisms have been described as causative agents. <sup>3, 19-21</sup>  
63 Marine fish are characterized by the presence of trimethylamine N-oxide (TMAO) in their flesh, though  
64 its role remains uncertain: osmoregulation, buoyancy regulation, cryoprotection, etc. <sup>22</sup> In cod (*Gadus*  
65 *morhua*), the mean TMAO content has been calculated between 60 and 120 mg nitrogen 100 g<sup>-1</sup>, de-  
66 pending on season or catching area. <sup>3</sup> TMAO can be catabolized in two principal ways: an exogenous  
67 and an endogenous pathway. The first pathway results in its reduction to trimethylamine (TMA) by  
68 TMAO reductase (EC 1.6.6.9), a molybdoenzyme presents on the periplasm of specific spoilage organ-  
69 isms. <sup>23</sup> The second pathway involves the action of endogenous TMAO demethylase (EC 4.1.2.32) and

70 leads to the production of dimethylamine (DMA) and formaldehyde in equimolar proportions; this en-  
71 zyme activity is particularly important in gadoid fish species.<sup>22</sup> Additional minor synthesis pathways  
72 were described for TMA, which might originate from the degradation of choline, acetylcholine, carnitine  
73 or betaines.<sup>24</sup> TMA and DMA are directly involved with quality and freshness issues as they result di-  
74 rectly from the spoilage of fish through TMAO catabolism. On the one hand TMA is responsible of the  
75 characteristic fishy-odour, with a rejection level in chilled fish comprised between 10 and 15 mg nitro-  
76 gen 100 g<sup>-1</sup><sup>3, 25</sup> while on the other hand DMA is produced soon after fish harvest, contributing to the  
77 overall flavour of fish, with no specific odour described.<sup>25</sup> In the case of freezing, DMA is also an indi-  
78 rect marker of flesh texture as it is produced together with formaldehyde, indeed methanal forms cross-  
79 links with proteins resulting in a marked toughening of fish flesh.<sup>3</sup>

80 Historically; several methods have been developed to quantify TMA and DMA using capillary electro-  
81 phoresis (CE), high-performance liquid chromatography (HPLC) or gas chromatography (GC).<sup>24, 26, 27</sup>  
82 Unfortunately, these methods usually necessitate an onerous sample preparation step with the use of  
83 hazardous solutions, but some, such as CE and HPLC, are usually more informative than GC concerning  
84 quantities of TMAO and some cations. Headspace analysis can markedly reduce sample preparation  
85 time.<sup>26, 27</sup> More recently, solid-phase micro-extraction (SPME) headspace methods have been developed  
86 for quantification of volatile amines in fish.<sup>28-32</sup> Extraction steps together with separation of amines are  
87 usually well described and transferable, although two studies did not manage to clearly differentiate  
88 TMA and DMA signals.<sup>28, 29</sup> Concerning fibres, most of the studies used fibres coated with  
89 CAR/DVB/PDMS,<sup>30-32</sup> only one study used a non-available, laboratory-developed fibre.<sup>29</sup> Regrettably,  
90 very few published studies described the optimization step for the sample preparation.<sup>29, 30</sup> SPME meth-  
91 ods were applied to numerous fish species involving fresh water or mangrove fish<sup>29-31</sup> and marine fish,  
92<sup>28, 31, 32</sup> but except for one study,<sup>28</sup> it was not applied to the most consumed marine species in Europe.  
93 Among these studies, very few applied these methods to monitor the spoilage of fish.<sup>28, 30</sup> Finally, when  
94 used, comparative methods consist mostly of TVB-N analysis,<sup>28, 30, 32</sup> **TMA-N** assay being overlooked,  
95 and only one study used sensory tests.<sup>30</sup>

96 This paper deals with the development of a new SPME-GC-MS method that specifically quantifies  
97 TMA and DMA. This method, using a CAR/PDMS fibre, a new internal standard and fully commercial-  
98 ly available consumables, was applied; together with QIM score evaluation, TVB-N and TMA-N assays,  
99 to the concrete case of a spoilage study on cod (*Gadus morhua*). The SPME-GC-MS method was able to  
100 quantify partial volatile basic nitrogen (PVB-N), the sum of TMA-N and DMA-N concentrations and to  
101 define the DMA-N/ TMA-N ratio (DTR) used to characterize the early decrease in freshness.

## 102 EXPERIMENTAL

### 103 Chemicals

104 Concerning the chemicals used in this study, a C<sub>5</sub> - C<sub>15</sub> n-paraffin mix, and ammonium chloride, were  
105 acquired from Sigma-Aldrich (St-Quentin-Fallavier, France). Other chemicals were used as pure com-  
106 pounds solutions: trimethylamine hydrochloride (PubChem CID: 1146), dimethylamine hydrochloride  
107 (PubChem CID: 674), or internal standards: 2-phenylethylamine (PubChem CID: 1001), sec-butylamine  
108 (PubChem CID: 24874), N, N-diisopropylmethylamine (PubChem CID: 547370), 1,2-  
109 dimethylpropylamine (PubChem CID: 61236), all of which were purchased from Sigma-Aldrich. Potas-  
110 sium hydroxide pellets used to raise pH levels were also acquired from Sigma-Aldrich. Formaldehyde  
111 solution was purchased from VWR (Fontenay-sous-Bois, France). Trichloroacetic-acid (TCA), 4% boric  
112 acid with methyl red and bromocresol green indicator, 0.1 N Titrim® sulphuric acid and 10% sodium  
113 hydroxide solutions were purchased from Dislab (Lens, France). Finally, sodium chloride (NaCl) was  
114 acquired from Oxoid (Dardilly, France).

115 Solutions of pure compounds were prepared and diluted, if required, using a saturated NaCl solution at 4  
116 °C. Eleven mL of solution was then poured into 20 mL SPME vials from Chromoptic (Courtaboeuf,  
117 France), and a potassium hydroxide pellet (117 ± 14 mg, 85% purity) was added. Standard solutions  
118 were prepared on the day of analysis as follows, 10 µL of pure compound solution was added to 10 mL  
119 of bidistilled-grade water from Dislab and gently mixed, then 4.4 µL of standard solution was added in  
120 SPME vials. Finally, vials were hermetically sealed.

### 121 Fish sample preparation

122 As a first step of method optimization, the effects of NaCl concentration and of the solution's pH were  
123 evaluated. 50.0g of fish was blended with 100.0 mL of 30.0% (w/w) NaCl solution. After centrifugation  
124 at 10,000g for 10 min at 4 °C, 11 mL of supernatant was poured into SPME vials hermetically sealed  
125 after addition or not of a potassium hydroxide pellet.

126 During the storage study a single step of sample preparation was chosen, based on Regulation (EC) No.  
127 2074/2005, <sup>33</sup> to better compare SPME-GC-MS and steam distillation assays. Fillet was cut into 1 cm-  
128 edge cubes, which were then mixed together to obtain a matrix representative of the overall freshness  
129 state of fillet.  $100.0 \pm 0.2$  g of flesh was then put into a Waring blender and approximately 200 g of a 7.5  
130 % (w/w) TCA solution was added to ensure a weight ratio of 2:1. The whole was then blended and fil-  
131 trated through a 5 µm cut-off filter paper, the filtrate being used for both steam distillation and SPME-  
132 GC-MS assays.

### 133 **SPME-GC-MS procedure**

134 Vials were managed automatically thanks to a CTC Analytics CombiPAL (Zwingen, Switzerland). First-  
135 ly, a sample was transferred to the extraction chamber, heated and shaken at 500 rpm for 10 min for the  
136 headspace equilibration. A 23 Ga, 75 µm, Carboxen®/Polydimethylsiloxane (CAR/PDMS) SPME fibre  
137 from Supelco (Saint-Germain-en-Laye, France) was then inserted into the headspace and volatile ad-  
138 sorption was carried out. Times and temperatures of extraction were tested from 1 to 40 min at 35 °C  
139 and 50 °C in order to determine the best temperature and extraction time. Once the volatiles had been  
140 extracted, the SPME fibre was transferred to a Merlin Microseal injector from Supelco, where volatiles  
141 were desorbed at a temperature of 210 °C for 1 min. Finally, after each injection SPME fibre was heated  
142 to 300 °C, under a helium flow, for 20 min in the fibre conditioner device. Two types of liner were tested  
143 during this study: regular SPME glass liner and deactivated SPME glass liner from Restek (Lisses,  
144 France). Volatile compounds were separated and identified using a GC-MS QP2010 Plus from Shimad-  
145 zu (Noisel, France). The GC device was successively equipped with two capillary columns; an SLB-5ms  
146 capillary column 60 m x 0.25 mm x 0.25 µm from Sigma-Aldrich and a Stabilwax DB capillary column  
147 30 m x 0.25 mm x 0.25 µm from Restek. Helium was used as the carrier gas for both, with respective

148 flows of 1.78 mL min<sup>-1</sup> and 1.20 mL min<sup>-1</sup>. The GC conditions applied with SLB-5ms were the same as  
149 described in a previous study.<sup>34</sup> The Stabilwax DB capillary column was used under the following con-  
150 ditions: the oven temperature was initially set at 60 °C for 5 min, then increased to 210 °C at 15  
151 °C.min<sup>-1</sup> and maintained for 5 min. To optimize the method, an experimental plan-like approach was  
152 used on two levels of three parameters: split value (splitless or split of 5), injection temperature (150 °C  
153 and 210 °C) and initial oven temperature (45 °C and 60 °C). To determine the impact of these parame-  
154 ters on peaks, two shape indicators were used: tailing factor (TF) and full width at half maximum  
155 (FWHM). MS conditions were set as follows: interface temperature equal to the final oven temperature  
156 to avoid any recondensation, 70 eV of ionization voltage and for the early development steps, a mass  
157 range from 33 to 200 m/z for total ion chromatogram (TIC) tracing. In the later phases, single ion moni-  
158 toring (SIM) was preferred with specific ions of TMA, DMA and internal standard (42 m/z, 44 m/z, 45  
159 m/z, 55 m/z, 58 m/z, 59 m/z and 72 m/z). Volatile compounds were identified using the NIST08 spectral  
160 database and comparison of linear retention indices (LRI) with those of the corresponding pure chemi-  
161 cals.

## 162 **Shelf-life assessment**

163 Eleven gutted, whole cod (*Gadus morhua*) were purchased at a local fish retailer in Boulogne-sur-Mer  
164 (France). Fish were caught in the north-east Atlantic, during spring, and were packed into two polysty-  
165 rene boxes, covered by crushed ice and insulated by a plastic film. Fish were acquired from the auction  
166 of the day to ensure the best freshness grade. Based on QIM attributes, the initial quality of raw fish was  
167 very good; skin was bright and flesh was greyish, flat eyes clearly revealing a black pupil thanks to a  
168 transparent cornea, with only the gills displaying a slight discoloured aspect. Fish preparation was car-  
169 ried out at the innovation platform *PFI-Nouvelles Vagues* (Boulogne-sur-Mer, France). In a room with  
170 ambient temperature maintained at 10 °C, each fish was filleted with skin. Three fillets were placed in  
171 new polystyrene boxes, taking care to place the two fillets of any given fish in separate boxes. After  
172 insulation with plastic film, boxes were filled with fresh crushed ice and placed in a cold room at +2.0  
173 °C. The day of fillet preparation was the day of the first analysis and was considered as D01 of the study.



174 On days D01, D04, D07, D09, D11, D14 and D16, a single box was withdrawn and the three fillets were  
175 analysed.

176 On the day of analysis each of the fillets was processed in the same way, as follows. Firstly, the fillet  
177 was subjected to sensory analysis, carried out by three judges, using QIM score evaluation as described  
178 by Cardenas Bonilla *et al.*<sup>35</sup> After fish preparation as previously described, 1 mL of filtrate was con-  
179 served for SPME-GC-MS analysis and the remainder was used for total volatile basic nitrogen (TVB-N)  
180 and TMA-N assays, in duplicate, by steam distillation as previously described.<sup>34</sup> Briefly, before the tube  
181 to be placed in a Gerhardt Vapodest distillation unit (Les Essarts-le-Roi, France), 25 mL of filtrate were  
182 loaded in tube. In case of TMA-N assays, with 20 mL of formaldehyde were added. Then, 6 mL of 10%  
183 NaOH was poured and the distillation was performed. The distillate was collected into a beaker contain-  
184 ing 10 mL of 4% boric acid, before titration with a 0.1 N sulfuric acid solution. According to Regulation  
185 (EC) No. 2074/2005,<sup>33</sup> a solution of NH<sub>4</sub>Cl at 1.91g.L<sup>-1</sup> was used as a control for steam distillation as-  
186 says. Results were considered as valid for recovery rates comprised between 98% and 101%. For  
187 SPME-GC-MS, the filtrate was diluted with a saturated NaCl solution from 1/100 to 1/4000 depending  
188 on the day of analysis concerned. On each day, filtrate was analysed in triplicate as follows: 11 mL of  
189 diluted filtrate was poured into SPME vials, 4.4 µL of 1,2-dimethylpropylamine standard solution was  
190 added together with one potassium hydroxide pellet and vials were hermetically sealed.

### 191 **Descriptive statistics, inferential statistics and modelling**

192 All of the statistical work was carried out using R software 3.1.1 (<http://www.r-project.org>). Except  
193 where otherwise indicated, error bars shown on bar charts and line graphs illustrate twice the standard-  
194 error value (standard deviation of the sampling distribution), thus showing the 95% confidence limits of  
195 the measurement. When least significant difference (LSD) tests are performed, two results tagged with  
196 different letters are significantly different with an error risk of 5%. ANOVA were carried out after care-  
197 fully establishing normality of the distribution using Shapiro-Wilks test and distribution homoscedastici-  
198 ty using Bartlett's test, all under the independent sample hypothesis. Interdependence of the values

199 yielded by this research was evaluated by calculating Pearson's correlation or Spearman's correlation  
200 coefficients depending on the linear relationships between them.

201 TMA production being a reflection of the specific spoilage organisms' metabolism activity, modelling of  
202 **TMA-N** by a microbial growth model was tested. Using a modified Gompertz model,<sup>36</sup> a non-linear  
203 regression was performed with R software (**Supporting information – Algorithm S1**). The three pa-  
204 rameters  $A$ ,  $\mu_m$  and  $\lambda$  were entered as input variables in accordance with the observed data and an itera-  
205 tive process was followed to refine the parameters in order to fit the model to observed data. The **DMA-**  
206 **N/TMA-N** ratio (DTR) was modelled against QIM using an exponential equation with another non-  
207 linear regression (**Supporting information – Algorithm S2**), **the data structure suggesting this type of**  
208 **approach**. In both cases a special care was taken to enter the most probable parameter values to reduce  
209 the risk of reaching local minima during the iterative process.

## 210 RESULTS AND DISCUSSION

### 211 Sample preparation optimization

212 In a first step of method optimization, special attention was applied to the chemical conditions of the  
213 sample. **In absence of potassium hydroxide** (KOH), pH values of solutions were lower than 8.0 and no  
214 detectable peak was recorded on chromatogram. **It was already known that TMA and DMA are only**  
215 **volatiles in their conjugate base form**<sup>28</sup> and have respective pKa of 9.76 and 10.64.<sup>37</sup> **Addition** of KOH  
216 in the solution **increased** the pH above **12.0 and clear peaks appeared** on chromatograms. NaCl tended to  
217 increase both TMA and DMA recovery with a clear improvement of the peak area for TMA and a slight-  
218 er increase concerning DMA which is in accordance with previous observations.<sup>29</sup> It was thus decided  
219 to retain the use **of a saturated solution of NaCl, with** the addition of a KOH pellet into the vial for the  
220 preparation of samples.

### 221 Optimization of headspace extraction

222 The second group of parameters optimized concerned the conditions for headspace extraction, mostly  
223 concerning time and temperature of extraction as, these have already been described as key parameters,  
224 although few studies include a documented optimization step.<sup>30</sup> Two temperatures (35 °C and 50 °C)

225 and seven extraction times (1, 2, 5, 10, 20, 30 and 40 min) were tested, as illustrated in **Figure 1**. Com-  
226 pared with similar extraction times at 50 °C, bar graphs clearly show that an extraction temperature of  
227 35 °C produced peaks higher intensities from time of 5 min and longer (p-value < 0.001). Concerning  
228 time of extraction, longer times led to higher peak intensities. Least significant difference tests tended to  
229 conclude that a significant increase of peak area was observed between 20 min and 30 min of extraction,  
230 whereas area increase between 30 min and 40 min was not significant. Regarding these results, extrac-  
231 tions at 35 °C for 30 min were chosen as the best compromise for extracting volatiles. Extraction by  
232 SPME is the result of two concomitant equilibria: first between matrix and headspace and then between  
233 fibre and headspace. Results giving smaller areas observed with an extraction temperature of 50 °C  
234 could be explained by the fact that equilibrium between fibre and headspace displaced towards the head-  
235 space, as previously reported.<sup>38</sup> Following this observation, increasing the time of extraction at a fixed  
236 low extraction temperature resulted in the adsorption of more volatile compounds on SPME fibre. The  
237 temperature of 35 °C was confirmed by two other studies,<sup>30,31</sup> but in contrast the time of extraction was  
238 slightly higher than the range from 5 to 22 min described elsewhere.<sup>28-32</sup> This difference could be ex-  
239 plained by the fact that a CAR/PDMS fibre was used instead of Carbox-  
240 en®/Polydimethylsiloxane/Divinylbenzene (CAR/PDMS/DVB) or Polydimethylsilox-  
241 ane/Divinylbenzene (PDMS/DVB) fibres, and in addition no saturation was noted after 10 min, as was  
242 observed by Chan *et al.*<sup>30</sup>

### 243 **Injection and separation optimizations**

244 A comparison of the above-mentioned columns concluded that Stabilwax-DB columns offer the best  
245 peak shapes (data not shown). Improvement of peak shapes could be explained by the phase coated on  
246 the Stabilwax-DB columns composed of Carbowax® polyethylene glycol, with higher polarity than 5%  
247 diphenyl/95% methyl-siloxane coated on the SLB-5ms columns. Apart from the phase coated on the  
248 columns, peak resolutions and shapes are also influenced by numerous parameters, including injector  
249 temperature, oven temperature or split value. **Table 1** reports ANOVA results assessing the impact of the  
250 three parameters tested on TMA and DMA peak shapes. Except for the initial temperature of the col-

251 umn, which has no significant impact on the tailing factor of TMA, the three parameters had a signifi-  
252 cant impact on both tailing factor and full-width at half-maximum of TMA and DMA. The shape of  
253 DMA peaks is significantly influenced by all the interactions between the parameters, whereas the shape  
254 of TMA peaks is more likely significantly influenced by the injection parameters, *i.e.* injector tempera-  
255 ture and split value. For each assessed parameter, the highest levels led to the best values concerning  
256 shape indicators. An injection at 210 °C with a split value of 5 and an initial column temperature of 60  
257 °C were thus chosen to perform the next analysis.

258 Deactivated SPME glass liner (data not shown) led to greater areas for both TMA and DMA; it was thus  
259 selected for analysis of volatile amines.

### 260 **Internal standard screening and quantification range**

261 To overcome signal variation, an internal standard was added for sample preparation. Four compounds,  
262 2-phenylethylamine, sec-butylamine, N,N-diisopropylmethylamine and 1,2-dimethylpropylamine were  
263 tested, as they share structures or chemical properties close to the amines studied. As special attention  
264 was paid to safety issues when selecting internal standards, sec-butylamine was rapidly discarded be-  
265 cause of its toxic, polluting and highly corrosive properties. Among the remaining compounds 1,2-  
266 dimethylpropylamine was the molecule that led to best linearity of the signal for both TMA and DMA  
267 (data not shown). Using 1,2-dimethylpropylamine as internal standard enabled a quantification range  
268 from 100 µg kg<sup>-1</sup> to 900 µg kg<sup>-1</sup> for TMA and from 150 µg kg<sup>-1</sup> to 1300 µg kg<sup>-1</sup> for DMA with good  
269 linearity characterized by respective determination coefficients of  $R^2_{\text{TMA}} = 0.98$  and  $R^2_{\text{DMA}} = 0.99$  (**Sup-**  
270 **porting information – Figure S1**). No further investigations were brought to LOD estimation, consid-  
271 ering that lower TMA and DMA values, observed in fish preparation, will rarely be lower than 1 mg kg<sup>-1</sup>.  
272 <sup>39</sup>

### 273 **Application to fish freshness assessment monitoring**

274 Sensory analysis results are show in **Figure S2 (Supporting information)**. A marked evolution is rec-  
275 orded from D01 to D11 with an average QIM score increase from 0 to 13; no significant increase being  
276 observed afterwards, this might reflect a final spoilage state of cod fillets. Throughout the study the av-

277 erage QIM score values were linear in comparison with conservation days with an acceptable determina-  
278 tion coefficient (adjusted  $R^2 = 0.97$ ); these results are consistent with those of previous published results.  
279 <sup>35</sup> Based on the Cardenas Bonilla *et al.* method, the rejection limit, corresponding to a QIM score of 8,  
280 was observed in the neighbourhood of Day 7. Priority has been given to mean QIM score values in the  
281 development below.

282 Results concerning steam distillation assays are presented on **Figure S3 (Supporting information)**.  
283 Unfortunately, a technical problem on D09 (Day 9) prevented the acquisition of workable data concern-  
284 ing biochemical analysis; this day does not appear in the charts. Based on TVB-N measurement and EC  
285 Regulation No. 2074/2005, <sup>33</sup> fillets were considered as spoiled between D04 and D07, probably after  
286 D06. These results are consistent with the sensory analysis and previous observation with the same  
287 methods of fillet processing and storage. <sup>35</sup> Comparison of TMA-N concentrations measured by steam  
288 distillation assay and SPME-GC-MS (**Figure 2 a**) led to highly correlated values ( $r = 0.98$ ;  $p$ -value <  
289 0.001). Moreover, there is a linear relationship between values of the TMA-N obtained by the two meth-  
290 ods (adjusted  $R^2 = 0.99$ ) (**Supporting information – Figure S4**), and no significant difference was ob-  
291 served between the two methods throughout the study ( $p$ -value = 0.42). In addition, TMA-N concentra-  
292 tion is highly correlated with both QIM values ( $r = 0.97$ ;  $p$ -value < 0.001) and days of storage ( $r = 0.93$ ;  
293  $p$ -value < 0.001). A modified Gompertz model enabled TMA-N production to be modelled accurately  
294 throughout the study and additional information was inferred from this model. Thanks to modelling, the  
295 lag phase of TMA-N production was estimated to be 4.8 days. During the exponential phase, the daily  
296 average increase of TMA-N concentration was 8.5 mg nitrogen 100 g<sup>-1</sup>. Finally, theoretical initial and  
297 final TMA-N concentrations in cod fillets were respectively estimated to 4.4 and 66.3 mg nitrogen 100  
298 g<sup>-1</sup>. Compared with steam distillation, PVB-N monitoring by SPME-GC-MS was capable of the specific  
299 detection of DMA-N, so it was possible to monitor concentration throughout the storage period (**Figure**  
300 **2 b**). Compared with TMA-N, DMA-N levels remained lower than 20 mg nitrogen 100 g<sup>-1</sup> in the flesh  
301 of cod fillet. Additionally, DMA-N correlation values are lower than those of TMA-N, but correlation  
302 factors remain acceptable for QIM values ( $r = 0.93$ ;  $p$ -value < 0.001) and days of storage ( $r = 0.88$ ;  $p$ -  
303 value < 0.001). Recorded concentrations of DMA-N follow the same linear trend mentioned in a previ-

304 ous study on gutted whole cods,<sup>40</sup> but values are almost ten times higher; it could be hypothesized that  
305 fish filleting might have discharged TMAO demethylase and induced an increase in its activity in com-  
306 parison with the one in whole fish.

### 307 **Proposition of the DTR ratio**

308 Despite acceptable correlations with QIM scores and number of days of storage throughout the storage  
309 period, neither TMA-N nor DMA-N evolved markedly in the early stages of fish freshness decrease. It  
310 was decided to further investigate the quantities of PVB-N compounds using ratio as described in an  
311 earlier study.<sup>10</sup> Many ratios were tested including: TMA-N/PVB-N, DMA-N/PVB-N, PVB-N/TMA-N  
312 and PVB-N/DMA-N, but none of them provided sufficiently accurate information. By contrast, the  
313 DMA-N/TMA-N ratio (DTR) led to interesting results; it illustrates the relative proportion of autolytic  
314 activity in the degradation of TMAO relative to the spoilage activity. Plotting DTR against QIM score  
315 values (Figure 3) showed a marked decrease of DTR values for QIM scores comprised between 0 and 7,  
316 corresponding to fish suitable for human consumption. DTR values were found to be homogeneous with  
317 an exponential function of QIM values (Figure 3). Globally, DTR values decreased from 0.9 to 0.2  
318 throughout storage, illustrating the increasing preponderance of the activity of specific spoilage organ-  
319 isms in comparison with autolysis. The limit of 0.2 corresponded to QIM values higher than the maxi-  
320 mum storage time estimated for the fillets of this study. It should be mentioned that three outlier values  
321 were recorded for a QIM value in the neighbourhood of 0 with DTR value slightly lower than 0.4; these  
322 values correspond to the analysis in triplicate of a single fillet performed on D01. These atypical values  
323 are the result of TMA-N values markedly superior to those for the other fish and might be the fingerprint  
324 of an early development of spoilage flora.

325 No recovery rate was calculated in this study, as it was in another studies,<sup>26</sup> to assess the matrix effect  
326 on volatile amine extraction and analysis method. Nonetheless, the SPME-GC-MS method was applied  
327 together with steam distillation assays, which is well characterized and used as a reference in Europe,<sup>33</sup>  
328 and TMA-N results from both methods were not significantly different.

329 The authors note that further storage studies, species, catch seasons, and fish processing techniques need  
330 to be analysed with this method in order to confirm the suitability of DTR in assessing fish freshness.  
331 The developed method made it possible to completely dispense with the use of formaldehyde, sulphuric  
332 acid and boric acid. For steam distillation assays, all these compounds require special care concerning  
333 the safety of technicians and waste management, because of their polluting, highly corrosive and toxic  
334 properties. In the future the use of TCA, which is polluting and corrosive, could be avoided; a procedure  
335 for fillet preparation with TCA solution replaced by a saturated NaCl solution should be considered.  
336 This method of sample preparation was not used in this study to enable a better comparison of SPME-  
337 GC-MS with steam distillation assays. Nonetheless, preliminary results (data not shown) demonstrated  
338 that the use of a saturated NaCl solution for fillet preparation did not affect TMA and DMA results rec-  
339 orded on chromatograms. The main drawback of the developed method is analysis time compared with  
340 steam distillation assays; nonetheless this is quickly offset by the specificity of the detection of volatile  
341 amines and the possibilities it opens up. Moreover, based on the results shown in **Figure 1**, it might be  
342 possible to reduce extraction times considerably by reducing dilutions during samples preparation; nev-  
343 ertheless, linearity of the signal would have to be checked beforehand.

## 344 CONCLUSION

345 A new SPME-GC-MS procedure for quantification of both TMA and DMA was developed. Special care  
346 was paid to optimize different parameters such as solution preparation, extraction, volatile compounds  
347 separation and signal normalization, with the use of a new internal standard. The developed method  
348 proved capable of analysing PVB-N, and quantifying of both TMA-N and DMA-N, in a sample within  
349 fifty minutes. PVB-N measurement by the SPME-GC-MS procedure was applied, together with refer-  
350 ence steam distillation assays and sensory analysis, to a shelf-life monitoring study on cod fillets con-  
351 served under melting ice. First results are encouraging, indeed TMA-N results using SPME-GC-MS and  
352 steam distillation assay were very highly correlated. Modelling of TMA-N production throughout the  
353 storage period was successfully carried out using a modified Gompertz model. Finally, the study of DTR  
354 evolution in regard to QIM score values made it possible to visualize the early freshness decrease of cod

355 fillets. The authors thus propose DTR as a new tool for monitoring the early freshness evolution in fish.  
356 The Authors also note that further investigations need to be carried out to confirm the reliability of the  
357 method; nevertheless, the use of hazardous chemicals can already be significantly reduced by adopting  
358 this SPME-GC-MS method.

#### 359 ACKNOWLEDGMENTS

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364 providing financial support for his PhD studies.

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- 478

Table 1: ANOVA results obtained to assess the impact of three parameters on the shapes of both trimethylamine and dimethylamine peaks.

	Trimethylamine <sup>a</sup>		Dimethylamine <sup>b</sup>	
	FWHM <sup>c</sup>	TF <sup>d</sup>	FWHM <sup>c</sup>	TF <sup>d</sup>
TI: Temperature of injection	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***
TC: Initial temperature of the column	0.0053**	0.74	< 0.0001***	< 0.0001***
S: Split value for injection	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***
TI x TC	0.0036**	0.05*	< 0.0001***	< 0.0001***
TI x S	< 0.0001***	< 0.0001***	0.0007***	< 0.0001***
TC x S	0.07	0.94	0.0012**	< 0.0001***
TI x TC x S	0.77	0.0023**	0.0003***	< 0.0001***

ANOVA described here were achieved after normalization of the factors using the log (X+1) normalization method. <sup>a</sup> Data of ANOVA with trimethylamine pure solution, for each combination of split levels, injection and column temperatures, 5 replicated measurements were performed. <sup>b</sup> Data of ANOVA with dimethylamine pure solutions, for each combination of split levels, injection and column temperatures, 5 replicated measurements were performed. <sup>c,d</sup> Influence of parameters and combination of parameters on full width half maximum <sup>c</sup> and tailing factor <sup>d</sup> shape indicators; asterisks illustrate the significance level of the parameters impacts, in accordance with probabilities (\*\*\* p<0.001, \*\* p<0.01, \* p<0.05).

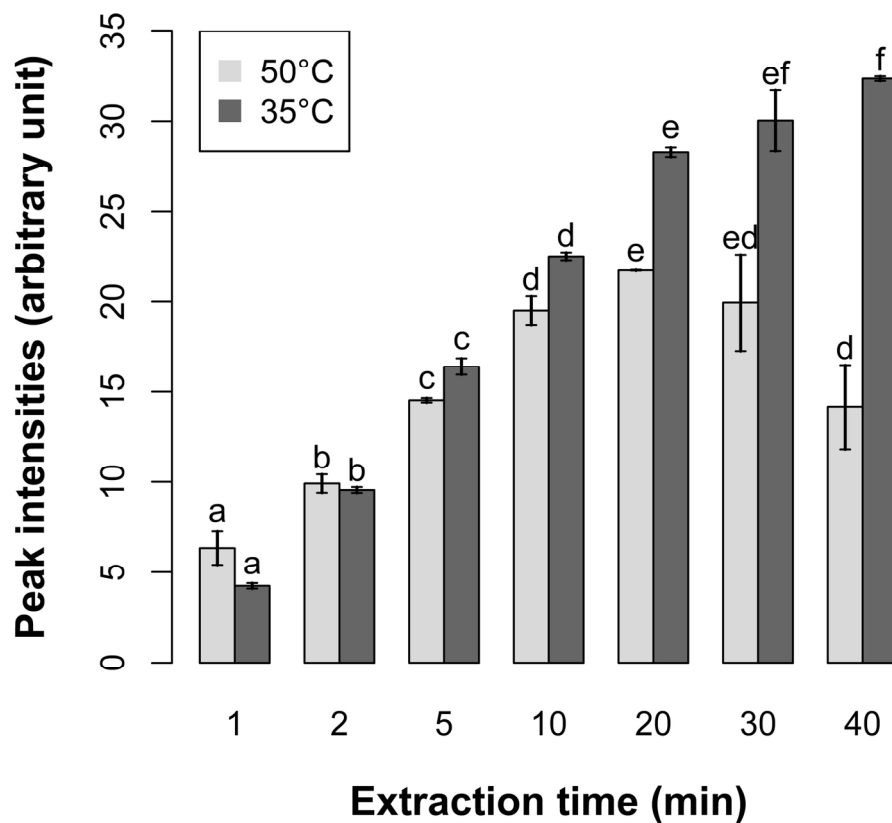


Figure 1: Bar plot of TMA peak intensities depending on extraction time and temperature of extraction from pure solutions. Error bars indicate the min/max peak intensities as measurements were carried out in duplicate. LSD tests were carried out in series (50°C and 35°C). Letters must be compared within a considered series.

79x79mm (600 x 600 DPI)

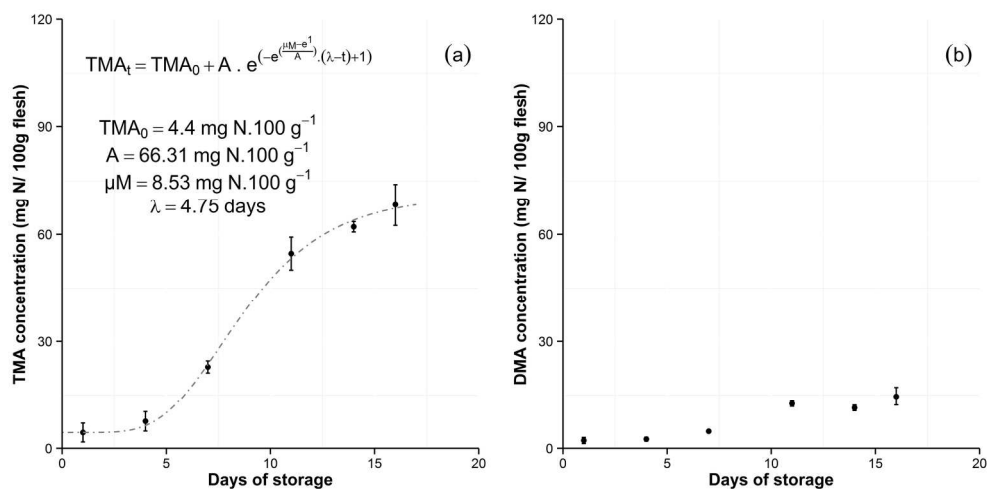


Figure 2: Evolution of mean TMA-N (a) and DMA-N (b) concentrations (mg N/100g) measured by SPME-GC-MS (n=9) throughout the storage period. Each dot reflects the mean value observed for three fillets analysed in triplicate. Concerning TMA-N production modelling, parameters, obtained after 20 iterative steps, correspond to maximum theoretical concentration (A), TMA-N concentration maximum daily increase during the exponential phase ( $\mu$ ), TMA-N concentration lag phase ( $\lambda$ ) and initial TMA-N concentration (TMA<sub>0</sub>).

95x47mm (600 x 600 DPI)

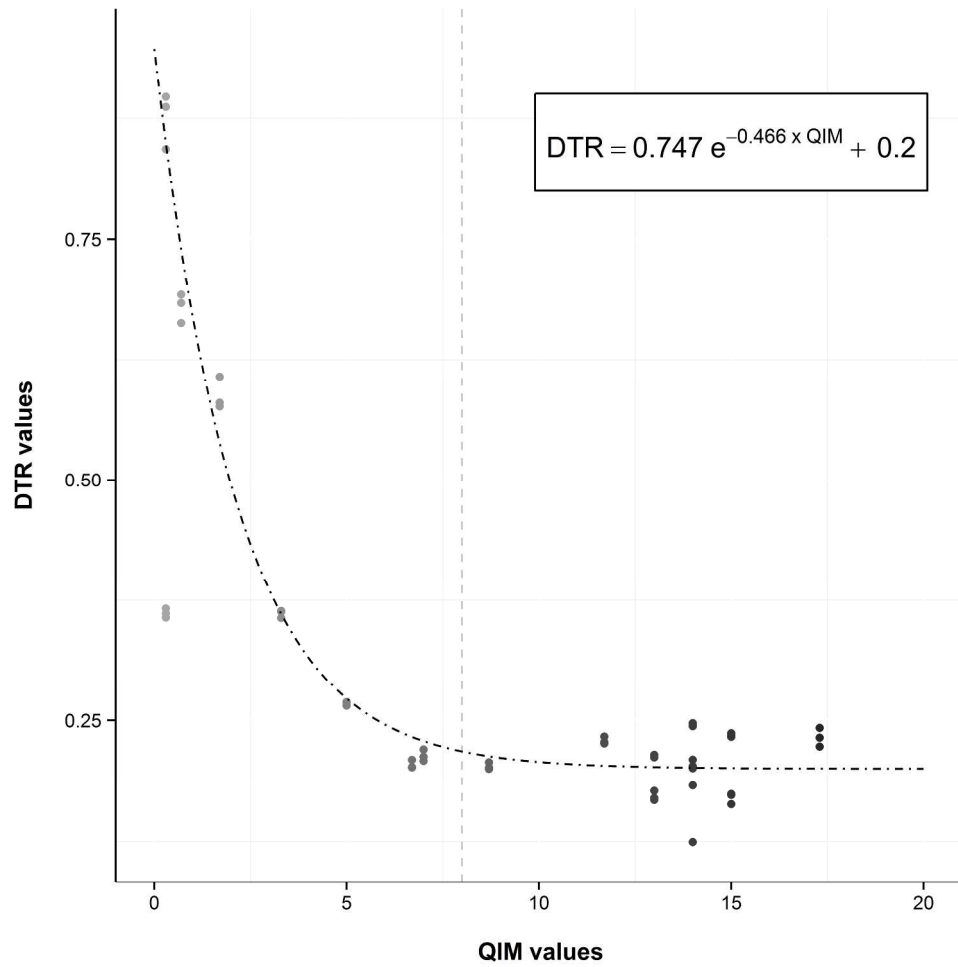


Figure 3: Dot plot showing the evolution of DMA-N/TMA-N ratio (DTR) values as a function of QIM values. Each dot corresponds to a single analysis repeat for a given fillet. The dark grey dot-dashed line illustrates the modelling of data using an exponential equation. The grey dashed line illustrates the estimated maximum QIM value regarding the work of Cardenas Bonilla et al. 35  
133x133mm (600 x 600 DPI)

## Development of SPME-GC-MS method for the specific quantification of dimethylamine and trimethylamine: use of a new ratio for the freshness monitoring of cod fillets.

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## Supporting information file

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### Algorithm S1: Modelling of TMA values

```

1
2
3
4
5 #Algorithm written by A.Dehaut
6 #adapted from the very interesting course of J.R Lobry available (in
7 #French) at http://pbil.univ-lyon1.fr/R/pdf/tdr46.pdf
8
9 #Assignment of the working directory in R_subfolder#
10
11 setwd('X:/R_folder/R_subfolder')
12
13 #Assignment of the file R_logistic, where header is text#
14
15 donnee<-read.csv2('R_logistic.csv',header=T)
16 attach(donnee)
17
18 #Function called "logistique" based on modified Gompertz model#
19
20 logistique <- function(t, param)
21 {
22     a <- param[1]
23     mu <- param[2]
24     l <- param[3]
25     b <- (mu*exp(1))/a
26     c <- (1-t)
27     return(est_TMA_min+a*exp(-exp(b*c+1)))
28 }
29
30 #####
31
32 #Definition of a new data frame with data on Days and TMA
33
34 da<-as.matrix(rbind(Days,TMA), dimnames = NULL)
35
36 #Definition of the function called "scelog" allowing finding the sum
37 #of squares of deviations #
38
39 scelog <- function(param, data)
40 {
41     tobs <- da[1,]
42     xobs <- da[2,]
43     xtheo <- sapply(tobs, logistique, param)
44     return(sum((xobs - xtheo)^2))
45 }
46
47 #####
48
49 #Use of the nlm function allowing finding the parameters that
50 #minimize the sum of squares of deviations #
51
52 nlm(f = scelog, p = c(a_est, mu_est, l_est), data = da)
53
54 #Recovery of the estimates of param a,mu and l.
55
56 est <- nlm(f = scelog, p = c(a_est, mu_est, l_est), data =
57 da)$estimate
58
59
60

```



## Algorithm S2: Modelling of the DTR values

```

1
2
3
4
5
6 #Algorithm written by A.Dehaut
7 #adapted from the very interesting course of J.R Lobry available (in
8 #French) at http://pbil.univ-lyon1.fr/R/pdf/tdr46.pdf
9
10 #Assignment of the working directory in R_subfolder#
11
12 setwd('X:/R_folder/R_subfolder')
13
14 #Assignment of the file R_logistic, where header is text#
15
16 donnee<-read.csv2('R_logistic.csv',header=T)
17 attach(donnee)
18
19 #Function called "exponentielle" #
20
21 exponentielle <- function(t, param)
22 {
23     a <- param[1]
24     b <- param[2]
25     return(DTR_min+a*exp(-(b*t)))
26 }
27
28 #####
29
30 #Definition of a new data frame with data on Days and TMA
31
32 da<-as.matrix(rbind(RDT,QIM), dimnames = NULL)
33
34 #Definition of the function called "scelog" allowing finding the sum
35 #of squares of deviations #
36
37 scelog <- function(param, data)
38 {
39     tobs <- da[2,]
40     xobs <- da[1,]
41     xtheo <- sapply(tobs, exponentielle, param)
42     return(sum((xobs - xtheo)^2))
43 }
44
45 #####
46
47 #Use of the nlm function allowing finding the parameters that
48 #minimize the sum of squares of deviations #
49
50 nlm(f = scelog, p = c(a_est, b_est), data = da)
51
52 #Recovery of the estimates of param a,mu and l.
53
54 est <- nlm(f = scelog, p = c(a_est, b_est), data = da)$estimate
55
56
57
58
59
60

```

**Outcomes from the nlm function after modelling of TMA<sub>Steam Distillation</sub> TMA<sub>SPME</sub> & DTR:**

```

      TMASteam Distillation
$minimum
[1] 1009.049
$estimate
[1] 56.802525  7.802283  4.149119
$gradient
[1] -8.810839e-07  1.293319e-05 -
3.263012e-05
$code
[1] 1
$iterations
[1] 20

      TMASPME
$minimum
[1] 1394.435
$estimate
[1] 66.310266  8.533023  4.746776
$gradient
[1] 0.0000207965  0.0001402129 -
0.0002908528
$code
[1] 1
$iterations
[1] 20

DTR (atypical fillet having been
      excluded)
$minimum
[1] 0.05380738
$estimate
[1] 0.7468669  0.4660460
$gradient
[1] -3.146580e-07 -2.852996e-07
$code
[1] 1
$iterations
[1] 3
```

Figure S1: TMA and DMA calibration curves

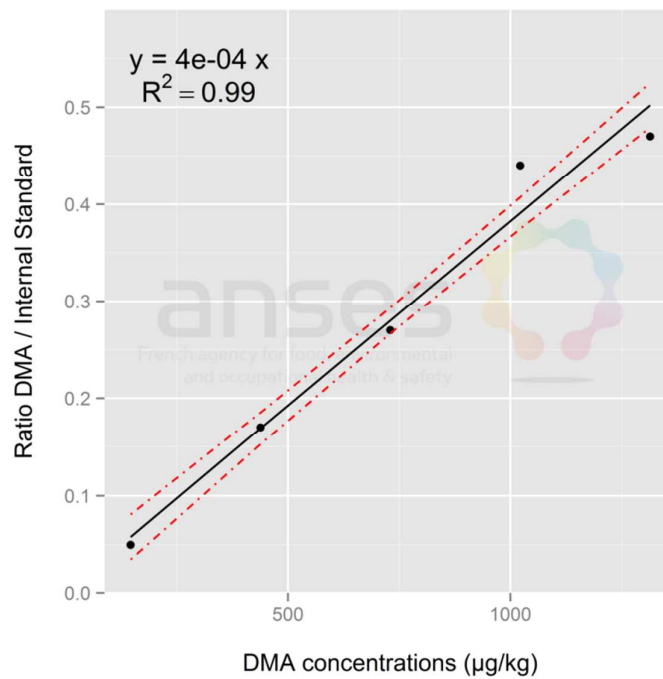
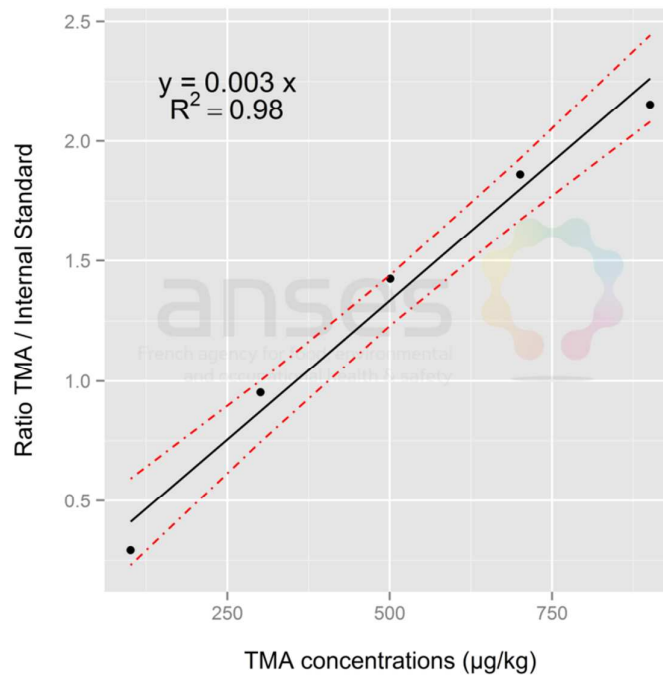
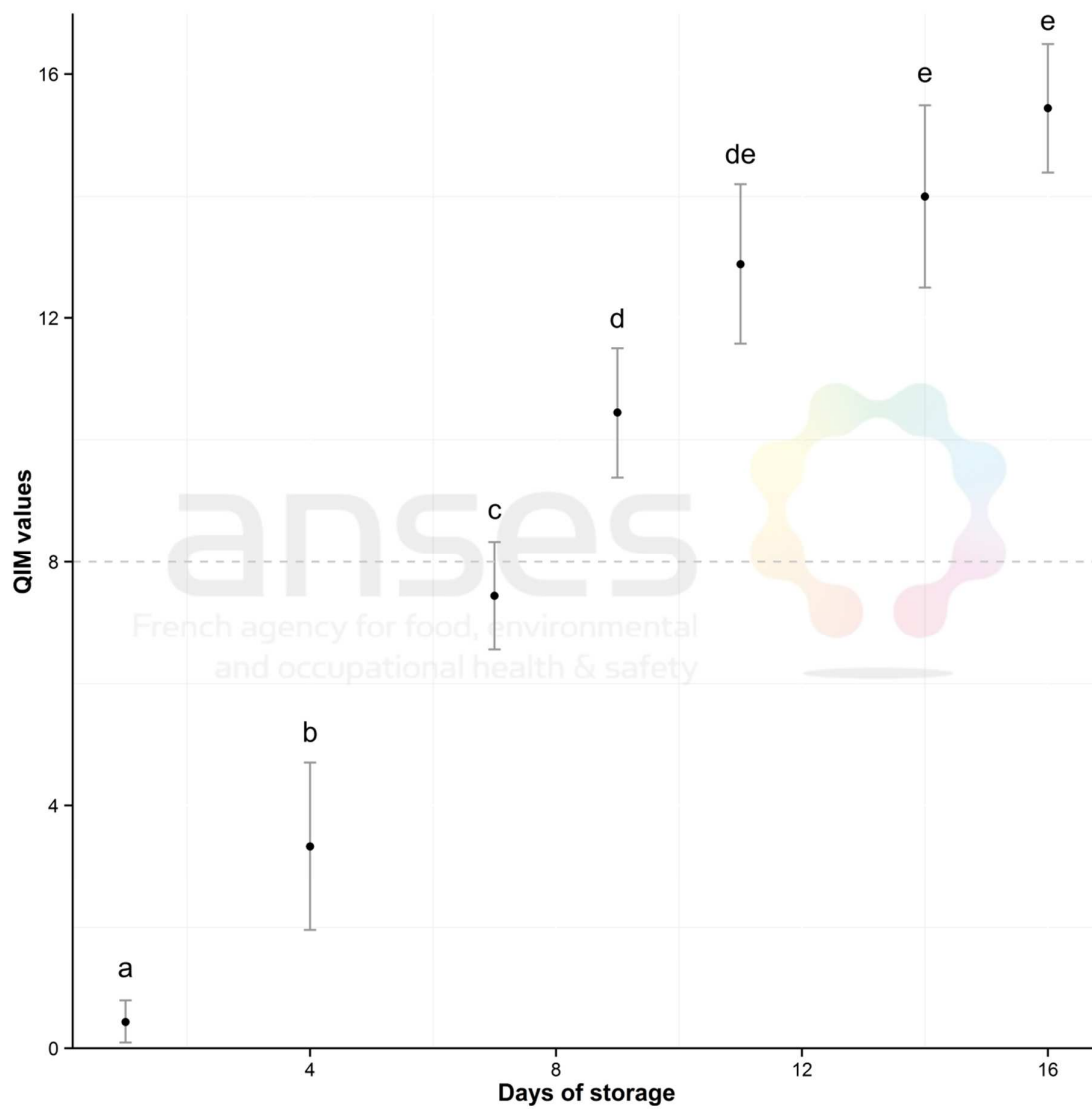
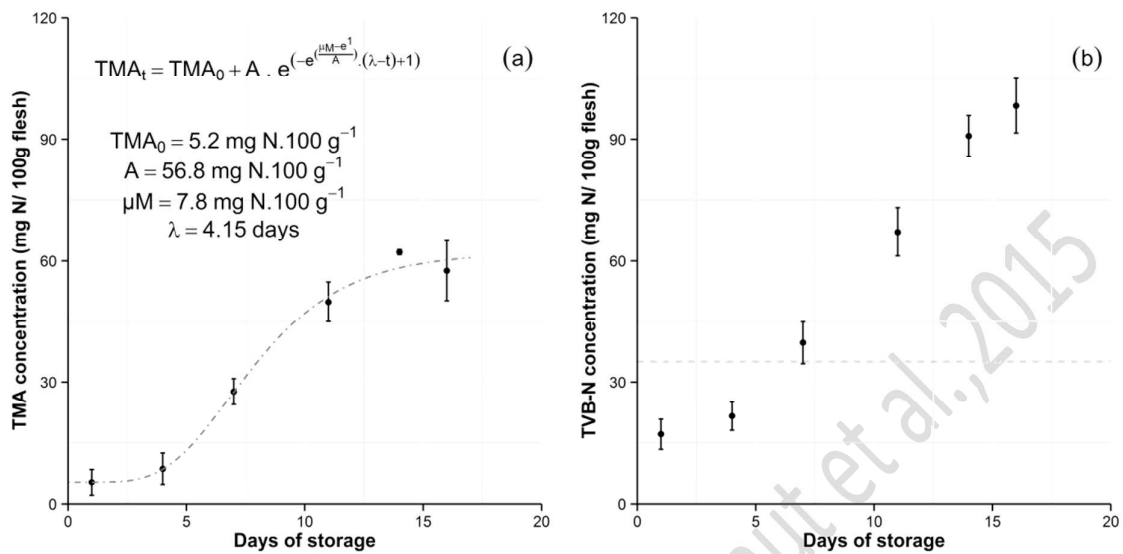


Figure S2: QIM score evolution throughout the storage



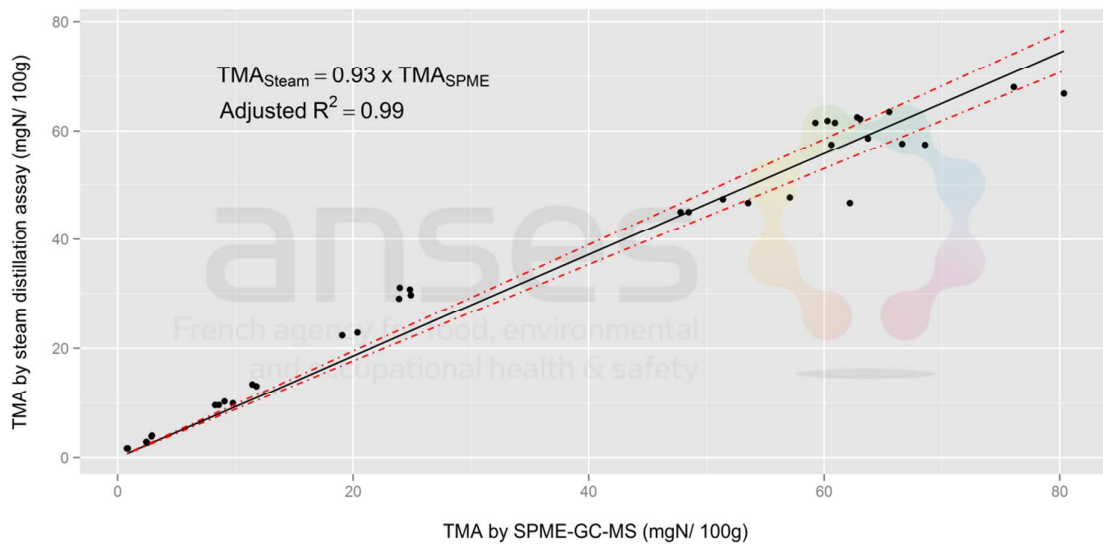
Dot plot showing the average QIM scores (n=9) of the three samples evaluated per storage day, by the panellists, throughout the storage. Error bars illustrate 95% confidence intervals of the mean (two standard errors). The grey dashed line illustrates the estimated maximum QIM value regarding the work of Cardenas Bonilla *et al.*<sup>35</sup> Letters over dots correspond to the results of LSD test.

Figure S3: Evolution of TMA-N and TVB-N evaluated by steam-distillation assays



Evolution of mean TMA (a), TVB-N (b) concentrations (mg N/100g) measured by steam-distillation assay (n=6); each dot reflects the mean value observed for three fillets analysed in duplicate. Concerning TVB-N, the grey dashed line refers to the limit value set by EC Regulation 2074/2005.

Figure S4: Relation between TMA<sub>steam distillation</sub> and TMA<sub>SPME</sub> values



Supporting informations Dehian  
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