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Simultaneous determination of ammonia, dimethylamine, trimethylamine and trimethylamine-N-oxide in fish extracts by capillary electrophoresis with indirect UV-detection

Maike Timm, Bo M. Jørgensen*

Danish Institute for Fisheries Research, Department of Seafood Research, Technical University of Denmark, Building 221, DK-2800 Kgs. Lyngby, Denmark

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Abstract

A capillary electrophoretic method with indirect UV detection is described for simultaneous determination of ammonia, dimethylamine (DMA), trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) in aqueous extracts of fish. A buffer consisting of 4 mM formic acid, 5 mM copper(II)sulfate and 3 mM crown ether 18-crown-6 enabled separation of the analytes in 5–10 min. The use of an extended light path capillary technique resulted in a good sensitivity and repeatability. The linear dynamic range, based on a hydrostatic injection at 50 mbar for 2 s, was from the detection limit to at least 2.5 mM. The detection limit for ammonia, DMA, TMA, and TMAO was less than 0.04 mM, corresponding to 2 mg nitrogen per 100 g fish. As an extra benefit, the method also provided a quantitative determination of potassium, sodium, calcium and magnesium ions.

Keywords: CE; Amines; TVN; TVB; DMA; TMA; TMAO

1. Introduction

Freshness is one of the most important criteria for quality of fish products (Howgate, 1982). But fish undergoes irreversible changes (colour, texture, smell) soon after harvest, resulting in quality loss. A remarkable post-mortem deteriorating chemical change is the gradual accumulation of certain volatile amine compounds in the flesh (Veciana-Nogues, Albala-Hurtado, Izquierdo-Pulido, & Vidal-Carou, 1996). These amines can also be precursors of cytotoxic N-nitrosamines (Monser & Greenway, 1996; Yao, Yang, Zhang, Xie, & Wei, 1998).

Trimethylamine-N-oxide (TMAO) is found in most marine fish and can reach high concentrations (Oetjen & Karl, 1999). It is reduced to trimethylamine (TMA) by spoilage bacteria (Malle, Eb, & Tailliez, 1986), giving rise to the characteristic pungent smell of iced fish, and TMA is therefore an indicator of spoilage (Pedrosa-Menabrito & Regenstein, 1990). During frozen storage of some fish species, when bacterial growth is inhibited, TMAO may be degraded by an intrinsic enzyme activity of the fish (Pedrosa-Menabrito & Regenstein, 1990; Simeonidou, Govaris, & Vareltzis, 1997). In gadoid fish, the enzymatic decomposition of TMAO generates equimolar amounts of dimethylamine (DMA) and formaldehyde (LeBlanc & LeBlanc, 1988). Production of DMA depends on the sub-zero temperature of storage (Sotelo, Gallardo, Pineiro, & Pérez-Martin, 1995) and is therefore useful as a frozen storage index.

In addition to TMA and DMA, the nitrogen base fraction contains varying concentrations of ammonia, amines from the decarboxylation of amino acids (García-Garrido & Luque de Castro, 1997) and other nitrogen compounds that become volatile when made alkaline (Pedrosa-Menabrito & Regenstein, 1990). Ammonia represents a major proportion of the volatile amines being a product from several enzymatic processes of spoilage (Gill, 1990).

Traditionally, the evaluation of fish quality has been based on organoleptic tests. Chemical assessment of spoilage of fishery products dates back to about a century ago when the determination of the volatile nitrogenous bases (TVB-N) was published as standard

* Corresponding author.
E-mail address: boj@dfu.min.dk (B.M. Jørgensen).

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method for the inspection of fish in Germany (König, 1910). Since then, TVB-N has been widely applied to assess the quality of fish and seafood (Malle & Tao, 1987). Likewise, TMA and DMA formed from TMAO have been used for decades as quality indicators (Oetjen & Karl, 1999).

Several methods for the determination of the listed substances have been proposed and reviewed (Brumley & Kelliher, 1997; García-Garrido & Luque de Castro, 1997; Gill, 1990; Malle & Tao, 1987; Siang & Tsukuda, 1989). Sample preparation is generally done by steam distillation of an alkalised sample (Keay & Hardy, 1972) or by leaching the sample with perchloric acid (PCA) or trichloroacetic acid (TCA; García-Garrido & Luque de Castro, 1997). The most common methods for estimation of TVB-N, DMA, TMA and TMAO (after reduction to TMA) are based on (steam) distillation (Malle et al., 1986; Malle & Tao, 1987; Sotelo et al., 1995; Stockemer & Kruse, 1985), Conway micro-diffusion and titration (Conway, 1947) or colorimetric measurements by the "picrate method" for TMA (Dyer, 1945) or DMA (Dyer & Mounsey, 1945). The described methods do not provide sensitive indices, however, because of their high variability (Pedrosa-Menabrito & Regenstein, 1990) and lack of specificity (Gill, 1990). And despite their inadequacy, giving results for one single substance only, these methods have remained popular, highly regarded by hygienists and extensively used (Malle et al., 1986).

More elaborate, instrumental methods have evolved through the years. At present, gas chromatography after head space purging or solvent extraction (Oetjen & Karl, 1999) and high performance liquid chromatography methods are the most widely adopted techniques for the measurement of primary and secondary amines in various matrices. The major advantages of the chromatographic techniques are higher sensitivity and specificity, and the simultaneous determination of several substances. However, these methods have some inherent problems related to the difficulty in handling low-molecular-mass amines due to their high water solubility and volatility, and also to the requirement for expensive materials and apparatus (Dabek-Zlotorzynska & Maruszak, 1998; Gill, 1990). Furthermore, conventional liquid chromatography is not easily applicable to aliphatic amines because of the lack of fluorescence or UV absorption making them unsuitable for fluorometric or photometric detection (Dabek-Zlotorzynska & Maruszak, 1998) and thereby requiring the use of more elaborate detection methods (Altria, Wallberg, & Westerlund, 1998), e.g. derivatisation (Parris, 1984). Yet some other methods in current use are based on enzymatic reactions or on ion selective electrodes or gas sensors (García-Garrido & Luque de Castro, 1997; Ellis, Pivarnik, & Thiam, 2000; Gill, 1990; Sadok, Uglow, & Haswell, 1996).

Recently, applications of capillary electrophoresis (CE) in the separation and determination of non-chromophoric inorganic cations and anions, based on indirect UV-detection, have been developed (Matchett & Brumley, 1997; Riviello & Harrold, 1993). CE has emerged as a powerful separation technique due to its high efficiency: short analysis time, minimal solvent and sample requirement, and large peak separation capacity (Chiou & Shih, 1998; Dabek-Zlotorzynska & Maruszak, 1998). Additionally, CE is an extremely versatile separation method because selectivity can be adjusted extensively by addition of different modifiers to aqueous buffers or by changing buffer pH (Dabek-Zlotorzynska & Maruszak, 1998). The indirect detection method is particularly effective in measuring substances like primary through quaternary amines without derivatisation (Matchett & Brumley, 1997). Beck and Engelhardt (1992) developed applications of imidazole and other chromophoric amines as background ions for indirect detection of aliphatic amines and alkali ions. Similarly, a separation of low-molecular-mass amines using indirect UV-detection with a quinine-based electrolyte was reported (Ma, Zhang, & Cooper, 1992; Riviello & Harrold, 1993).

The present paper describes a fast, sensitive and quantitative, simultaneous determination of ammonia, DMA, TMA and TMAO in fish extracts. The TVB-N value may subsequently be calculated as the sum of the three volatile amines. The method is a modification of that described in the Cation Solutions Kit (PN 5064-8206) from Agilent Technologies and that published by Riviello and Harrold (1993). It is optimised for amine analysis in fish extract, but in addition provides the content of the inorganic cations potassium, sodium, calcium and magnesium. An increased accuracy is obtained by including monomethylamine (MMA) as an internal standard.

2. Experimental

2.1. Reagents

Ammonium chloride, monomethylamine hydrochloride, dimethylamine hydrochloride, trimethylamine hydrochloride and trimethylamine-N-oxide, all of analytical grade, were used for preparing standards. A stock solution (each 5 mM) of ammonia, DMA, TMA and TMAO was made in aqueous hydrochloric acid (pH≈5.2). Dilutions were made with double distilled water yielding concentrations in the range from 0.025 to 2.5 mM. The internal standard, MMA, was added to a final concentration of 0.24 mM in each standard.

The buffer was prepared as a mixture of 4 mM formic acid (pH 3.0), 5 mM copper(II)sulfate and 3 mM crown ether 18-crown-6, all of the highest grade commercially

available. Buffer and standard solutions were stored at 4 °C between analyses.

2.2. Instrument

The instrument used was a Hewlett-Packard HP^{3D} capillary electrophoresis system equipped with a buffer replenishment system, UV/VIS diode array detector, an auto sampler and a HP fused silica capillary (effective length = 56 cm, total length = 64.5 cm, inner diameter = 50 μm) with extended light path (bubble cell). The software package HP Chem Station with manual position of the baseline was used for calculation of peak areas. Peak identification and quantification of the separated components were made with the aid of the internal and external standards.

2.3. Sample extraction

Minced cod muscle samples (25 g) were blended with 75 ml water and adjusted to pH 5.2 by dropwise addition of 0.01 M HCl. The solution was heated to 70 °C (with stirring) and immediately afterwards cooled to room temperature. The precipitate was removed by filtration through a standard paper filter. The filtered extracts were kept frozen at −30 °C until analysis.

2.4. CE procedure

A new capillary was conditioned with the running buffer only. Following the recommendations by the manufacturer, conditioning with strong bases or acids was left out. All liquids were filtered through membrane filters (Minisart® 0.45 μm) on the day of analysis in order to remove micro-particles that might block the flow through the capillary. Before starting a sequence, the capillary was flushed with buffer for 15 min, and between each run it was again flushed with buffer for 3 min. The temperature of the capillary was set to 25 °C. Standards or samples (diluted 1:10 with water containing the internal standard) were injected hydrodynamically (2 s, 50 mbar) with a subsequent buffer plug (3 s, 50 mbar). In-between, the capillary was twice dipped into water in order to avoid sample carry-over into the buffer. A voltage of 30 kV was applied with the positive polarity at the sample injection end. The separated compounds were detected in the indirect mode by measuring the difference in absorbance at 310 nm ± 20 nm and 215 nm ± 10 nm. The stop time was set to 10 min.

The running buffer was renewed at least each five to eight samples. When the replenishment system was in use, the tubes were rinsed at the beginning of each sequence. Once a week, cleaning the cathode to remove copper deposits, e.g. with 1 M HCl, is recommended.

3. Results and discussion

3.1. Optimisation of parameters

Copper(II)sulfate was chosen as the primary electrolyte constituent, because the cupric ion at pH 3 has an electrophoretic mobility similar to that of the analytes and appropriate spectral characteristics (Riviello & Harrold, 1993). Formic acid was used for adjustment of the pH to 3.0 in order to prevent hydrolysis of copper and to provide buffering capacity to the electrolyte. Increasing the voltage or temperature caused faster migration of the analytes. But voltage has an instrumentally fixed upper limit of 30 kV and unfortunately the baseline noise is proportional to the applied voltage (Riviello & Harrold, 1993), which influences the separation negatively. We decided to keep a temperature of 25 °C, considering the volatility and thermal lability of the analytes. This is in agreement with the choice by Gill (1990) who also expected alkyl amines to decompose at high temperatures.

Indirect UV-detection has the benefit of providing equal response factors for the analytes. Through displacement of the UV-absorbing copper ion in the sample zone, presence of non-absorbing substances is signalled as a reduction in the background absorbance. A suitable 'background ion' should have a high molar absorption coefficient at a wavelength where the analytes do not absorb. The mobility should be similar to the ions of interest, in order to avoid zone broadening by electro migration dispersion (Matchett & Brumley, 1997). The copper ion fulfills these requirements. Several signal wavelengths were examined, but compared with the wavelength suggested by the instrument manufacturer (310 nm; Hewlett-Packard, Pub. No. 12-5968-2977E) no increase in signal was obtained. Using a reference wavelength of 205 nm resulted in higher responses of the analytes, but also in an increase in baseline noise. As a consequence, the detection limit could not be improved. The same was true for direct detection at 215 nm. Therefore we chose the indirect mode with the wavelengths 310 nm for the signal and 215 nm for the reference. Several authors (Altria et al., 1998; Beck & Engelhardt, 1992; Chiou & Shih, 1998; Matchett & Brumley, 1997) have also applied indirect UV-detection for CE analyses. A popular choice for the UV-absorbing ion is imidazole, which has similar electrophoretic mobility as a range of metal ions (Altria et al., 1998). However, these methods also had drawbacks. The method of indirect detection is the least selective and sensitive, and therefore the one most subject to interferences, including a noisy, high background and a relatively high detection limit. It also imposes severe limitations on the choice of electrolytes, their concentrations, additives (Matchett & Brumley, 1997), and the extraction system.

The separation of ammonium and potassium using aqueous CE is considered difficult due to their identical electrophoretic mobilities (Riviello & Harrold, 1993). Additives like crown ethers can enhance resolution of such closely migrating analytes (Chiou & Shih, 1998; Matchett & Brumley, 1997). Crown ethers are cyclic polyethers, able to form stable and selective (inclusion) complexes with metals, ammonium cations and protonated amines under acidic conditions (Altria et al., 1998; Chiou & Shih, 1998). Due to different affinity the mobility of ions is changed to varying extents, resulting in different migration times (Altria et al., 1998; Riviello & Harrold, 1993). We did not experience problems with the separation of ammonia and potassium, but initially the TMA and calcium peaks did overlap to some extent. An increase of 18-crown-6 did not improve the separation of TMA and calcium. Perhaps this is due to the fact that the mobility change of secondary and tertiary amines with 18-crown-6 is smaller than that of primary amines (Chiou & Shih, 1998). Moreover, it seems as if the 18-crown-6 cannot complex very well at low pH values, which may be attributed to the partial protonisation of crown ether in acid (Chiou & Shih, 1998).

Dilution of the buffer gave dramatically worse peak shape and consequently also worse peak separation, although migration was performed slightly faster. Also, the migration order changed, which resulted in overlapping of peaks. These effects were mainly due to a modified electro-osmotic flow, caused by changes in pH, current, and effective charge at the wall of the capillary. The other alternative, using a stronger buffer than that of 4 mM, was avoided in order to limit the current and thereby the heat generation (Beck & Engelhardt, 1992). Separation of TMA and calcium was accomplished by increasing the copper(II)sulfate concentration to 5 mM at the expense of a slightly higher baseline noise.

The influence of the hydrodynamical injection parameters, duration and pressure, was also studied. Doubling the injection time while simultaneously reducing the pressure to half the value improved neither the sensitivity nor the repeatability, so we decided to continue with a moderate pressure of 50 mbar and a rather short injection time of 2 s. We also decided not to use the electro-kinetic injection mode as it discriminates between ions with different mobility. Sample carry-over into the running buffer was avoided by dipping the capillary into water after sampling. Additionally, a buffer plug was injected to improve separation by taking advantage of the stacking effect.

In combination with repeatability determinations, we studied the influence of frequency of buffer change and evaporation/concentration of analytes during the day, taking into account migration times and peak areas. Migration times became longer in relation to the buffer being in use for a longer time, but peak areas showed no such behaviour. Instead, peak area values were fluctuating and replenishment did not affect this behaviour. Nevertheless, we preferred to change buffer at least every fifth to eighth run to avoid baseline shifts. It seemed though, that peak areas generally decreased slightly during the day. Some evaporation might have occurred from vials that were kept at room temperature for a longer time period without being closed tightly anymore (due to the punched lids after samples had been analysed). A slow decomposition of TMAO apparently also took place under these conditions. Therefore, it is suggested to make use of the instrument's ability of cooling the sample tray.

The optimised electrolyte system containing 5.0 mM copper(II)sulfate, 4.0 mM formic acid and 3.0 mM 18-crown-6 at pH 3.0 permitted a full separation of the volatile amines ammonia, MMA, DMA and TMA, as well as TMAO and inorganic cations in less than 5 min. With a new, well-conditioned capillary, the retention times for ammonia, MMA, DMA, TMA and TMAO were 2.64, 3.21, 3.56, 3.87, and 4.45 min, respectively. Potassium, sodium, calcium and magnesium passed the detector cell at 3.08, 3.67, 3.97, and 4.25 min, respectively. A representative electropherogram of a fish extract with amine standards added is shown in Fig. 1.

The application of CE for determining non-chromophoric amines has not been extensively investigated so far. Riviello and Harrold (1993) reported the copper(II) electrolyte system (4.0 mM) to give a maximal resolution at an 18-crown-6 concentration of 3-5 mM, and they also claimed the separation to be complete within 5 min. However, as they focussed on the determination of metal ions, no electropherogram was shown with amine compounds included. Aliphatic amines and inorganic cations were also separated with a buffer system of 5 mM imidazole, pH 4.5 (Beck & Engelhardt, 1992). For this system a different order of migration resulted, but the amines tested were well separated from the inorganic cations, and the separation was completed within 4 min. In another publication, separation of aliphatic C1- to C4-substituted primary, secondary, and tertiary amines and alkanolamines in water was studied by free zone capillary electrophoresis with indirect UV-detection (Matchett & Brumley, 1997). Altria et al. (1998) on the other hand, used a non-aqueous medium (methanol with 1% glacial acetic acid and 2 mM 18-crown-6) with indirect detection to determine a range of metal ions or small non-chromophoric amines. The lower viscosity of some organic solvents allows higher mobility and potentially faster separations, but the lower detection sensitivity is a disadvantage of many non-aqueous media as they have higher background UV-absorbance than water. This problem can be circumvented by using indirect UV-detection or a mass spectrometer detector. Since currents are typically lower in non-aqueous media, less heat is produced, which has a positive

impact on the separation efficiency. Chiou and Shih (1998) also describe buffer compositions with 30–50% methanol and N-methylimidazole as the UV-absorbing ion. The only detection which is not based on an indirect mode, was published by Bobbitt, Jackson, and Hendrickson (1998). They used a borate buffer pH 9.5 for chemiluminescence detection with in situ generated $Ru(bpy)_3^{3+}$.

Other methods than CE require more time for each single run, especially when sample pre-processing is necessary. Whereas early GC-procedures had problems with overlapping peaks of the amines, the GC method after extraction with benzene according to Pérez Martin, Franco, Molist, and Gallardo (1987) shows a chromatogram where baseline resolution is achieved. Retention times of 1.03 and 1.35 min for DMA and TMA are very low and the run is completed after ca. 8 min where the solvent peak is eluted. But like the method by Manthey (1988), where DMA and TMA are detected after 1.1 and 1.8 min, the complete procedure includes a cooling step and therefore the total analysis time amounts to 13–20 min (Lundstrom & Racicot, 1983; Manthey, 1988). Similarly, an HPLC gradient-elution for the separation of Fmoc-derivatives of amines and amino acids ends up with a total run-time of 15 min (Teerlink, Hennekes, Mulder, & Brulez, 1997).

3.2. Repeatability and quantitative characteristics

Instrument repeatability was explored in 10 consecutive electropherograms of a mixed standard solution of 0.5 mM. The migration times showed good repeatability: the relative standard deviation (RSD) for migration time was 0.1–0.3% when the buffer was renewed after 5 runs, but 0.02–0.06% when replenishment was performed between all runs. The run-to-run repeatability of peak areas was 3.0–5.0% (RSD) with DMA, TMA and TMAO showing the best results and ammonia the highest variation. When buffer was changed between each run the values further decreased to approximately 2%. Consequently, a frequent replenishment is recommended although it lengthens the time of analysis.

For a CE system coupled to laser-induced fluorescence detection, Dabek-Zlotorzynska and Maruszak (1998) evaluated the quantitative applicability of the method by running seven replicates of the standard (100 μg/l) after derivatisation. Each amine showed high repeatability in terms of the corrected peak area or migration time. The RSD values of the corrected peak areas were between 2.5–5.0%, in agreement with our results. But migration times with RSD less than 0.5% were obtained only when the capillary was rinsed after each run.

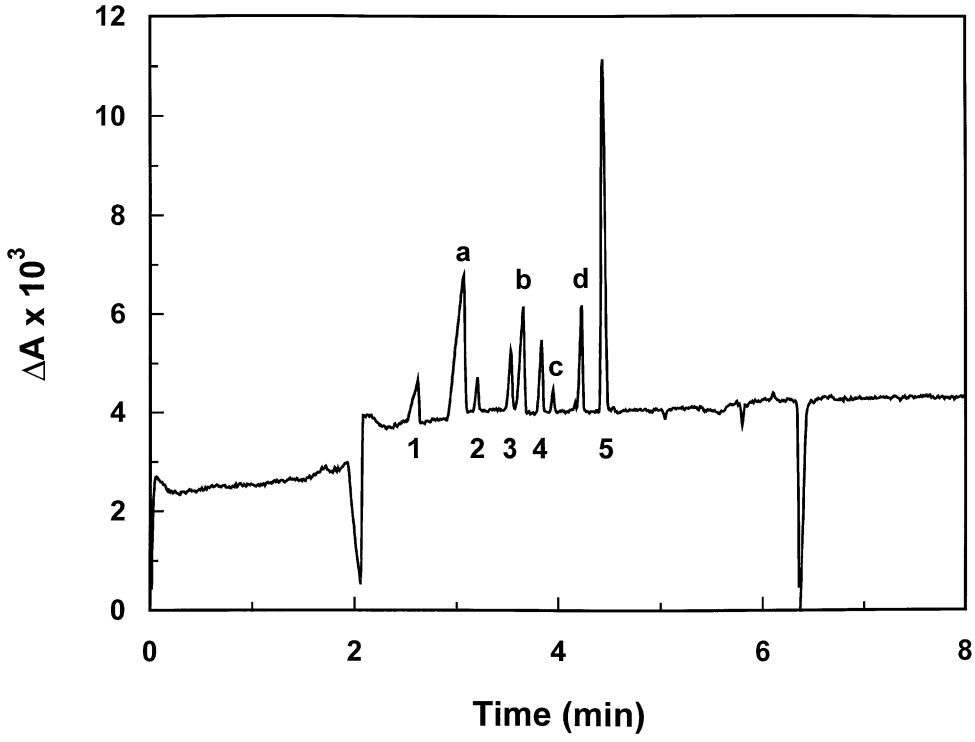

Fig. 1. Electropherogram of a fish extract with amines added. A sample was prepared by mixing 50 μl cod fillet extract with 450 μl amine solution [0.5 mM ammonium chloride, 0.5 mM dimethylammonium chloride (DMA), 0.5 mM trimethylammonium chloride (TMA), 0.5 mM trimethylamine-N-oxide (TMAO), and 0.24 mM methylammonium chloride (MMA)]. Preparation of the fish extract and the electrophoresis conditions were as described in the Experimental section. Assignment of peaks: 1, ammonia; 2, MMA; 3, DMA; 4, TMA; 5, TMAO; a, K^+; b, Na^+; c, Ca^{2+}; d, Mg^{2+}.

With the gas chromatographic method by Veciana-Nogues et al. (1996) relative standard deviations of retention times ranged from 1.1–2.2%. Eight determinations of volatile amines on the same fresh sample were carried out. The results of the within-day precision study reached an RSD of 5.5–6.2%. Pérez Martin et al. (1987) determined the mean of five values with coefficients of variation less than 4% for a GC method with benzene extraction, whereas Manthey (1988) calculated an RSD of 3.5–5.0% for DMA and 2.3% for TMA with comparable parameters. And Oetjen and Karl (1999) found repeatability values between 1.5 and 7.0% ($n=6$).

Also the HPLC methods published reached comparable values. Parris (1984) determined ammonia and volatile amines in meat tissue as fluorescent dansyl derivatives by RP-HPLC with an RSD of 6.3%. Teerlink et al. (1997) reported a derivatisation with Fmoc to yield a within- and between-run precision to be better than 6%. Monser and Greenway (1996) even found that the relative standard deviation for 1 mM TMA was 2.2% and for 5 mM MMA and DMA was 1.5 and 3.1%, respectively ($n=6$). The lowest values though, were reported by Yao et al. (1998) for an ion chromatography method where the RSD was in the range 0.7–1.2%.

To determine the day-to-day repeatability of the application, we tested the capillary over a period of 5 days performing a sequence of 10 runs each day. The buffer was renewed each five runs. The RSD was less than 3% for migration time and less than 10% for peak area, except for ammonia. Dabek-Zlotorzynska and Maruszak (1998) also found the RSD of migration time to be less than 4%. Although these figures in many cases may be acceptable, a considerable improvement was gained by including an internal standard. MMA is well suited for this purpose as it is not present in most fish species. The internal standard facilitated peak identification and corrected for analysis parameter variability, e.g. volume of sample injected. Furthermore, the internal standard repeatability was an indicator for analytical performance.

Linear calibration curves were obtained with standards between 0.025 and 2.5 mM, corresponding to 0.03–7 mg nitrogen per 100 ml (Fig. 2). Higher concentrations, e.g. 5 mM, were left out of consideration as peak broadening occurs in indirect detection mode when the concentration of the analyte approaches that of the background ion (Matchett & Brumley, 1997).

A similar linearity has been reported for the GC and HPLC methods, but the ranges analysed by the different authors vary strongly. Veciana-Nogues et al. (1996) prepared standard curves in the range of 0.2–22 mg nitrogen per 100 ml. Lundstrom and Racicot (1983) analysed free amines in the range 2.0–1000 μg/ml, corresponding to amine contents of 0.1–87 mg nitrogen per 100 g. Oetjen and Karl (1999) found the linearity of calibration curves to be good for three amines in the range between 2.5 and 12 mg. And Abdul-Rashid, Riley, Fitzsimons, and Wolff (1991) showed their method to be

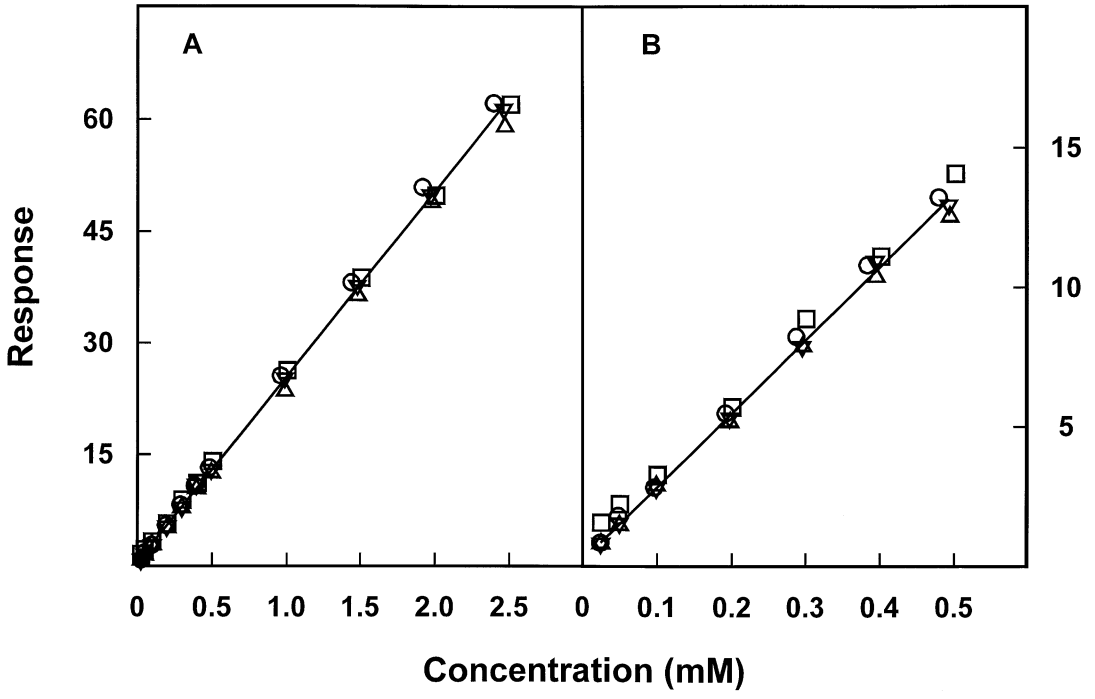

Fig. 2. Standard curves. Series of solutions containing the substances ammonium chloride (□), dimethylammonium chloride (△), trimethylammonium chloride (▽), and trimethylamine-N-oxide (○), in concentrations from 0.025 mM to 2.5 mM in water, were analysed. The electrophoresis procedure was as described in the Experimental session. The response is defined as peak area divided by retention time and its unit is 10^{-3}. Results from the entire concentration range are shown in panel A and those from the lower fifth of the concentration range in panel B.

linear over the concentration range 1 μM–0.7 mM by calibration with a GC/NPD-system. Concerning the HPLC methods, Monser and Greenway (1996) obtained calibration graphs for TMA in the range 0.1–10 and 5–50 mM for MMA and DMA. The range for TMA was at the levels expected in fish samples, but DMA was measured at unrealistically high concentrations. Teerlink et al. (1997) showed standard curves with a linear relationship between peak area and concentration in the range 1 μM–0.48 mM for Fmoc-derivatives of the amines. And Sahasrabuddhey, Jain, and Verma (1999) obtained a linear calibration graph for 0.1–10 mg/l analytes in environmental aqueous samples after derivatisation.

The detection limit, defined as recommended by IUPAC (Currie, 1999) was approximately 0.01 mM for all relevant amines. The quantification limit was below 0.04 mM for a 10-times diluted fish extract, corresponding to 2 mg nitrogen per 100 g fish. This is sufficient for the method to be of practical value, since it is far below the level where fish are rejected by sensory panels (Dalgaard, Gram, & Huss, 1993).

Aliphatic amines could also be separated in a buffer with 5 mM imidazole (pH 4.5) as the light absorbing compound, with the detection limit stated to be around 0.4 ppm (Beck & Engelhardt, 1992). When CE is coupled to laser-induced fluorescence (LIF) detection of amount of substance at the attomole level has been achieved (Dabek-Zlotorzynska & Maruszak, 1998). Currently, LIF seems to be one of the most sensitive detection methods available for CE with a detection limit about 1 nM and thus better than the detection limits obtained by GC or HPLC (Dabek-Zlotorzynska & Maruszak, 1998). Brumley and Kelliher (1997) also determined aliphatic amines after derivatisation, using fluorescein isothiocyanate with CE/LIF detection and calculated "determinative levels" down to 10 ppb as well as "practical detection limits" of 30–100 ppb.

For the gas chromatographic method by Oetjen and Karl (1999), detection limits were calculated as three times the standard deviation divided by the sensitivity (slope of the calibration) and were below or equal to 0.3 mg (MMA), 0.05 mg (DMA) and 0.01 mg (TMA) nitrogen per 100 g. Determination limits were calculated as nine times the standard deviation divided by the sensitivity (slope) and were below or equal to 0.9, 0.2 and 0.03 mg nitrogen per 100 g, respectively. Veciana-Nogues et al. (1996) calculated their determination limits to be lower than 0.04 (TMA), 0.11 (DMA) and 0.17 mg nitrogen per 100 g (MMA), respectively. Abdul-Rashid et al. (1991) pre-concentrated MMA, DMA and TMA from water by micro-diffusion, then measured by GC/NPD. Detection limits (four times the standard deviation of the blank) were in the picogram range for all three analytes (61, 450, 590 pg per 5 μl injection).

The lowest measurable amount of TMA that could be detected using the HPLC method by Monser and Greenway (1996) was 0.1 mM (equivalent to 1.2 mg nitrogen per 100 g fish), based on two times the baseline noise. With HPLC after Fmoc-derivatisation, Teerlink et al. (1997) found the detection limit at a signal/noise ratio of three to be 0.02 μmol/l with the lower limit of determination at 0.1 μmol/l. In environmental aqueous samples, the HPLC determination after derivatisation gave 0.2 μg/l as limit of detection for ammonia and 0.3–0.6 μg/l for MMA and DMA (Sahasrabuddhey et al., 1999). For the ion chromatography method by Yao et al. (1998), detection limits (signal to noise ratio = 3) were 0.02, 0.05, 0.13, and 0.6 μg/ml for ammonia, MMA, DMA and TMA, respectively.

3.3. Application of the method to fish samples

Cod extracts, prepared according to the standard procedure of our laboratory, were used for studying the applicability of the CE method. Dilution of the extracts was necessary due to the high concentration of sodium and potassium ions and a slight tendency to foam. The cost is, of course, a proportional increase in the quantification limit. This might be avoided by including an adequate sample preprocessing step, but at the expense of the simplicity and high throughput rate of the method.

On a trial basis, standards were diluted with water, buffer, hydrochloric acid (pH 5.2 and 0.025 M), trichloroacetic acid (TCA; 6% and 0.6%), and perchloric acid (PCA; 6 M and 0.6 M) and subjected to the CE analysis. Water and buffer did not show any differences, but with increasing acidity, the peak areas decreased dramatically. With 6% TCA and 6 M PCA detection of all peaks was completely suppressed. According to these results, a suitable extraction method cannot involve deproteinisation with the common reagents, TCA or PCA. The present procedure instead makes use of hydrochloric acid for adjustment of pH to the pK_a of the major fish muscle proteins. After a brief heating, the precipitated protein is removed by filtration.

Analytical accuracy was evaluated through recovery studies using a cod extract, which contained no MMA, DMA or TMA, but 12.5 mg ammonia nitrogen per 100 g and 102 mg TMAO nitrogen per 100 g. (We previously confirmed that the method was not notably influenced by the fish matrix.) The extract was diluted 10 times with three different standard mixtures containing ammonia, DMA, TMA and TMAO each in concentrations of 0.1, 0.5 and 1.0 mM and 0.24 mM MMA as internal standard (correcting for possible deviations in amount of injected sample). Recoveries were best for the 0.5 mM standard, varying between 99 and 108%. The 1.0 mM standard showed a slightly higher variation (97–111%). The volatile amines in the 0.1 mM standard were recovered 95–124%, the wider range probably caused by the concentration approaching the detection

limit. And TMAO showed an apparent recovery of 237% due to the very small amount of standard added to the high amount that originally was present in the fish extract.

The best mean recoveries ranging from 97.2 to 99.1% were obtained with the gas chromatographic method by Veciana-Nogues et al. (1996) who tested two addition levels by the standard addition method. Oetjen and Karl (1999) used trout flesh, which was homogenised and spiked with different levels of MMA, DMA and TMA. No MMA, DMA or TMA could be detected in the trout flesh itself. In agreement with our results, recovery rates for the three amines varied between 91 and 106% within the relevant concentration range of 2.5–14.5 mg nitrogen per 100 g. In recovery experiments from frozen minced red hake containing four various levels of DMA or TMA, Lundstrom and Racicot (1983) found that benzene as a solvent yielded better recoveries (100–110% for DMA and 94–98% or TMA) than n-amyl alcohol (60–65% for DMA and 95–109% for TMA). Similarly, Manthey (1988) determined recovery rates of 94–103% for DMA and TMA. In contrast to Lundstrom and Racicot (1983), Pérez Martin et al. (1987) found recoveries of only 80.4% for DMA and 85.4% for TMA after benzene extraction when three levels of mixture were added to blended hake flesh. The lowest values were published by Abdul-Rashid et al. (1991), who found that although the repeatability of their GC/NPD method was good, the recovery was low for MMA (57.6%) and DMA (45.5%), whereas it was 73.8% for TMA. This was considered acceptable though taking the high volatility of the amines into consideration.

Using an HPLC method, Monser and Greenway (1996) obtained good recoveries of 99.7–102.0% for fish extracts. By spiking a number of serum samples ($n = 3$–6) at three different levels, Teerlink et al. (1997) calculated the recovery with the HPLC method after Fmoc-derivatisation to be 99–107%. Also the ion chromatography method by Yao et al. (1998) yielded recoveries varying from 92–108%.

The applicability of the present CE-based method for quantitative determination of ammonia, DMA, TMA, and TMAO in fish was also tested on samples from a storage experiment. Cod fillets that had been vacuum-packed and blast frozen (−35 °C for 4 h) were assessed regularly throughout 12 months of storage at −20 and −30 °C. The results were compared with those provided by the modified micro-diffusion method previously used in our laboratory. No significant differences (t-test, $\alpha = 0.05$) were found in most cases (taking into account that the micro-diffusion method determines DMA as TMA). The content of total volatile bases (TVB-N; micro-diffusion method) was a few percent higher than the sum of ammonia, DMA and TMA as measured by the present CE-based method. This might be due to the formation of other volatile bases in small amounts during spoilage of the fish (Pedrosa-Menabrito & Regenstein, 1990).

4. Conclusion

All of the methods previously published suffer from certain drawbacks, reviewed by Dabek-Zlotorzynska and Maruszak (1998), Lundstrom and Racicot (1983), and Shewan, Gibson, and Murray (1971). Besides the fact that traditional methods measure only one substance (e.g. DMA, TMA or TMAO) or an undefined sum value (TVB-N), different problems occur for the single-substance determinations. Application of the colorimetric measurement of TMA is limited, due to interferences by secondary amines, such as DMA, because of the incomplete reaction with formaldehyde (García-Garrido & Luque de Castro, 1997; Ruiter & Weseman, 1976; Sadok et al., 1996). Inter-laboratory tests of TVB-N determination showed systematic errors between participating laboratories both with a codex method and with the 'home' methods (Vyncke, Luten, Brünner, & Moermans, 1987), which might result from incomplete recovery of TVB-N and/or breakdown of protein during analysis (Botta, Lauder, & Jewer, 1984). Steam distillation is not convenient for the routine analysis of large numbers of samples, micro-diffusion requires a lengthy incubation period (Gill, 1990) and both also suffer from interference by DMA (Brumley & Kelliher, 1997). Electrodes have the great advantage of on-site portability and application without need for extensive training requirements (Pivarnik, Thiam, & Ellis, 1998), but for ammonia, enzyme assays are preferable as the ammonia-selective electrode also responds to volatile amines (Brumley & Kelliher, 1997; Parris, 1984). The enzymatic determination is specific, providing results for that single substance only.

An advantage of chromatographic and electrophoretic techniques is that the amines are physically separated and can be individually and simultaneously quantified. Problems of interference are diminished, making the methods specific and providing more information from the samples. Nevertheless, these techniques also have disadvantages besides their requirement for expensive specialised equipment and experienced technical staff. Analysis of volatile amines by GC has mainly been hampered by loss of sample response. To avoid the tedious steam-distillation step, head-space procedures or organic extraction have been used. But head-space methods are not suitable for non-volatile substances such as TMAO, and the repeatability is not satisfactory without an automatic vaporising device being available (Fiddler, Doerr, & Gates, 1991; Veciana-Nogues et al., 1996). Also, TMAO present in the samples is able to form TMA by degradation or thermal decomposition (Oetjen & Karl, 1999). Another serious limitation in GC

analysis is adsorption between the aliphatic amines and the chromatographic support or adsorbent, resulting in severe peak tailing and memory effects (ghosting phenomena) causing a low repeatability (Fiddler et al., 1991). These problems may be circumvented by derivatisation prior to GC (Teerlink et al., 1997). Pre-column derivatisation is also essential for analysis of aliphatic amines by HPLC. But it is very difficult to find a single reagent that can be applied for all amines including ammonia and TMAO, especially because tertiary amines in general are extremely difficult to derivatise (Cobo, Silva, & Pérez-Bendito, 1997; Kruse & Stockemer, 1989). Additionally, the reaction has to be selective, quantitative, fast and not cause interferences. For CE-based methods, most of these problems are avoided. The largest difficulty is to obtain a sufficiently high and repeatable detector response due to the very short light path (the diameter of the capillary). A stable CE-system, optimised buffers, frequent buffer replenishment and extended light path devices are useful remedies in this context.

We have developed a method for simultaneous determination of ammonia, MMA, DMA, TMA and TMAO to replace the labourious and time-consuming Conway micro-diffusion method. Based on CE-separation and indirect detection, easy-prepared extracts were used without further sample conditioning (e.g. derivatisation). The TVB-N value could be calculated to a sufficient precision from the results of the single amines. Additionally, sodium, potassium, calcium and magnesium ions could be determined. The analytical precision and repeatability were comparable to those of other published methods. We used MMA as an internal standard, but for certain purposes (e.g. screening) it would not be necessary. In the case of samples of crustaceans it is recommended to leave out or replace this internal standard, because MMA may be present in those samples (Kruse & Stockemer, 1989). It has even been suggested that MMA may be used to assess spoilage of crustaceans (Veciana-Nogues et al., 1996).

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References

Abdul-Rashid, M. K., Riley, J. P., Fitzsimons, M. F., & Wolff, G. A. (1991). Determination of volatile amines in sediment and water samples. *Analytica Chimica Acta*, *252*, 223–226.

Altria, K. D., Wallberg, M., & Westerlund, D. (1998). Separation of a range of cations by nonaqueous capillary electrophoresis using indirect and direct detection. *Journal of Chromatography B*, *714*, 99–104.

Beck, W., & Engelhardt, H. (1992). Capillary electrophoresis of organic and inorganic cations with indirect UV detection. *Chromatographia*, *33*, 313–316.

Bobbitt, D. R., Jackson, W. A., & Hendrickson, H. P. (1998). Chemiluminescent detection of amines and amino acids using in situ generated $Ru(bpy)_3^{3+}$ following separation by capillary electrophoresis. *Talanta*, *46*, 565–572.

Botta, J. R., Lauder, J. T., & Jewer, M. A. (1984). Effect of methodology on total volatile basic nitrogen (TVB-N) determination as an index of quality of fresh Atlantic cod (*Gadus morhua*). *Journal of Food Science*, *49*, 734–750.

Brumley, W. C., & Kelliher, V. (1997). Determination of aliphatic amines in water using derivatization with fluorescein isothiocyanate and capillary electrophoresis/laser-induced fluorescence detection. *Journal of Liquid Chromatography & Related Technologies*, *20*, 2193–2205.

Chiou, C.-S., & Shih, J.-S. (1998). Application of crown ethers as modifiers for the separation of amines by capillary electrophoresis. *Analytica Chimica Acta*, *360*, 69–76.

Cobo, M., Silva, M., & Pérez-Bendito, D. (1997). Chemiluminescent measurement of tertiary amines using the continuous-addition-of-reagent technique. Determination of trimethylamine in fish samples. *Analytica Chimica Acta*, *356*, 51–59.

Conway, E. J. (1947). *Microdiffusion analysis and volumetric error*. London: Crosby Lockwood & Sons.

Currie, L. A. (1999). Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC recommendations 1995). *Analytica Chimica Acta*, *391*, 105–126.

Dabek-Zlotorzynska, E., & Maruszak, W. (1998). Determination of dimethylamine and other low-molecular-mass amines using capillary electrophoresis with laser-induced fluorescence detection. *Journal of Chromatography B*, *714*, 77–85.

Dalgaard, P., Gram, L., & Huss, H. H. (1993). Spoilage and shelf life of cod fillets packed in vacuum or modified atmospheres. *International Journal of Food Microbiology*, *19*, 283–294.

Dyer, W. J. (1945). Amines in fish muscle. I. Colorimetric determination of trimethylamine as the picrate salt. *Journal of the Fish Research Board of Canada*, *6*, 351–358.

Dyer, W. J., & Mounsey, Y. A. (1945). Amines in fish muscle. II. Development of trimethylamine and other amines. *Journal of the Fish Research Board of Canada*, *6*, 359–367.

Ellis, P. C., Pivarnik, L. F., & Thiam, M. (2000). Determination of volatile bases in seafood using the ammonia ion selective electrode: collaborative study. *Journal of AOAC International*, *83*, 933–943.

Fiddler, W., Doerr, R. C., & Gates, R. A. (1991). Gas chromatographic method for determination of dimethylamine, trimethylamine, and trimethylamine oxide in fish-meat frankfurters. *Journal of the Association of Official Analytical Chemists*, *74*, 400–403.

García-Garrido, J. A., & Luque de Castro, M. D. (1997). Determination of trimethylamine in fish by pervaporation and photometric detection. *The Analyst*, *122*, 663–666.

Gill, T. A. (1990). Objective analysis of seafood quality. *Food Reviews International*, *6*, 681–714.

Howgate, P. F. (1982). Quality assessment and quality control. In A. Aitken, I. M. Mackie, J. H. Merritt, & M. L. Windsor (Eds.), *Fish handling & processing, 2nd ed* (pp. 177–186). Edinburgh: Her Majesty's Stationary Office.

Keay, J. N., & Hardy, R. (1972). The separation of aliphatic amines in dilute aqueous solution by gas chromatography and application of this technique to the quantitative analysis of tri- and dimethylamine in fish. *Journal of the Science of Food and Agriculture*, *23*, 9–19.

Kruse, R., & Stockemer, J. (1989). Einsatzmöglichkeiten der Dampfraum-Gaschromatographie bei der Untersuchung von Fischen and Garnelen: Bestimmung von Mono-, Di- und Trimethylamin. *Archiv für Lebensmittelhygiene*, *40*, 87–89.

König, J. (1910). *Untersuchung von Nahrungs—und Genußmitteln und Gebrauchsgegenständen*. Berlin: Springer.

LeBlanc, E. L., & LeBlanc, R. J. (1988). Effect of frozen storage temperature on free and bound formaldehyde content of cod (*Gadus morhua*) fillets. *Journal of Food Processing and Preservation*, *12*, 95–113.

Lundstrom, R. C., & Racicot, L. D. (1983). Gas chromatographic determination of dimethylamine and trimethylamine in seafoods. *Journal of the Association of Official Analytical Chemists*, *66*, 1158–1163.

Ma, Y., Zhang, R., & Cooper, C. L. (1992). Indirect photometric detection of polyamines in biological samples separated by high-performance capillary electrophoresis. *Journal of Chromatography*, *608*, 93–96.

Malle, P., & Tao, S. H. (1987). Rapid quantitative determination of trimethylamine using steam distillation. *Journal of Food Protection*, *50*, 756–760.

Malle, P., Eb, P., & Tailliez, R. (1986). Determination of the quality of fish by measuring trimethylamine oxide reduction. *International Journal of Food Microbiology*, *3*, 225–235.

Manthey, M. (1988). Fisch als Lebensmittel—Gaschromatographische Bestimmung von Dimethyl- und Trimethylamin in Fisch und Fischerzeugnissen. *Informationen für die Fischwirtschaft*, *35*, 131–135.

Matchett, W. H., & Brumley, W. C. (1997). Preconcentration of aliphatic amines from water determined by capillary electrophoresis with indirect UV detection. *Journal of Liquid Chromatography & Related Technologies*, *20*, 79–100.

Monser, L. I., & Greenway, G. M. (1996). Liquid chromatographic determination of methylamines. Determination of trimethylamine in fish samples using a porous graphitic carbon stationary phase. *Analytica Chimica Acta*, *322*, 63–68.

Oetjen, K., & Karl, H. (1999). Improvement of gas chromatographic determination methods of volatile amines in fish and fishery products. *Deutsche Lebensmittel-Rundschau*, *95*, 403–407.

Parris, N. (1984). An improved fluorometric method for the determination of ammonia and volatile amines in meat tissue by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, *32*, 829–831.

Pedrosa-Menabrito, A., & Regenstein, J. M. (1990). Shelf-life extension of fresh fish—a review. Part III—fish quality and methods of assessment. *Journal of Food Quality*, *13*, 209–223.

Pérez Martin, R. I., Franco, J. M., Molist, P., & Gallardo, J. M. (1987). Gas chromatographic methods for the determination of volatile amines in seafoods. *International Journal of Food Science and Technolology*, *22*, 509–514.

Pivarnik, L. F., Thiam, M., & Ellis, P. C. (1998). Rapid determination of volatile bases in fish by using an ammonia ion-selective electrode. *Journal of AOAC International*, *81*, 1011–1022.

Riviello, J. M., & Harrold, M. P. (1993). Capillary electrophoresis of inorganic cations and low-molecular-mass amines using a copper-based electrolyte with indirect UV detection. *Journal of Chromatography A*, *652*, 385–392.

Ruiter, A., & Weseman, J. M. (1976). The automated determination of volatile bases (trimethylamine, dimethylamine and ammonia) in fish and shrimp. *Journal of Food Technology*, *11*, 59–68.

Sadok, S., Uglow, R. F., & Haswell, S. J. (1996). Determination of trimethylamine in fish by flow injection analysis. *Analytica Chimica Acta*, *321*, 69–74.

Sahasrabuddhey, B., Jain, A., & Verma, K. K. (1999). Determination of ammonia and aliphatic amines in environmental aqueous samples utilizing pre-column derivatization to their phenylthioureas and high performance liquid chromatography. *Analyst*, *124*, 1017–1021.

Shewan, J. M., Gibson, D. M., & Murray, C. K. (1971). The estimation of trimethylamine in fish muscle. In R. Kreuzer (Ed.), *Fish inspection and quality control* (pp. 183–186). London: Fishing News (Books) Ltd.

Siang, N. C., & Tsukuda, N. (1989). Laboratory tests and equipment for quality assessment of chilled and frozen fish. *INFOFISH International*, *6*, 24–27.

Simeonidou, S., Govaris, A., & Vareltzis, K. (1997). Effect of frozen storage on the quality of whole fish and fillets of horse mackerel (*Trachurus trachurus*) and Mediterranean hake (*Merluccius mediterraneus*). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, *204*, 405–410.

Sotelo, C. G., Gallardo, J. M., Pineiro, C., & Pérez-Martin, R. (1995). Trimethylamine oxide and derived compounds' changes during frozen storage of hake (*Merluccius merluccius*). *Food Chemistry*, *53*, 61–65.

Stockemer, J., & Kruse, R. (1985). Neue Methoden zur Bestimmung von TVB-N bzw. TMA-N in Fischen und Fischerzeugnissen. *Archiv für Lebensmittelhygiene*, *36*, 116–117.

Teerlink, T., Hennekes, M. W. T., Mulder, C., & Brulez, H. F. H. (1997). Determination of dimethylamine in biological samples by high-performance liquid chromatography. *Journal of Chromatography B*, *691*, 267–276.

Veciana-Nogues, M. T., Albala-Hurtado, M. S., Izquierdo-Pulido, M., & Vidal-Carou, M. C. (1996). Validation of a gas-chromatographic method for volatile amine determination in fish samples. *Food Chemistry*, *57*, 569–573.

Vyncke, W., Luten, J., Brünner, K., & Moermans, R. (1987). Determination of total volatile bases in fish: a collaborative study by the West European Fish Technologist's Association (WEFTA). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, *184*, 110–114.

Yao, S., Yang, X., Zhang, H., Xie, Y., & Wei, W. (1998). Determination of ammonia and aliphatic amines in food by ion chromatography with double-cell bulk acoustic wave detection. *Journal of AOAC International*, *81*, 1099–1103.