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Validation of Selective Ion Flow Tube Mass Spectrometry for Fast Quantification of Volatile Bases Produced on Atlantic Cod (*Gadus morhua*)

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Selective ion flow tube mass spectrometry (SIFT-MS) is a direct mass spectrometric technique that allows qualitative and quantitative analysis of a large number of volatile organic compounds. Because of its speed and ease of use, this nondestructive technique could be considered as a practical tool for quality control. This research focuses on the possibilities of direct headspace sampling by SIFT-MS for the quantification of the volatile basic nitrogen content (TVB-N) of fish fillets. These volatile bases [trimethylamine (TMA), dimethylamine (DMA), and ammonia] give additional information in conjunction with the sensory scoring and microbiological analysis about the quality of the fish fillets. This research validates in a first part the SIFT-MS method for the quantification of the volatile bases in mixed cod samples. With regard to the investigated linearity, repeatability, reproducibility, recovery, limit of detection, and limit of quantification, SIFT-MS appeared to be an adequate technique for measuring volatile bases spiked on cod. In the second part of this research, the technique was validated for the analysis of volatile bases on cod fillets during a storage experiment under ice. A good correlation was obtained between the proposed direct headspace sampling and traditional methods. The sensitivity of the SIFT-MS method can be improved when cod fillets are made more alkaline (pH > 11) during sampling.

KEYWORDS: SIFT-MS; Atlantic cod (*Gadus morhua*); TVB-N; TMA-N

INTRODUCTION

Quality estimation of raw fishery products is nowadays a necessity for good functioning quality systems in fish-processing companies. Fish and fishery products are very susceptible to irreversible quality losses during storage due to chemical, but mainly microbiological, degradation. To assess the degree of spoilage of fish and fishery products, fast, accurate, objective, and nonambiguous measurements are needed. An often used parameter to determine fish spoilage, next to sensory scoring, is the quantification of typical odorous basic nitrogen compounds present in the fish tissue. $(CH_3)_3N$ (trimethylamine or TMA), $(CH_3)_2NH$ (dimethylamine or DMA), and NH_3 (ammonia) are products of autolytical and microbiological degradation and are collectively known as the total volatile basic nitrogen (TVB-N) fraction.

Facultative anaerobic bacteria such as *Shewanella putrefaciens*, *Acinetobacter*, and *Photobacterium phosphoreum*, recovered on iced fish, are able to reduce trimethylamine-*N*-oxide (TMAO) to TMA (*1*). TMAO has an osmoregulatory function in marine fish species, and the amount of TMAO present in the muscle tissue depends on the species, season, and marine environment (*2*). TMA is known to have a typical "fishy" odor. Some gadoid fish species are able to reduce TMAO in an autolytic pathway to DMA and formaldehyde (*3*, *4*). When bacterial growth is reduced, as under freezing conditions, production of DMA is an important factor involving quality losses and is considered as a frozen storage index (*5*). Ammonia is generally a bacterial degradation product, produced by the decarboxylation of amino acids in the fish muscle tissues (*1*). Ammonia is also released in Elasmobranch fish species, even in the early stages of storage, because of a fast enzymatic ammonia production originating from ureum (*6*).

The determination of the TVB-N in a fish sample is known to be the most common chemical parameter applied, because of its simplicity to evaluate the microbiological spoilage degree of fish and meat products. According to European directive 95/149/EEC, this indicator can be used if sensorial methods raise doubts about the freshness of the food product. Critical limits have been set for groups of seafood species and are expressed in milligrams of TVB-N per 100 g of tissue, for example, 35 mg of TVB-N/100 g of tissue and 15 mg of TMA-N/100 g of tissue for cod stored under ice (*1*, *7*). Fresh cod normally has <20 mg of N/100 g of TVB and 3 mg of N/100 g of TMA (*1*). Generally, the most common methods for TVB-N estimation are based on steam distillation of an alkalized sample (*8*−*10*). TMA analysis is

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generally performed according to the picrate method principle (*11*) or by microdiffusion and titration (*12*). Through the years, new technologies have been developed and are still being developed for the quantification of TMA, DMA, and ammonia. Different gas chromatography methods have been reported in the literature for the measurement of primary and secondary amines (*5*, *13*−*15*). Timm et al. reported a capillary electrophoretic method with indirect UV detection for the determination of amines (*5*). The research group of Menéndez et al. developed a method for the determination of the volatile bases based on a sample introduction system coupled to an ion mobility spectrometer (*16*). Sadok et al. and Ruiz Capillas et al. described a method for the determination of TMA-N and TVB-N with flow injection analysis (*17*, *18*). Some other methods currently used are based on enzymatic reactions (*19*), on selective electrodes (*20*), on gas sensors (*21*−*23*), and on pH-sensitive color indicators (*17*, *24*). The fact that some of the new methods are too complex, too difficult in use, too inaccurate, too time-consuming, and too expensive resulted in a further use of the traditional methods in the fish industry.

The aim of this study is to introduce the application of selective ion flow tube mass spectrometry (SIFT-MS) as a measuring technique for the quantification of volatile bases in fish. This technique is developed for online quantification of volatile organic compounds (VOCs) based on mass spectrometry. Its speed allows a high throughput of samples to be analyzed. Recently, SIFT-MS has found its way into many different research fields, mainly involving medical and environmental applications, but the technique has also been described as a tool used for the determination of quality parameters on food products (*25*−*27*). To date, this technique has not yet been validated for measuring amines in a fish matrix.

In the current study, validation of the presented application of SIFT-MS was performed in two sections. The accuracy of the methodology was validated in the first section using standards of the volatile bases spiked on a fish matrix. The second section describes the validation of the applicability of SIFT-MS by using the method in a shelf-life experiment on a well-studied product, Atlantic cod (*Gadus morhua*), stored under ice and comparing the obtained results with the microbiological growth, TMA-N, and TVB-N measurements performed with the traditional methodology.

MATERIALS AND METHODS

Reagents and Chemicals. Trimethylamine hydrochloride (Fluka, Steinheim, Germany), dimethylamine hydrochloride (Aldrich, Steinheim, Germany), and ammonia hydroxide (Sigma, Steinheim Germany), of analytical grade, were used for preparing standards spiked in mixed cod fillets. The alkaline solution was prepared as a mixture of 0.4 M KCl (Sigma) and 0.4 M NaOH (Fluka) dissolved in distilled water.

Raw Material. Atlantic cod (*G. morhua*) was gutted, skinned, and filleted under good manufacturing practices at a local fish-processing company at the Ostend harbor (Belgium) and subsequently transported to the Laboratory of Food Microbiology and Food Preservation (LFMFP) in Ghent (Belgium) in Styrofoam boxes under a layer of crushed ice. Cod fillets used for the optimization of the method were immediately frozen (−24 °C) and were used within 14 days of storage. On the day of use, they were thawed, mixed, spiked, and analyzed at 4 °C. The fillets obtained for the storage study were cut into pieces of 100.0 ± 0.5 g, wrapped in sterile plastic bags, and stored under ice inside a cooling unit with a temperature set at 1.0 ± 1.0 °C. The temperature of the fillets was monitored every 30 min using a Testo 177 temperature data logger and did not exceed 0.5 °C during a storage period of 12 days.

SIFT-MS Analysis. On the sampling day, portioned cod fillets of 100 ± 0.5 g were packed in high-barrier film bags (NX90, Euralpack, Wommelgem, Belgium) filled with 900 mL of inert N_2 gas as headspace using a Multivac A300/42 (Hagenmüller, Wolfert-schwenden, Germany) packaging unit. The barrier film is 90 μm thick and has an oxygen transmission rate of 2.0 $cm^3/m^2 \cdot d \cdot bar$ (23 °C, 85% RH). Samples were stored for 60 min at 4 °C before measuring, to allow the liquid and gas phases to equilibrate.

The selected ion flow tube mass spectrometer (Voice 100, Syft Technologies) sampled 125 ± 8 mL of headspace through a septum on the sampling bag during a time period of 60 s (1.6 ± 0.1 Torr L s^{-1}). VOCs of interest are introduced through a heated inlet into the flow tube, where reactions with precursor ions H_3O^+, NO^+, and O_2^+ result in ionized masses. Quantification of the VOCs occurs by using the reaction rate coefficients of the reaction between the precursor ions and the VOCs. This measuring technique is already well described in the literature (*28*, *29*). To ensure that the instrument is performing correctly, at the beginning of each day on which analytical work was performed, an automated check test was executed, which comprises the flow, the temperature, the quadrupole performance, and single-point accuracy and precision determinations using a certified standard containing benzene, ethylbenzene, ethylene, hexafluorobenzene, isobutene, octafluorotoluene, perfluorohexane, perfluoro-2-methyl-2-pentene, perfluoroheptane, and octadecafluorooctane. A method was built to target volatile bases in a well-defined cod matrix. Quantification of the amines was based on measuring the following ionized masses: $(CH_3)_3N \cdot H^+$ (m/z 60), $(CH_3)_2N \cdot H^+$ (m/z 46) and NH_4^+ (m/z 18) as reaction products with H_3O^+; $(CH_3)_3N^+$ (m/z 59) and $C_2H_6N^+$(m/z 44) as reaction products with NO^+; $C_2H_6N^+$ (m/z 44) and NH_3^+ (m/z 17) as reaction products with O_2^+. The selected product ions of these reactions are monitored by a mass spectrometer located at the downstream end of the flow tube. The ratio of the product ion count to the precursor ion count provides a quantitative measure for the amount of VOC in the headspace.

TVB-N and TMA-N Determination. The TVB-N content of the cod samples was determined using the steam distillation method according to Antonacopoulos and Vyncke (*10*). TMA analyses were performed on the basis of the Dyer spectrophotometric method (*11*), as modified by Boskou and Debevere (*30*).

Microbiological Analysis. From each fillet, 30 g of sample was collected aseptically in a stomacher bag and diluted 10 times in physiological saline peptone solution (PPS, 0.85% NaCl, 0.1% peptone). After the sample had been homogenized for 1 min in a stomacher (Lab Blender 400), the appropriate successive 10-fold dilutions in PPS were performed. The total viable aerobic count was determined on the nutrient-rich marine agar (Difco, le Pont de Claix, France) using the pour plating technique. The plates were aerobically incubated for 4 days at 22 °C. The number of H_2S-producing bacteria was enumerated by pouring plates with Iron Agar Lyngby (Oxoid, Hampshire, U.K.) containing L-cysteine (Fluka) in accordance with the manufacturer's preparation instructions. H_2S-producing bacteria appeared on the plates as black colonies due to the precipitation of ferrous sulfide in the agar.

pH Measurements. The pH was measured by a pH-electrode (InLab 427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected with a pH-meter (SevenEasy, Mettler Toledo GmbH).

Validation of SIFT-MS Method as a Tool for Measuring Volatile Bases. Linearity was tested on standard solutions of trimethylamine, dimethylamine, and ammonia, spiked in the range of 0−30 mg of N on 100 g of mixed cod fillet. The analytical procedure was performed in duplicate, and eight data points were used each time.

For the limit of detection (LOD) and limit of quantification (LOQ), 10 nonspiked fish samples were analyzed. The LOD and LOQ were calculated using the IUPAC equations (*31*).

$$\mathrm{LOD} = x_{\mathrm{bl}} + 3 \times \mathrm{SD}_{\mathrm{bl}}$$

$$\mathrm{LOQ} = x_{\mathrm{bl}} + 6 \times \mathrm{SD}_{\mathrm{bl}}$$

with x_{bl} = mean of 10 samples and $\mathrm{SD}_{\mathrm{bl}}$ = standard deviation of the 10 samples.

For the determination of the recovery (R) of the volatile bases, mixed cod already containing 10 mg of N/100 g was spiked with 10 mg of N/100 g. Measurements were performed on samples at 4 °C. To calculate the recovery, the following equation was used (*32*):

$$R = [Q_{\mathrm{base}}(\mathrm{O+S}) - Q_{\mathrm{base}}(\mathrm{O})]/Q_{\mathrm{base}}(\mathrm{S})$$

with $Q_{base}(S)$ = the quantity of base added, $Q_{base}(O)$ = the quantity of base in the original sample, and $Q_{base}(O + S)$ = the quantity of base recovered from the spiked sample.

To assess the repeatability of the SIFT-MS method, five identically spiked mixed cod (10 mg of N/100 g) samples were subsequently analyzed at a temperature of 4 °C. Reproducibility was assessed by measuring five identically spiked mixed cod samples (10 mg of N/100 g) on different days of analysis.

Three samples within the range of 0−30 mg of N/100 g of cod were tested by determining the TMA headspace concentration of three cod samples on GC-MS together with SIFT-MS. These samples were analyzed to assess the accuracy of the method within the range of interest, and the result was used to calculate the Z score.

$$Z = (x - X)/\sigma$$

with x = the reported result, X = the assigned value, and σ = the target value for standard deviation.

Validation of the SIFT-MS Method as a Tool for Measuring Volatile Bases Applied in a Storage Study of Cod Fillets. During 12 days of storage under ice (≤0.5 °C), evolutions of the TMA-N and TVB-N contents produced on the Atlantic cod fillets were monitored. On days 0, 3, 6, 9, 10, and 12 the TMA-N and TVB-N contents of six randomly selected samples were measured. The first three samples were analyzed according to the classical methods: TMA-N analysis according to the Dyer spectrophotometric method (*11*), TVB-N analysis according to the method of Antonacopoulos and Vyncke (*10*), and determination of the total viable aerobic plate count. On the other three samples the concentrations of volatile bases present in the headspace of the sample bags were measured using the SIFT-MS method. The correlation between the methods was determined on the basis of these measurements.

In a second phase, to increase the sensitivity of the SIFT-MS measurements, the measured sampling bags were injected through a septum with 10 mL of an alkaline buffer (0.4 M KCl; 0.4 M NaOH). The samples were equilibrated for 10 min at 4 °C on a shaking unit, to maximize the contact between the fillet and the alkaline solution. This resulted in a pH increase of the surface of the cod fillets to a pH > 11. Subsequently, the samples were measured again using the same SIFT-MS method.

Statistical Analysis. On each measuring day, three samples were randomly selected and analyzed with different methods. Data were subjected to one-way analysis of variance to assess significant ($p < 0.05$) differences between concentrations measured on different days. The software used to explore statistical significances on the obtained results was S-Plus 8.0 for Windows.

RESULTS

Validation of SIFT-MS Method as a Tool for Measuring Volatile Bases. In a first phase, the method for measuring volatile bases in cod fillets with SIFT-MS as measuring technique was validated. Standard curves were obtained for trimethylamine, dimethylamine, and ammonia at pH 7.0 ± 0.2 and a temperature of 4 °C. The equations of the linear regressions were found to be $y = 192.72x + 776.23$ ($R^2 = 0.968$) for TMA, $y = 58.50x + 177.77$ ($R^2 = 0.953$) for DMA, and $y = 43.83x + 217.83$ ($R^2 = 0.942$) for NH_3 with parameters x and y being, respectively, the concentration of the base added in milligrams of N per 100 g of fish and the concentration of the base measured in the headspace of the sampling bag in micrograms per cubic meter.

The implemented analytical SIFT-MS method had in the headspace measurements a LOD of 826, 386, and 278 $\mu g/m^3$ and a LOQ of 878, 460, and 290 $\mu g/m^3$ for, respectively, TMA, DMA, and NH_3. Recoveries of 1.01, 0.98, and 1.03 were obtained for the respective bases. The relative standard deviation of five subsequently repeated measured samples for TMA was 3.8%. The reproducibility for TMA measurements was assessed with a relative standard deviation of 8.7%. The accuracy of the method was validated for trimethylamine using samples that were analyzed on GC-MS and compared to the SIFT-MS result. A Z score of 0.73 ± 0.22 was achieved. No false-positive results could be observed by measuring these three compounds individually or together.

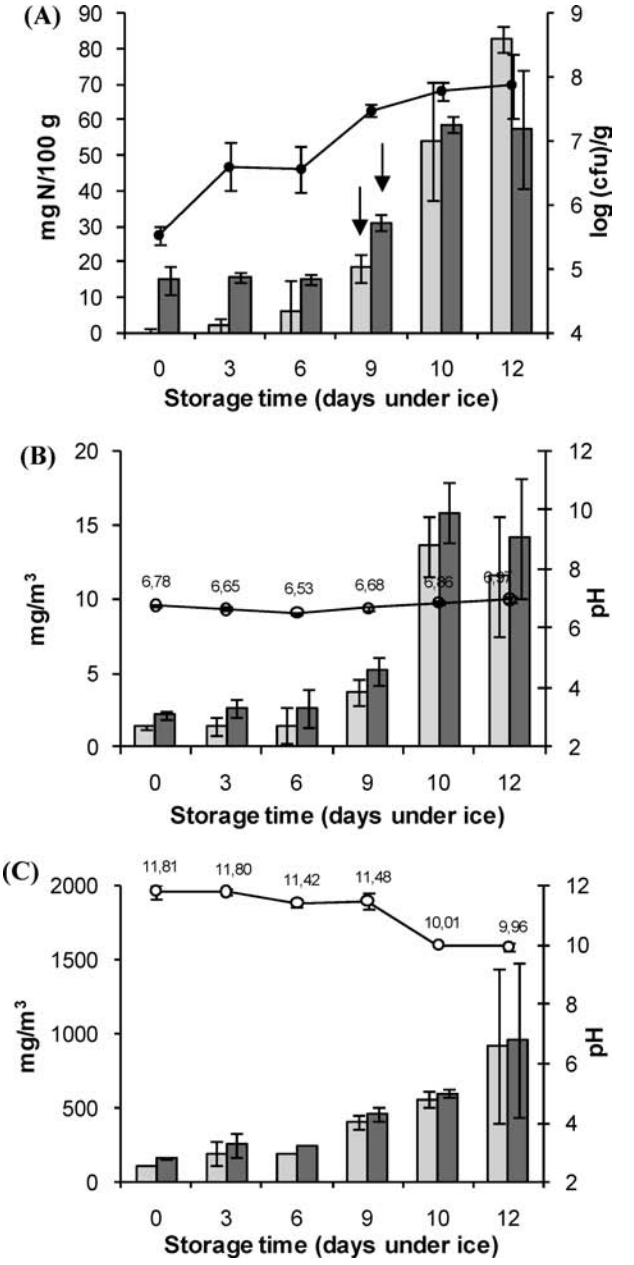

Figure 1. (**A**) Microbiological changes in the total viable aerobic count on marine agar (●) during the storage of cod fillets under ice. The bars indicate the TVB-N measurements according to the steam distillation technique (dark gray) and TMA measurements according to the spectrophotometric method of Dyer (light gray), during the storage of cod fillets under ice. The arrows indicate the moments at which the limits of acceptability for TVB-N (35 mg of N/100 g of cod) and for TMA (15 mg of N/100 g of cod) were reached. Error bars indicate 95% confidence interval ($n = 3$). (**B**, **C**) Evolution of TVB (dark gray) and TMA (light gray) concentrations (mg/m^3) measured with SIFT-MS (**B**) in the headspace of cod fillets stored under ice [The pH evolution of the cod fillets (○) during the storage experiment is indicated on the secondary y axis] and (**C**) in the headspace of cod fillets stored under ice to which 10 mL of buffer (0.4 M KCl; 0.4 M NaOH) alkaline solution was added [the pH evolution (○) on the surface of cod fillets after the addition of 10 mL of buffer (0.4 M KCl; 0.4 M NaOH) is indicated on the secondary y axis]. The TVB-N concentrations are considered as the sum of NH_3, DMA, and TMA concentrations. Error bars indicate 95% confidence interval ($n = 3$).

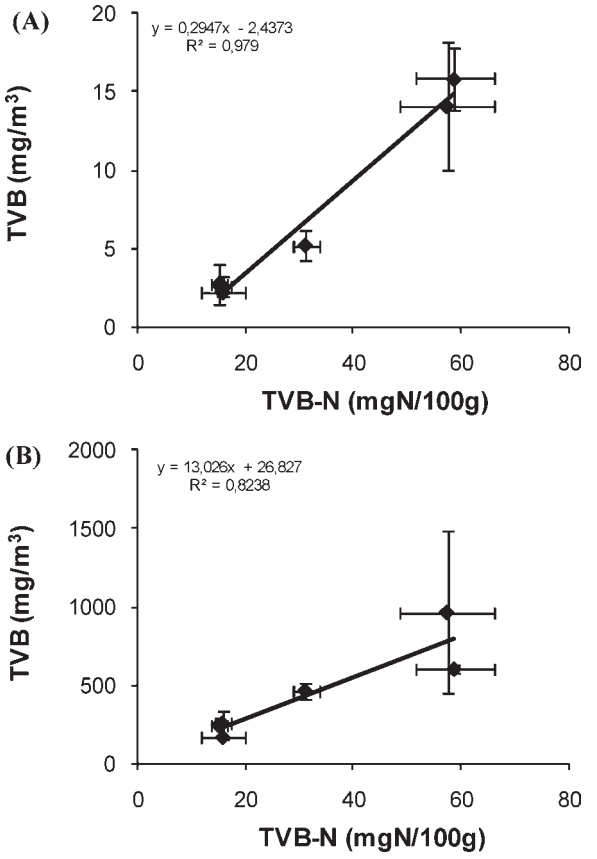

Figure 2. Correlation between TVB-N values measured with the steam distillation method (mg of N/100 g) and the TVB-N values obtained from SIFT-MS headspace measurements (mg/m^3) on (**A**) cod fillets of the storage trial and (**B**) the alkalized cod fillets of the storage trial. Error bars indicate 95% confidence interval (n = 3).

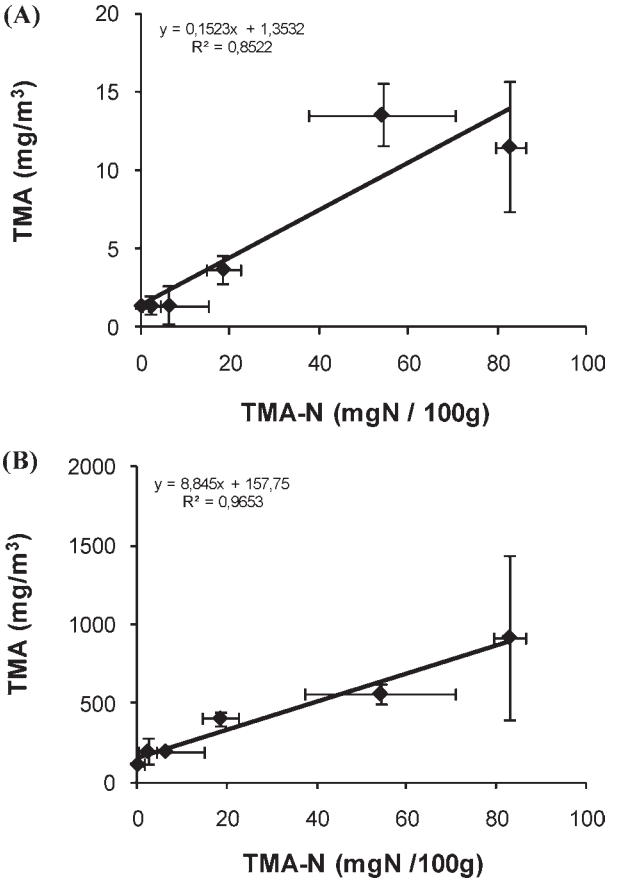

Figure 3. Correlation between TMA-N values measured with the steam distillation method (mg of N/100 g) and the TMA values obtained from SIFT-MS headspace measurements (mg/m^3) on (**A**) cod fillets of the storage trial and (**B**) the alkalized cod fillets of the storage trial. Error bars indicate 95% confidence interval (n = 3).

Validation of the SIFT-MS Method as a Tool for Measuring Volatile Bases Applied in a Storage Study of Cod Fillets. Evolution of the microbial growth (log cfu/g) on cod fillets aerobically stored under ice is shown in **Figure 1A**. The total viable aerobic count at the beginning of the storage experiment was 5.5 ± 0.1 log cfu/g on marine agar plates. After 9 days of storage, the total viable aerobic count exceeded 7 log cfu/g. The number of H_2S-producing bacteria was found to be 2.5 ± 0.2 log cfu/g at day 0, 4.4 ± 0.2 log cfu/g at day 6, and 5.7 ± 0.2 log cfu/g at day 9. The evolution of TMA-N and TVB-N (mg of N/100 g) present in the cod fillets during storage under ice is also presented in **Figure 1A**. TVB-N values remained around 15 mg of N/100 g of cod for fillets stored during the first 6 days and then increased to 31.2 ± 2.5 mg of N/100 g of cod for fillets stored for 9 days under ice. Implementing statistical t tests showed that the increase was significant between the sixth and ninth days of storage under ice. The TVB-N value reached a value between 57 and 59 mg of N/100 g of cod at day 12. The TMA-N content increased steadily from 0 to 6.2 ± 8.8 mg of N/100 g during the first 6 days of storage and subsequently increased to 18.4 ± 3.9 mg of N/100 g at day 9. At day 12, the TMA-N value reached 82.8 ± 3.6 mg of N/100 g. A significant difference was observed between the TMA-N value at days 0 and 9.

The evolution of volatile TMA, DMA, and ammonia concentrations present on cod fillets stored under ice was followed over time using SIFT-MS. The results are given in **Figure 1B**. In the first 6 days of the storage experiment, no significant increase of TMA, DMA, or ammonia was observed. From 9 days of storage on, a significant increase of the TMA concentration was observed. At days 9, 10, and 12 the average concentrations were, respectively, 3725 ± 894, 13562 ± 2009, and 11521 ± 4110 μg/m^3. DMA concentrations increased slightly over 12 days of storage. The initial measured concentration present at day 0 was 442 ± 200 μg/m^3, which increased to 743 ± 309 μg/m^3 at day 6, to 817 ± 206 μg/m^3 at day 9, and to 970 ± 53 μg/m^3 after 12 days of storage under ice. A significant ammonia increase was observed between 9 and 10 days of storage. Before the ninth day of storage, the concentration of ammonia stayed constant and no significant increases were measured. pH evolutions during the storage experiment are illustrated in **Figure 1B**. During storage under ice, the pH initially decreased the first 6 days of storage followed by an increase from day 9 on. **Figure 2A** illustrates the correlation between the results obtained via the classical TVB-N determination and the results obtained via SIFT-MS. The correlation coefficient was found to be 0.98. Analogue **Figure 3A** illustrates the correlation between the results obtained via the classical TMA-N measurements and the results obtained via SIFT-MS headspace measurements with a correlation coefficient of 0.85.

Alkalization of the surface of the cod fillets to a pH above the pK_a values of the volatile amines resulted in forcing these metabolites into the headspace. In that way the sensitivity of measuring changing concentrations of volatile bases during the storage experiment was expected to increase. The measured headspace concentrations after adding the alkaline (0.4 M KCl; 0.4 M NaOH) solution are given in **Figure 1C**. The overall TVB-N concentration was approximately 100 times higher than the original samples measured without the alkaline solution. On day

9, the TMA concentration more than doubled from 195 mg/m^3 at day 6 to 403 mg/m^3 at day 9. This increase in the TMA concentration continued over the following days and resulted in concentrations of 560 mg/m^3 TMA on day 10 and 918 mg/m^3 TMA on day 12. The increase in TMA concentrations is the biggest contribution to the parallel increase in TVB concentrations. DMA and ammonia concentrations stayed steady during the whole storage experiment. The measured pH was >11 except for the samples measured at days 10 and 12. The correlations between the classical TVB-N and TMA-N measurements in relation to the SIFT-MS headspace measurements are shown in, respectively, **Figures 2B** and **3B** with $R^2 = 0.82$ and $R^2 = 0.97$.

DISCUSSION

Validation of SIFT-MS Method as a Tool for Measuring Volatile Bases. The developed method for SIFT-MS for the analysis of volatile bases was validated by determining parameters such as linearity, sensitivity, reproducibility, repeatability, recovery, and accuracy. On the basis of the correlation coefficients of the standard curves made for TMA, DMA, and NH_3 at a fixed pH of 7, generally it could be concluded that linearity within the range of 0−30 mg of N/100 g of sample was acceptable, certainly when the matrix effect of the mixed cod and very small pH deviations between samples are borne in mind. Comparing the LODs and LOQs obtained for the volatile bases in cod with the LOQs obtained in water (33, 128, and 14 μg/m^3 for TMA, DMA, and NH_3, respectively), these limits for cod can be considered as high. However, because the LOD and LOQ are influenced by the measurement time, the LOD and LOQ values obtained using this presented 60 s method were still low enough compared to the concentrations retrieved in the storage experiment. Also recovery, repeatability, and reproducibility were adequate for this purpose and are in line with other newly developed techniques (*5*). Comparison of the concentration of SIFT-MS TMA measurements in cod samples with GC-MS indicated that the instrument is measuring analytically correct and that in these cases no false-positive results were present. Further optimization of the investigated parameters is still possible, but would affect the sampling speed. Then again, decreasing measuring time is also possible, but this would affect the investigated parameters in a negative sense. In summary, the presented methodology appears to be satisfactory for the application of measuring volatile bases in fish within the concentration range of interest using SIFT-MS technology.

Validation of the SIFT-MS Method as a Tool for Measuring Volatile Bases Applied in a Storage Study. The point of sensory rejection for cod fillets stored under ice is, according to the literature, reached at a typical total viable psychrotrophic bacterial count of 10^7−10^8 cfu/g (*33*). In the presented study, this microbiological limit was reached after 9 days of storage (**Figure 1A**). At this day, limits of acceptability for TVB-N (35 mg of N/100 g) and TMA (15 mg of N/100 g) were reached (*1*). Therefore, day 9 could be considered as the moment of sensory rejection. Larsen et al. described a shelf life of 9−12 days of storage for cod stored under ice (*7*). At day 12 higher values for TMA-N were obtained compared to TVB-N. Theoretically, this is not possible. The values were obtained using two different techniques; therefore, we hypothesize that at least one of the techniques is not giving a linear response at higher concentration ranges. Further investigation is needed to confirm this hypothesis. Results from microbiological analysis further showed that the number of H_2S-producing bacteria was found to be 5.7 ± 0.2 log cfu/g after 9 days of storage. *S. putrefaciens* is known to be a typical H_2S-producing spoiler in cod stored under ice. Because of a difference of 1.8 log with the total viable psychrotrophic bacterial count, this typical spoiler for cod stored under ice was not found to be the dominating organism. Dalgaard et al. described a yield factor for TMA of $10^{-9.5\pm0.2}$ mg of N TMA/cfu *S. putrefaciens* (*7*, *34*). Thus, according to the microbiological results in this shelf-life study, *S. putrefaciens* is responsible for a maximum of 0.01 ± 0.01 mg of N TMA/100 g of cod after 9 days of storage and, consequently, compared to 18.4 ± 3.9 mg of N TMA/100 g retrieved, it could be concluded that TMAO present on the fillets was not reduced by *S. putrefaciens*. Larsen et al. conducted a similar storage trial with cod stored aerobically under ice. The dominant spoilage bacterium was found to be *P. phosphoreum*. The yield factor for TMA for *P. phosphoreum* was determined to be $10^{-8.0\pm0.3}$ mg of N TMA/cfu (*7*, *34*). If it is considered that *P. phosphoreum* is the dominating spoilage organism, it would be responsible for approximately 30.19 ± 22.97 mg of N TMA /100 g of cod. Although, with this consideration in mind, an underestimation of the total viable psychrotrophic count is possible due to the fact that the high temperature of pour plating (approximately 45 °C) and the incubation temperature of 22 °C are not optimal for enumeration of this spoilage organism (*35*). *Pseudomonas* spp. typically cause sweet, malty, fruity, and onion-like odors contributed by alcohols, carbonyls, esters, and sulfur compounds. Nevertheless, van Spreekens also reported the possibility of *Pseudomonas putrefaciens* and *Pseudomonas fluorescens* producing TMA (*36*), but no recently published studies could confirm these results. The initial TVB-N content of the cod fillets in the beginning of the storage was lower than 20 mg of N/100 g of fish, which is acceptable for fresh cod according to Debevere et al. (*1*). After 12 days of storage, the TMA-N content exceeded the TVB-N content significantly. This indicates the variability of the classical methods in higher concentration ranges and the inadequacy of comparing results obtained by the two traditional methods in higher concentration ranges.

The results of the SIFT-MS measurements are in line with the classical measurements. After 9 days, at the moment of sensory rejection, the SIFT-MS measurements clearly indicate a significantly higher headspace concentration for TMA at day 9 compared to the previous measuring days (**Figure 1B**). The concentration of ammonia also increases from 9 days of storage on. Spoilage bacteria utilize TMAO as a terminal hydrogen acceptor, allowing further growth; in the next phase, ammonia is produced because of decarboxylation of amino acids (*37*, *38*). The production of the bases on the surface of the cod fillets caused an increase in pH of 0.45 after an initial decrease of 0.25 during the first 6 days. The increase in TMA in the headspace is more explicit when the fish fillets are made more alkaline by adding 10 mL of buffering solution (**Figure 1C**). Also, in similar studies, alkalization of the sample is generally used to increase sensitivity for the analysis of volatile bases in headspace measurements (*16*). The Henderson−Hasselbalch equation describes how pH of biological and chemical systems is determined by acids and bases. Under stronger basic conditions, they occur as free amines and readily go into the vapor phase (*10*). Yet, caution should be taken obtaining VOC concentrations using an elevated pH of the matrix. High VOC concentrations may cause nonlinearity in the signal as the SIFT-MS generally operates in the range that does not cause more than 10−20% reduction in the precursor ion signal. In this study, this was the case for TMA concentrations obtained at days 9, 10, and 12 by alkalization of the sample, assuming a lower accuracy for these measurements. Therefore, an alkalization of the fish matrix to a pH > 11 to increase sensitivity is of use when low TVB-N concentrations are present in the fish matrix. An alternative is to use alkaline buffers causing the pH of the cod matrix to be > 7 and < 11. **Figure 1C** also shows the average measured pH after addition

of the 0.4 M KCl−0.4 M NaOH solution. The lower pH obtained at sampling days 10 and 12 results in an underestimation of the total volatile bases when these samples are comapred to earlier days of sampling. This is why the ammonia concentration appears to drop, but, in fact, the concentrations are increasing when these results are compared with the concentrations shown in **Figure 1B**. Therefore, stress is put on the importance of pH control when generally volatile bases are measured in a headspace.

On the basis of all TVB-N measurements performed in this storage trial, a good correlation ($R^2 = 0.98$) was found between the classical steam distillation technique and the proposed technique for measuring TVB-N without alkalization (**Figure 2A**). This clearly illustrates the applicability of the technique as an alternative for the classical TVB-N measurements. According to the obtained equation, the limit of acceptability of 35 mg of N/100 g in fish flesh coincides under these experimental conditions (temperature, pH, volume headspace) with 7.8 mg/m^3 volatile bases measured in the headspace. Changing the pH of the fillets to >11 increases sensitivity, but because of the varying pH, the resulting correlation coefficient was found to be lower.

Clearly, traditional methods can provide useful measures to evaluate fish freshness and spoilage. However, these measurements are laborious and time-consuming and are thus not convenient for the routine analysis of large numbers of samples. Traditional methods are focused to one substance (e.g., TMA, DMA, or NH_3) or to an undefined sum value for volatile bases (TVB-N) (*5*). Vyncke et al. showed in an interlaboratory study that systematic errors occurred between participating laboratories in measuring TVB-N both with a codex method and with the home methods (*39*). This was caused by an incomplete recovery of TVB-N and/or breakdown of protein during analysis (*5*, *9*, *39*).

The advantage of using SIFT-MS for TVB-N determination is that it is a very fast technique. Within a minute, a simultaneous quantification of the concerned bases is established. Moreover, next to the volatile bases, also other spoilage metabolites or quality parameters such as formaldehyde, can be quantified within the same timeframe. Olafsdottir et al. stated that amines contribute only 29% of the metabolites produced in chilled cod. Also, ketones, alcohols, acids, aldehydes, esters, and sulfur metabolites are produced at the moment of sensory rejection (*40*). The proposed method is a nondestructive measuring technique that is also applicable for MAP packed fish and fishery products because of the inability of the technique to detect N_2, CO_2, and O_2. For measuring spoilage metabolites in MAP packed fish, this can be considered as an advantage. This fact would make the methodology also applicable for outgoing quality control on MAP packed fish and fishery products. There are, however, some disadvantages involved with SIFT-MS measurements. The technique is based on measuring masses in full scan mode and in single ion monitoring (SIM) mode. Measuring in SIM mode implies that the measured sample is already well-defined and well controlled to avoid false-positive results. Defining the headspace of the food matrix is possible by performing a mass scan for each reagent ion, and so determining the VOCs present in the headspace, or by performing analysis in combination with GC-MS. If one is measuring in an undefined matrix, not knowing the VOCs present in that headspace, masses of the unknown VOCs may overlap and thus amplify the signal of masses of interest, although SIFT-MS takes into account only the signal of the lowest measured masses from different precursors to counter this problem. A second drawback of this technique, and most probably the most important drawback for the fish industry, is the price of the equipment, maintenance expenses, and the salary of a skilled technician. However, this methodology could be very useful for research or food control laboratories.

The method developed in this research appears to be a relevant method for TVB-N measurements using SIFT-MS. The method was validated on standards spiked on a cod matrix. The linearity, repeatability, reproducibility, accuracy, and limits of detection and quantification obtained in this research were sufficient for the application of this method. The applicability of this measuring technique was validated in a storage trial of cod stored under ice and proved to give results similar to those obtained with the classical methods currently used in the industry for incoming quality control.

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