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.

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

Alexandre DEHAUT,^a Simon DUTHEN,^a Thierry GRARD,^b Frédéric KRZEWINSKI,^c Assi N'GUESSAN,^d Anne BRISABOIS^a & Guillaume DUFLOS^{a,*}

^a ANSES, Laboratoire de Sécurité des Aliments – Département des Produits de la Pêche et de l'Aquaculture, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-Mer, France.

^b Université du Littoral Côte d'Opale – ANSES, Équipe Biochimie des Produits Aquatiques, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-Mer, France

^c Université de Lille 1, Unité de Glycobiologie Structurale et Fonctionnelle, UGSF - Bâtiment C9, 59650 Villeneuve d'Ascq, France

^d Université de Lille 1, Laboratoire Paul Painlevé – UMR CNRS 8524, Cité Scientifique, 59650 Villeneuve d'Ascq, France

* Corresponding author: E-mail: guillaume.duflos[at]anses.fr Phone: +33 321 992 500 Fax: +33 321 991 725

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ABBREVIATIONS

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

ABSTRACT

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

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

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

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

INTRODUCTION

Fish is increasingly consumed around the world despite disparities recorded between continents.¹ It is a very convenient food, with good nutritional properties but is nevertheless highly perishable, with a short shelf-life ranging from a few days to around twenty days in cold storage, depending on fish species and packaging.^{2,3} Freshness estimation is a key issue concerning this food matrix, as in fish there is a close link between freshness and overall quality.⁴ Consumers are more and more concerned about the quality of what they eat, with specific attentions to price and freshness.⁵ Accurate indicators and tools characterizing the decrease in freshness are therefore required. To date, numerous techniques have been developed including sensory,^{6,7} physical,^{8,9} biochemical¹⁰⁻¹³ and microbial^{14,15} methods, but sensory analysis remains the most widely used technique for freshness estimation by the industry.¹⁶ Unfortunately, these techniques have certain drawbacks. Concerning sensory analysis, the need for a constantly trained, objective and available sensory panel can be problematic.¹⁷ Physical methods usually require whole fish or fillets with skin, which makes them less efficient with processed fish. The main problem encountered with chemical analysis is their lack of reliability during the early stages of freshness decay.³ Finally, microbial methods require very long processing periods and these methods are highly variable regarding numerous parameters.¹⁸

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

leads to the production of dimethylamine (DMA) and formaldehyde in equimolar proportions; this enzyme activity is particularly important in gadoid fish species.²² Additional minor synthesis pathways were described for TMA, which might originate from the degradation of choline, acetylcholine, carnitine or betaines.²⁴ TMA and DMA are directly involved with quality and freshness issues as they result directly from the spoilage of fish through TMAO catabolism. On the one hand TMA is responsible of the characteristic fishy-odour, with a rejection level in chilled fish comprised between 10 and 15 mg nitrogen 100 g⁻¹^{3, 25} while on the other hand DMA is produced soon after fish harvest, contributing to the overall flavour of fish, with no specific odour described.²⁵ In the case of freezing, DMA is also an indirect marker of flesh texture as it is produced together with formaldehyde, indeed methanal forms cross-links with proteins resulting in a marked toughening of fish flesh.³

Historically; several methods have been developed to quantify TMA and DMA using capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) or gas chromatography (GC).^{24, 26, 27} Unfortunately, these methods usually necessitate an onerous sample preparation step with the use of hazardous solutions, but some, such as CE and HPLC, are usually more informative than GC concerning quantities of TMAO and some cations. Headspace analysis can markedly reduce sample preparation time.^{26, 27} More recently, solid-phase micro-extraction (SPME) headspace methods have been developed for quantification of volatile amines in fish.²⁸⁻³² Extraction steps together with separation of amines are usually well described and transferable, although two studies did not manage to clearly differentiate TMA and DMA signals.^{28, 29} Concerning fibres, most of the studies used fibres coated with CAR/DVB/PDMS,³⁰⁻³² only one study used a non-available, laboratory-developed fibre.²⁹ Regrettably, very few published studies described the optimization step for the sample preparation.^{29, 30} SPME methods were applied to numerous fish species involving fresh water or mangrove fish²⁹⁻³¹ and marine fish,^{28, 31, 32} but except for one study,²⁸ it was not applied to the most consumed marine species in Europe. Among these studies, very few applied these methods to monitor the spoilage of fish.^{28, 30} Finally, when used, comparative methods consist mostly of TVB-N analysis,^{28, 30, 32} TMA-N assay being overlooked, and only one study used sensory tests.³⁰

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

EXPERIMENTAL

Chemicals

Concerning the chemicals used in this study, a C₅ - C₁₅ n-paraffin mix, and ammonium chloride, were acquired from Sigma-Aldrich (St-Quentin-Fallavier, France). Other chemicals were used as pure compounds solutions: trimethylamine hydrochloride (PubChem CID: 1146), dimethylamine hydrochloride (PubChem CID: 674), or internal standards: 2-phenylethylamine (PubChem CID: 1001), sec-butylamine (PubChem CID: 24874), N, N-diisopropylmethylamine (PubChem CID: 547370), 1,2-dimethylpropylamine (PubChem CID: 61236), all of which were purchased from Sigma-Aldrich. Potassium hydroxide pellets used to raise pH levels were also acquired from Sigma-Aldrich. Formaldehyde solution was purchased from VWR (Fontenay-sous-Bois, France). Trichloroacetic-acid (TCA), 4% boric acid with methyl red and bromocresol green indicator, 0.1 N Titrimorm® sulphuric acid and 10% sodium hydroxide solutions were purchased from Dislab (Lens, France). Finally, sodium chloride (NaCl) was acquired from Oxoid (Dardilly, France).

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

Fish sample preparation

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

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

SPME-GC-MS procedure

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

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

Shelf-life assessment

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

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

On the day of analysis each of the fillets was processed in the same way, as follows. Firstly, the fillet was subjected to sensory analysis, carried out by three judges, using QIM score evaluation as described by Cardenas Bonilla *et al.*³⁵ After fish preparation as previously described, 1 mL of filtrate was conserved for SPME-GC-MS analysis and the remainder was used for total volatile basic nitrogen (TVB-N) and TMA-N assays, in duplicate, by steam distillation as previously described.³⁴ Briefly, before the tube to be placed in a Gerhardt Vapodest distillation unit (Les Essarts-le-Roi, France), 25 mL of filtrate were loaded in tube. In case of TMA-N assays, with 20 mL of formaldehyde were added. Then, 6 mL of 10% NaOH was poured and the distillation was performed. The distillate was collected into a beaker containing 10 mL of 4% boric acid, before titration with a 0.1 N sulfuric acid solution. According to Regulation (EC) No. 2074/2005,³³ a solution of NH₄Cl at 1.91 g.L⁻¹ was used as a control for steam distillation assays. Results were considered as valid for recovery rates comprised between 98% and 101%. For SPME-GC-MS, the filtrate was diluted with a saturated NaCl solution from 1/100 to 1/4000 depending on the day of analysis concerned. On each day, filtrate was analysed in triplicate as follows: 11 mL of diluted filtrate was poured into SPME vials, 4.4 µL of 1,2-dimethylpropylamine standard solution was added together with one potassium hydroxide pellet and vials were hermetically sealed.

Descriptive statistics, inferential statistics and modelling

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

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

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

RESULTS AND DISCUSSION

Sample preparation optimization

In a first step of method optimization, special attention was applied to the chemical conditions of the sample. In absence of potassium hydroxide (KOH), pH values of solutions were lower than 8.0 and no detectable peak was recorded on chromatogram. It was already known that TMA and DMA are only volatiles in their conjugate base form²⁸ and have respective pKa of 9.76 and 10.64.³⁷ Addition of KOH in the solution increased the pH above 12.0 and clear peaks appeared on chromatograms. NaCl tended to increase both TMA and DMA recovery with a clear improvement of the peak area for TMA and a slighter increase concerning DMA which is in accordance with previous observations.²⁹ It was thus decided to retain the use of a saturated solution of NaCl, with the addition of a KOH pellet into the vial for the preparation of samples.

Optimization of headspace extraction

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

and seven extraction times (1, 2, 5, 10, 20, 30 and 40 min) were tested, as illustrated in **Figure 1**. Compared with similar extraction times at 50 °C, bar graphs clearly show that an extraction temperature of 35 °C produced peaks higher intensities from time of 5 min and longer ($p\text{-value} < 0.001$). Concerning time of extraction, longer times led to higher peak intensities. Least significant difference tests tended to conclude that a significant increase of peak area was observed between 20 min and 30 min of extraction, whereas area increase between 30 min and 40 min was not significant. Regarding these results, extractions at 35 °C for 30 min were chosen as the best compromise for extracting volatiles. Extraction by SPME is the result of two concomitant equilibria: first between matrix and headspace and then between fibre and headspace. Results giving smaller areas observed with an extraction temperature of 50 °C could be explained by the fact that equilibrium between fibre and headspace displaced towards the headspace, as previously reported.³⁸ Following this observation, increasing the time of extraction at a fixed low extraction temperature resulted in the adsorption of more volatile compounds on SPME fibre. The temperature of 35 °C was confirmed by two other studies,^{30,31} but in contrast the time of extraction was slightly higher than the range from 5 to 22 min described elsewhere.²⁸⁻³² This difference could be explained by the fact that a CAR/PDMS fibre was used instead of Carboxen®/Polydimethylsiloxane/Divinylbenzene (CAR/PDMS/DVB) or Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fibres, and in addition no saturation was noted after 10 min, as was observed by Chan *et al.*³⁰

Injection and separation optimizations

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

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

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

Internal standard screening and quantification range

To overcome signal variation, an internal standard was added for sample preparation. Four compounds, 2-phenylethylamine, sec-butylamine, N,N-diisopropylmethylamine and 1,2-dimethylpropylamine were tested, as they share structures or chemical properties close to the amines studied. As special attention was paid to safety issues when selecting internal standards, sec-butylamine was rapidly discarded because of its toxic, polluting and highly corrosive properties. Among the remaining compounds 1,2-dimethylpropylamine was the molecule that led to best linearity of the signal for both TMA and DMA (data not shown). Using 1,2-dimethylpropylamine as internal standard enabled a quantification range from 100 µg kg⁻¹ to 900 µg kg⁻¹ for TMA and from 150 µg kg⁻¹ to 1300 µg kg⁻¹ for DMA with good linearity characterized by respective determination coefficients of $R^2_{\text{TMA}} = 0.98$ and $R^2_{\text{DMA}} = 0.99$ (Supporting information – Figure S1). No further investigations were brought to LOD estimation, considering that lower TMA and DMA values, observed in fish preparation, will rarely be lower than 1 mg kg⁻¹.³⁹

Application to fish freshness assessment monitoring

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

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

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

ous study on gutted whole cods,⁴⁰ but values are almost ten times higher; it could be hypothesized that fish filleting might have discharged TMAO demethylase and induced an increase in its activity in comparison with the one in whole fish.

Proposition of the DTR ratio

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

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

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

CONCLUSION

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

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

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

	Trimethylamine ^a		Dimethylamine ^b	
	FWHM ^c	TF ^d	FWHM ^c	TF ^d
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. ^a Data of ANOVA with trimethylamine pure solution, for each combination of split levels, injection and column temperatures, 5 replicated measurements were performed. ^b Data of ANOVA with dimethylamine pure solutions, for each combination of split levels, injection and column temperatures, 5 replicated measurements were performed. ^{c,d} Influence of parameters and combination of parameters on full width half maximum ^c and tailing factor ^d 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 (μ), TMA-N concentration lag phase (λ) and initial TMA-N concentration (TMA_0).

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

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.

Alexandre Dehaut, ^a Simon Duthen, ^a Thierry Grard, ^b Frédéric Krzewinski, ^c Assi N'Guessan, ^d Anne Brisabois ^a & Guillaume Duflos ^{a,*}

^a ANSES, Laboratoire de Sécurité des Aliments - Département des Produits de la Pêche et de l'Aquaculture, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-Mer, France

^c Université du Littoral Côte d'Opale - ANSES, Équipe Biochimie des Produits Aquatiques, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-mer, France

^b Université de Lille 1, Unité de Glycobiologie Structurale et Fonctionnelle - UGSF - Bâtiment C9, 59650 Villeneuve d'Ascq, France

^d Université de Lille 1, Laboratoire Paul Painlevé – UMR CNRS 8524, Cité Scientifique, 59650 Villeneuve d'Ascq, France

*Corresponding author: E-mail: guillaume.duflos[at]anses.fr - Phone: +33 321 992 500 - Fax: +33 321 991 725

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Algorithm S1: Modelling of TMA values

```

#Algorithm written by A.Dehaut
#adapted from the very interesting course of J.R Lobry available (in
#French) at http://pbil.univ-lyon1.fr/R/pdf/tdr46.pdf

#Assignment of the working directory in R_subfolder#

setwd('X:/R_folder/R_subfolder')

#Assignment of the file R_logistic, where header is text#

donnee<-read.csv2('R_logistic.csv',header=T)
attach(donnee)

#Function called "logistique" based on modified Gompertz model#

logistique <- function(t, param)
{
  a <- param[1]
  mu <- param[2]
  l <- param[3]
  b <- (mu*exp(1))/a
  c <- (1-t)
  return(est_TMA_min+a*exp(-exp(b*c+1)))
}

#####

#Definition of a new data frame with data on Days and TMA

da<-as.matrix(rbind(Days,TMA), dimnames = NULL)

#Definition of the function called "scelog" allowing finding the sum
of squares of deviations #

scelog <- function(param, data)
{
  tobs <- da[1,]
  xobs <- da[2,]
  xtheo <- sapply(tobs, logistique, param)
  return(sum((xobs - xtheo)^2))
}

#####

#Use of the nlm function allowing finding the parameters that
minimize the sum of squares of deviations #

nlm(f = scelog, p = c(a_est, mu_est, l_est), data = da)

#Recovery of the estimates of param a,mu and l.

est <- nlm(f = scelog, p = c(a_est, mu_est, l_est), data =
da)$estimate

```

Algorithm S2: Modelling of the DTR values

```

#Algorithm written by A.Dehaut
#adapted from the very interesting course of J.R Lobry available (in
#French) at http://pbil.univ-lyon1.fr/R/pdf/tdr46.pdf

#Assignment of the working directory in R_subfolder#

setwd('X:/R_folder/R_subfolder')

#Assignment of the file R_logistic, where header is text#

donnee<-read.csv2('R_logistic.csv',header=T)
attach(donnee)

#Function called "exponentielle" #

exponentielle <- function(t, param)
{
  a <- param[1]
  b <- param[2]
  return(DTR_min+a*exp(-(b*t)))
}

#####

#Definition of a new data frame with data on Days and TMA

da<-as.matrix(rbind(RDT,QIM), dimnames = NULL)

#Definition of the function called "scelog" allowing finding the sum
of squares of deviations #

scelog <- function(param, data)
{
  tobs <- da[,2]
  xobs <- da[,1]
  xtheo <- sapply(tobs, exponentielle, param)
  return(sum((xobs - xtheo)^2))
}

#####

#Use of the nlm function allowing finding the parameters that
minimize the sum of squares of deviations #

nlm(f = scelog, p = c(a_est, b_est), data = da)

#Recovery of the estimates of param a,mu and l.

est <- nlm(f = scelog, p = c(a_est, b_est), data = da)$estimate

```


Outcomes from the nlm function after modelling of TMA_{Steam Distillation} TMA_{SPME} & DTR:

TMA _{Steam Distillation}				TMA _{SPME}			
\$minimum				\$minimum			
[1] 1009.049				[1] 1394.435			
\$estimate				\$estimate			
[1] 56.802525 7.802283 4.149119				[1] 66.310266 8.533023 4.746776			
\$gradient				\$gradient			
[1] -8.810839e-07 1.293319e-05 -3.263012e-05				[1] 0.0000207965 0.0001402129 -0.0002908528			
\$code				\$code			
[1] 1				[1] 1			
\$iterations				\$iterations			
[1] 20				[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.*³⁵ 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_{steam distillation} and TMA_{SPME} values