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PII: S0308-8146(96)00031-3

*Food Chemistry*, Vol. 57, No. 4, pp. 569–573, 1996

0308-8146/96 $15.00+0.00

*Analytical, Nutritional and Clinical Methods Section*

# Validation of a gas-chromatographic method for volatile amine determination in fish samples

**M. T. Veciana-Nogues,* M. S. Albala-Hurtado, M. Izquierdo-Pulido & M. C. Vidal-Carou**

*Unidad de Nutrición y Bromatologia, Facultad de Farmacia, Universidad de Barcelona, Av. Joan XXIII, s/n. 08028, Barcelona, Spain*

(Received 19 October 1995; accepted 26 January 1996)

A gas-chromatographic (GC) procedure is described for the determination of volatile amines in fish. The method includes a first step of volatile amine extraction with 0.6 N perchloric acid, followed by alkalinization with 65% (w/w) KOH and a subsequent toluene extraction. Dimethylamine (DMA) and trimethylamine (TMA) were well resolved; moreover, monomethylamine (MMA) did not interfere in the determination of DMA and TMA, and can also be useful in assessing spoilage of crustaceans. The suitability of toluene as a solvent for extraction is demonstrated. Linearity, precision and recovery were satisfactory. Sensitivity limits were lower than 0.038, 0.110 and 0.167 mg N per 100 g for TMA, DMA and MMA, respectively. In addition, contents of DMA and TMA in fresh and frozen hake samples were studied. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

Most marine species of fish and shellfish, in their metabolic processes, produce trimethylamine oxide (TMAO), which has an important role in osmoregulation. The TMAO content in fish varies with species and usually decreases postmortem. TMAO is reduced by bacterial enzymes to trimethylamine (TMA) (Castell *et al.*, 1971; Lundstrom & Racicot, 1983; Krzymien & Elias, 1990), whereas endogenous enzymes reduce TMAO to dimethylamine (DMA) and formaldehyde. Only when bacterial growth is halted (e.g. by freezing) does the DMA content increase (Huss, 1988). Therefore, TMA is related to bacterial fish spoilage in fresh or iced fish and DMA can be used as a quality index of frozen fish Krzymien & Elias, 1990; Gallardo *et al.*, 1991; Civera *et al.*, 1995).

The classical procedure to determine DMA in fish is the colorimetric method proposed by Dyer & Mounsey (1945) using copper dimethyldithiocarbamate. Another colorimetric method (Dyer, 1945) (or modifications) is probably the best known and the most widely used procedure. The current official (AOAC) method for TMA determination is also based on Dyer's method. Recently, flow injection procedures to optimize routine TMA analysis have been described (Leon *et al.*, 1994). The interference problems associated with colorimetric methods for TMA limit their application to those fish species that do not produce significant quantities of DMA (Lundstrom & Racicot, 1983). The use of gas chromatography (GC) avoids problems of interference, and more information can be obtained from samples per unit of time because TMA and DMA are determined simultaneously.

The first GC method was described by Hughes in 1959 (Lundstrom & Racicot, 1983), although several modifications have been proposed. Most procedures involve extraction of volatile amines from fish with trichloroacetic acid (TCA) or perchloric acid (PCA), followed by neutralization of extracts and, in the early procedures, steam distillation of amines into hydrochloride or sulphuric acid. Finally, the salts of amines are freeze-dried, redissolved and injected into the GC system. To avoid the tedious steam-distillation step, head-space procedures (Miller *et al.*, 1972; Manthey, 1988; Fiddler *et al.*, 1991; Kruse & Stockemer, 1989) or organic extraction of the neutralized fish extract have been used (Tokunaga *et al.*, 1977; Lundstrom & Racicot, 1983; Perez-Martín *et al.*, 1987; Krzymien & Elias, 1990).

Head-space methods were not used in our procedure because of their lack of repeatability when automatic vaporizing devices are not available. Thus, our objective was to test the feasibility of an experimental procedure based on the extraction of volatile amines with perchloric acid, followed by alkalinization and organic solvent extraction, and subsequent injection into a GC system. Monomethylamine (MMA), which can be used to assess spoilage of crustaceans (Kruse & Stockemer, 1989), was also included in our work, because it can interfere in DMA and TMA determinations.

*To whom correspondence should be addressed.

The proposed procedure was studied in terms of linearity, precision, recovery and sensitivity. In addition, the volatile amine contents in fresh and frozen hake samples from Spanish markets were measured and the amine contents of frozen hake samples from different sources compared.

## MATERIAL AND METHODS

### Samples

Fresh hake (*Merluccius merluccius* or *Merluccius capensis*) samples ($n = 27$) from Barcelona markets, frozen hake samples ($n = 26$) from Barcelona markets, and control hake samples ($n = 37$), which were frozen on a fishing boat, were delivered directly to our laboratory and kept at −20 °C until analysis. Samples of control hake were kept frozen (−20 °C) for 2 years. This period is close to the maximum storage time of frozen fish in commercial distribution networks.

### Chromatographic conditions

A Hewlett-Packard 5890 Series II gas chromatograph equipped with packed glass column (2 m × $\frac{1}{4}$ inch × 2 mm) and flame ionization detector was used. Peak areas and retention times were recorded on a Hewlett-Packard 3396A Series II integrator, and chromatograms were stored using a Hewlett-Packard 99114B disk unit. The chromatographic conditions and the temperature gradient program are shown in Table 1. The glass column was packed with 200 cm of Carbowax 20M + 0.8% KOH on Carbopack B (Teknokroma, Spain). The column was preconditioned overnight at 220 °C. The injection port was fitted with disposable glass liners to prevent amine decomposition on hot metal surfaces and to facilitate removal of alkaline residues.

### Sample preparation

Muscle tissue from the fish samples (10 g) was minced and blended with 12 ml of 0.6 N PCA. After centrifugation (10 min at 3000 rpm), liquid was removed by filtration through a Whatman No. 1 filter. The solid residue was homogenized with 10 ml of 0.6 N PCA and discarded after centrifugation (10 min at 3000 rpm) and filtration. The filtrates were combined and the volume was made up to 25 ml with 0.6 N PCA. If analyte contents were greater than 50 mg N per 100 g, a decrease in the ratio of sample weight/volume of PCA used was necessary by increasing the volume of PCA. Then, 0.1 ml of an internal standard (10 mg N per 100 ml of n-propylamine (PA) in PCA) was added to 1 ml of standard solutions or to fish extracts in test-tubes.

Toluene (1 ml) and KOH (1 ml, 65%, w/w) were added to all tubes. The tubes were stoppered and incubated in a water-bath (10 min, 60 °C). After incubation, they were shaken on a mechanical tube rotator (2 min). Aliquots of the toluene layer were transferred into closed vials, and 1 µl of each was injected into the GC system.

**Table 1. Gas chromatography working conditions**

| | |
|---|---|
| Carrier gas flow (column) | Helium, 23 ml $min^{-1}$ |
| Injector temperature | 190 °C |
| Detector (FID) temperature | 190 °C |
| Oven temperature | |
| Temperature 1 (time) | 75 °C (5 min) |
| Rate (75–150 °C) | 2.5 min |
| Temperature 2 (time) | 150 °C (10 min) |
| FID hydrogen flow | 30 ml $min^{-1}$ |
| FID air flow | 430 ml $min^{-1}$ |

FID, flame ionization detection.

### Calculations

Standard curves in 0.6 N PCA were prepared in the following range: 0.20–22.00 mg N per 100 ml for TMA-N and DMA-N, and 0.24–28.0 mg N per 100 ml for MMA-N. Correlation coefficients ($r$) and linear regression of ratio peak analyte area/peak internal standard area against amine concentration were calculated. Contents of DMA, TMA and MMA in PCA extracts were obtained directly by interpolation from calibration curves. The concentration of each amine in fish muscle tissue ($C_f$) was calculated using the formula:

$$C_f = C \times 25/W$$

where $W$ is the weight of homogenate and $C$ is the concentration from the calibration curve of each analyte.

## RESULTS AND DISCUSSION

Benzene is traditionally used for volatile amine extraction after KOH neutralization of fish extract. The use of toluene was proposed by Krzymien & Elias (1990) but its use is not widespread. A preliminary assay to study suitability of both organic solvents was carried out using standard solutions of MMA, DMA and TMA. The standard solutions were extracted in triplicate with benzene or toluene. Similar results (Table 2) were obtained for both solvents; however, toluene was chosen because it is cheaper and less toxic than benzene.

Figure 1 shows the good resolution obtained with a fresh fish sample (A) and with 2 mg N per 100 ml standard solutions of MMA, DMA and TMA (B). Analytes were well identified on the basis of retention time by comparison with standard solutions. Relative standard deviations (RSDs) of retention times ranged from 1.05% to 2.15%. Perez-Martín *et al.* (1987) proposed the use of Chromosorb 103 precolumns to hold-back organic solvent used in the extraction. This precolumn was not necessary with the proposed temperature

**Table 2. Relative response (analyte area/analyte internal standard area) of monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA) standards after extraction with toluene (T) or benzene (B)**

| MMA-N | | | DMA-N | | | TMA-N | | |
|---|---|---|---|---|---|---|---|---|
| μg g$^{-1}$ | B | T | μg g$^{-1}$ | B | T | μg g$^{-1}$ | B | T |
| 28 | 0.03 | 0.02 | 22 | 0.20 | 0.20 | 22 | 0.30 | 0.30 |
| 56 | 0.05 | 0.04 | 44 | 0.33 | 0.35 | 44 | 0.70 | 0.70 |
| 140 | 0.06 | 0.06 | 110 | 0.48 | 0.55 | 110 | 1.20 | 1.30 |
| 280 | 0.15 | 0.15 | 220 | 1.22 | 1.25 | 220 | 2.70 | 2.80 |

Results are mean of three determinations.

program (Table 1). The initial oven temperature of 75 °C used is lower than the temperature proposed by other authors who follow isothermal programs (115–120 °C).

The detector response was linear for the range of amines assayed. Least squares analysis gave a correlation coefficient of 0.9967, 0.9985 and 0.9991 for MMA, DMA and TMA, respectively. The coefficient of determination ($r^2$) was better than 99.35% for all standard curves. The linearity of the GC method was verified by analysis of variance of regression, and the results obtained were always satisfactory ($p < 0.001$).

Results of a within-day precision study are shown in Table 3. Eight determinations of volatile amines on the same fresh fish sample were carried out on the same day, using the same reagents and apparatus. Because only TMA-N and DMA-N were detected, a sample was spiked using 1 ml of a standard solution of MMA (1 mg N per 100 ml) RSDs obtained were always acceptable according to Horwitz's formula for intralaboratory studies (Horwitz, 1982*a*,*b*).

Recovery was tested by the standard addition method with two addition levels. The addition levels used and results obtained are shown in Table 4. By statistical analysis (Cochran's test), we verified that accuracy did not depend on the volatile amine content in the sample. Then, by considering the mean recovery for each amine, we also verified by Student's *t*-test that there were no significant statistical differences between mean recovery found and the theoretical value of 100%. Mean recoveries ranged from 97.18% to 99.05%.

The determination limit (DtL) was calculated according to the Long & Winefordner (1983) criterion. A blank was prepared by adding 0.1 ml of internal standard (10 mg N per 100 ml) to 0.6 N PCA. The solution was alkalinized and extracted with toluene as described in the methods. The determination limit was lower than 0.038, 0.110 and 0.167 mg N per 100 g for TMA-N, DMA-N and MMA-N, respectively.

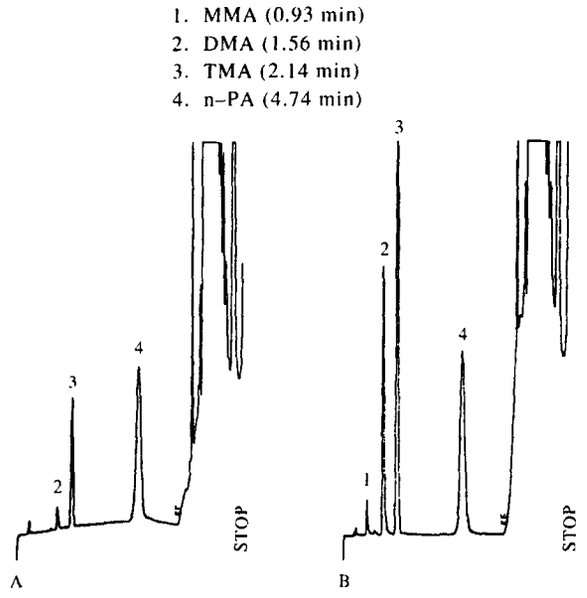


**Fig. 1.** Gas chromatogram of volatile amines obtained in fresh fish (A) and in a 2 mg N per 100 ml standard solution of MMA, DMA and TMA (B).

### Volatile amine contents of fish samples

Table 5 shows the results of the volatile amine determination on fresh and frozen hake samples. MMA-N was not detected in any sample. Contents of TMA-N in control frozen samples were always very low (0.05 ± 0.07 mg TMA-N per 100 g). These results confirmed that the earlier freezing, avoiding microbial growth, prevents TMA-N formation, and showed that TMA-N does not increase after long storage periods (2 years) at −20 °C. DMA-N contents (3.10 ± 1.63 mg DMA-N per 100 g) were higher than the TMA-N contents because of the endogenous enzymatic production of this amine during frozen storage.

DMA-N content in samples from the market ranged from not detected to 2.05 mg DMA-N per 100 g in fresh hake and from 0.33 to 11.70 mg DMA-N per 100 g in frozen hake. The average DMA-N levels in

**Table 3. Precision of method for volatile amine determination in fish**

| Amine | $X \pm SD^a$ | RSD$^b$ | RSDH$^c$ |
|---|---|---|---|
| MMA | 1.05 ± 0.58 | 5.50 | 5.61–7.48 |
| DMA | 0.86 ± 0.53 | 6.16 | 5.78–7.17 |
| TMA | 0.71 ± 0.42 | 5.93 | 5.94–7.93 |

[a]Mean and standard deviation in mg N per 100 g ($n = 8$).
[b]Relative standard deviation (%).
[c]Range of relative standard deviation (%) according to Horwitz's formula for intralaboratory studies.

**Table 4. Recovery of method for volatile amine determination in fish**

| Volatile amine | Content[a] | Content after addition | | Cochran's test ($G_{exp}$)[d] | Recovery (%) | Student's test ($t_{exp}$)[e] |
|---|---|---|---|---|---|---|
| | | Level I[b] | Level II[c] | | | |
| MMA-N | ND | 5.75 ± 0.53 | 10.28 ± 0.72 | 0.3127 | 98.61 ± 8.03 | 1.3813 |
| DMA-N | 0.83 ± 0.05 | 6.21 ± 0.43 | 11.36 ± 0.52 | 0.5999 | 97.18 ± 5.95 | 1.3357 |
| TMA-N | 0.72 ± 0.04 | 6.48 ± 0.16 | 11.52 ± 0.38 | 0.7777 | 99.05 ± 6.84 | 0.3925 |

[a]Initial average contents in mg N per 100 g ($n = 8$).
[b]5.50 mg N per 100 g.
[c]11.00 mg N per 100 g.
[d]Cochran's test: $C_{Tab(2.7;0.05)} = 0.8332$.
[e]Student's test: $t_{Tab(15;0.001)} = 3.9665$.
ND, not detected.

**Table 5. Contents of DMA-N and TMA-N in fresh and frozen hake samples (mg N per 100 g)**

| | Fresh (market)[a] | Frozen (market)[b] | Control[c] |
|---|---|---|---|
| DMA-N | 0.62 ± 0.58[d] (ND–2.05)[e] | 3.81 ± 3.09 (0.33–11.79) | 3.10 ± 1.63 (0.77–8.73) |
| TMA-N | 1.65 ± 1.87 (ND–8.17) | 0.72 ± 1.28 (ND–5.95) | 0.05 ± 0.07 (ND–2.30) |

[a]Fresh hake samples from market ($n = 27$).
[b]Frozen hake samples from market ($n = 26$).
[c]Frozen control hake samples ($n = 37$).
[d]Mean value and standard deviation.
[e]Range in parentheses.
ND, not detected.

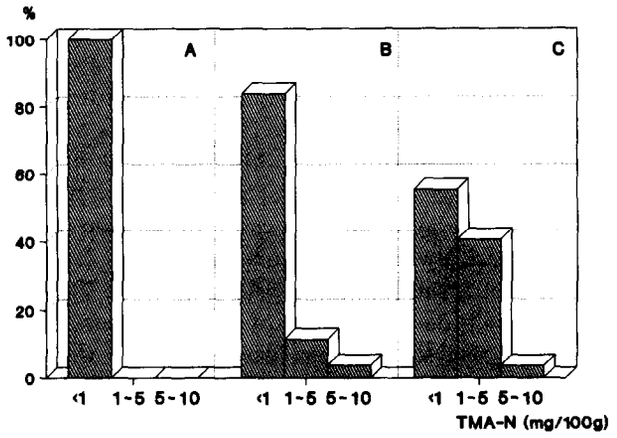


**Fig. 2.** Percentage of samples with high (5–10 mg N per 100 g), medium (1–5 mg N per 100 g) or low (< 1 mg N per 100 g) levels of TMA-N.

frozen samples from the market were similar to the average content of control samples, which were stored for 2 years. The coefficient of variation was higher in samples from the market (81.1%) than in control samples (52.6%). This finding agrees with an increase in DMA-N when fluctuations in freezing temperatures occurred (Leblanc *et al.*, 1988).

TMA-N contents were, in general, higher and more variable in samples from the market (fresh and frozen) than in control samples. Contents ranged from not detected to 8.17 mg TMA-N per 100 g in fresh samples and from not detected to 5.95 mg TMA-N per 100 g in frozen hake. These values agree with data previously reported by other authors for Spanish market hake samples (Ruiz-Martinez *et al.*, 1988; Gallardo *et al.*, 1991; Leon *et al.*, 1994; Sotelo *et al.*, 1995).

Values of volatile amine concentration in hake were not normally distributed; therefore, the Mann–Whitney *U*-test for non-parametric data was used. Significance was assigned at a level of $p < 0.05$. Average contents of TMA-N in fresh hake (1.61 ± 1.87 mg TMA-N per 100 g) were statistically higher ($p < 0.001$) than in frozen samples from the market (0.72 ± 1.28 mg TMA-N per 100 g). In contrast, frozen samples showed average values of DMA-N (3.81 ± 3.09 mg DMA-N per 100 g) statistically higher ($p < 0.001$) than fresh samples (0.61 ± 0.05 mg DMA-N per 100 g).

Several maximum allowable limits for TMA-N contents in fresh fish have been reported. Fish samples showing TMA-N lower than 1 mg TMA-N per 100 g could be rated as excellent quality grade, according to Castell's criterion (Castell *et al.*, 1958). Despite differences between fish species, most authors recommend maximum levels ranging from 10 to 15 mg TAM-N per 100 g. However, specifically for hake, maximum levels of 5 mg TMA-N per 100 g have been suggested (Ludorff & Meyer, 1978).

According to the above criteria to classify the fish freshness on the basis of the TMA-N content, Fig. 2 shows the percentages of samples with low, medium or high TMA-N contents. All control samples, 84.6% of fresh hake samples and 55.5% of frozen hake samples could be graded as excellent quality. Relatively high TMA-N contents were found only in 4.6% of the frozen samples from market suggesting that storage conditions were not suitable. It has been reported that TMA-N can increase when the storage temperature is above −20 °C (Sotelo *et al.*, 1995).

A validated, simple and rapid GC procedure for the simultaneous determination of MMA, DMA and TMA is proposed, avoiding interference associated with the traditional colorimetric procedures. In addition, we provide data for volatile amine contents in samples of fish species that produce significant quantities of DMA

(*Merlucius merlucius* and *Merlucius capensis*) and which are widely consumed in Spain.

## ACKNOWLEDGEMENTS

We thank J. Jesus Abós Gimeno (BSc Biology) for the provision of control samples and the Comisión Interministerial de Ciencia y Tecnología (Project ALI89-0630-C03-01) of the Ministerio de Educación y Ciencia (Spain) and the Comissió Interdepartamental de Recerca i Innovació Tecnològica of the Generalitat de Catalunya (Spain) for their financial assistance in this study.

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