Trimethylamine and Total Volatile Basic Nitrogen Determination by Flow Injection/Gas Diffusion in Mediterranean Hake (*Merluccius merluccius*)[†]

S. Baixas-Nogueras, S. Bover-Cid, M. C. Vidal-Carou,* M. T. Veciana-Nogués, and A. Mariné-Font

Departamento de Nutrició i Bromatologia, CeRTA, Facultat de Farmàcia, Universitat de Barcelona, Avinguda Joan XXIII s/n, 08028 Barcelona, Spain

The reliability of flow injection/gas diffusion (FIGD) methods to determine trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) in hake was studied in order to find an alternative and accurate, simple, cheap, and rapid method for non-protein nitrogen determination. FIGD methods involved extracting volatile amines with 7.5% trichloroacetic acid, followed by the injection of the extracts into the FIGD manifold, previously adjusted for TMA-N or TVB-N determinations. Each determination took ∼2 min. Reliability was satisfactory in linearity, precision, recovery, and sensitivity. There was good correlation ($p < 0.001$) between FIGD and the classic official methods, for both TMA-N and TVB-N determinations, and also between FIGD and the gas chromatographic procedure described for TMA-N. These results proved that FIGD methods are simpler, cheaper, and faster than current official procedures. To check the suitability of FIGD procedures over a wide range of analyte concentrations, changes of both TMA-N and TVB-N and the *P* ratio values throughout the ice storage of hake were monitored. The usefulness of each of these potential freshness indicators for hake is discussed.

Keywords: *Trimethylamine; hake; FIGD; volatile basic nitrogen*

INTRODUCTION

The generally accepted method for the assessment of quality in seafood products is sensory analysis. Advantages of sensory analysis are its simplicity and rapidity. However, there are also several disadvantages, such as the extensive training required for the panel members to avoid subjectivity. These drawbacks have catalyzed research into the development of objective methods using chemical indicators to reinforce the conclusions reached by sensory analysis. A joint project among different European countries (EU FAIR CT.96.3253) has been trying to develop flow injection/gas diffusion methods (FIGD) for trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) in fish. Flow injection analysis (FIA) seems to be a good alternative tool for optimizing routine analyses, mainly because it offers the advantages of simplicity, low cost, and rapidity (*1*). It has been described as an objective method with the same advantages as sensory analysis (*2–4*).

Trimethylamine oxide (TMAO) is a natural and nontoxic substance, generally involved in the osmoregulatory function of marine species of fish and shellfish (*5*). The TMAO content in fish varies within species and usually decreases after death. TMAO is mainly reduced by bacterial enzymes to TMA-N, which is largely responsible for the characteristic off-odor of dead marine fish (*6–8*). During fish storage, the increase in TMA-N has been widely correlated with a decrease in TMAO concentration (*9–11*).

During marine fish spoilage, along with TMA-N, ammonia and other basic nitrogenous compounds are also produced and together make up total volatile basic nitrogen (TVB-N). Thus, the most common chemical parameters for assessing the freshness of fish are the determination of both TVB-N and TMA-N. Although the significance and limitations of these chemical indices have been underscored by several authors, they have been widely used as a freshness index because of their close correlation with the organoleptic score (*12–14*). In addition, the determination of both TVB-N and TMA-N enables another criterion, known as the *P* ratio, to be calculated. This is the percentage quotient between TMA-N and TVB-N, which, according to Malle and Poumeyrol (*15*), is less affected by the various factors acting on both the TMA-N and TVB-N contents and provides relatively similar values in several different fish species.

The colorimetric method of Dyer (*16*) and its further modifications are probably the best known and most widely used procedures to determine TMA-N. The current official method [AOAC 971.14 (*17*)] for determining TMA-N in fish, also based on Dyer's method, involves a reaction of the TMA-N with picric acid to form a UV-colored complex. Several methods have been developed to improve the TMA-N analyses, including steam distillation (*18*), the Conway microdiffusion procedure (*19*), gas chromatography (GC) (*20*), liquid chromatography (LC) (*21*), and a TMA-N-specific ion electrode (*22*), as well as microbiological determinations or techniques based on biosensor or enzymatic methodologies (*23*). Nowadays, there is an official European

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* Author to whom correspondence should be addressed (telephone + 34-93-402 45 13; fax + 34-93-402 18 96; e-mail mcvidal@farmacia.far.ub.es).

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method for TVB-N determination, which involves steam distillation of an acidic fish extract and its further titration with 0.01 N HCl. However, there is no official European method for TMA-N.

The aim of this study was to validate FIGD procedures for the determination of both TMA-N and TVB-N, in terms of linearity, precision, recovery, and sensitivity, and to compare them with the respective official methods (EU method for TVB-N and AOAC method for TMA-N). In addition, the determination of TMA-N by FIGD will also be compared with a gas chromatography method, which is currently a faster and automated alternative. FIGD equipment and conditions required for TMA-N analysis have been previously described by Sadok et al. (*24*), but no data are available on FIGD's reliability for TMA-N in hake or on its suitability for TVB-N determination in any fish species. TMAO levels vary among fish species, so it is necessary to set maximum tolerable levels for both TMA-N and TVB-N in every fish species. However, the development of those compounds is strongly dependent on the fish storage conditions, this fact being the reason to monitor both parameters for grading fish freshness. The fish species chosen for this study was hake (*Merluccius merluccius* var. *mediterraneus*) because it is widely consumed in Spain and because there are relatively few references on the suitability of chemical indices to grade its freshness. In addition, the study aimed to examine the usefulness of the *P* ratio (percent) as an index of the freshness of hake, in line with the objectives of the European Project "Qualpoiss 2" FAIR CT.97.3253. Thus, data on the evolution of TMA-N, TVB-N, and *P* ratio values during the storage of hake samples on ice not only will provide information about the suitability of FIGD methodologies but also aim to contribute to the study of the best chemical indicators to evaluate hake freshness.

MATERIALS AND METHODS

Samples and Sample Preparation. Hake (*M. merluccius* var. *mediterraneus*) samples were caught off the Mediterranean coast (Vilanova i la Geltrú) near Barcelona, acquired from local Barcelona markets, and delivered directly to our laboratory. According to Botta's (*25*) sensorial criterion for hake, samples were graded as being of excellent quality. Some of the samples were immediately treated to obtain the fish extract, whereas another sample fraction was stored in flake ice (0 °C) inside a refrigerator set at 4 °C, in a self-draining box, until analysis. The melted ice was replaced daily. Samples were taken throughout the storage (at 0, 1, 2, 3, 5, 7, 9, 11, 14, and 16 days) to find the evolution of the chemical indicators studied. Samples with different volatile amine contents were needed to check whether the correlation of results from FIGD techniques and gas chromatography or reference methods occurs in a wide range of volatile amine content.

Muscle tissue from fish samples was minced, and 25 g was homogenized with 50 mL of 7.5% trichloroacetic acid in an Ultra-turrax homogenizer (Ika Labortechnik, T25 basic, Staufen, Germany) for 2 min. Then the mixture was filtered through a Whatman No.1 filter paper (Whatman, Kent, U.K.), and the extract was stored at −20 °C until the TMA-N and TVB-N determinations. This sample preparation procedure was the same for the TMA-N and TVB-N FIGD determinations and for the corresponding reference methods.

For the gas chromatography method, performed following the method of Veciana-Nogués et al. (*20*), 0.6 N perchloric acid was used to get the total extraction of the nonvolatile amines.

Standards and Reagents. All chemicals used were of analytical grade, and ultrapure water, obtained from a Milli-Q System (Millipore, Madrid, Spain), was used to dissolve them.

Standards. The standards used were trimethylamine hydrochloride (Sigma Aldrich, Madrid, Spain), ammonium chloride (Panreac, Barcelona, Spain), and *n*-propylamine hydrochloride (Sigma Aldrich).

Reagents for sample preparation included a 7.5% trichloroacetic acid solution (Panreac) and a 0.6 N perchloric acid solution (Panreac).

Reagents for the determination of TMA-N and TVB-N by FIGD included 20% formaldehyde (Panreac), 1 M sodium hydroxide solution (Panreac), and 0.3 g L^{-1} Bromthymol Blue solution (BTB) (Sigma Aldrich).

Reagents used for the determination of TMA-N by the AOAC method were 20% formaldehyde (Panreac), toluene (Panreac), saturated potassium carbonate solution (Panreac), anhydrous sodium sulfate (Panreac), and 0.02% picric acid solution (Fluka Chemica, Madrid, Spain).

Reagents for the determination of TVB-N by the UE method included phenolphthalein solution (Sigma Aldrich, Madrid, Spain), 40% sodium hydroxide solution (Quimivita S.A., Barcelona, Spain), 4% boric acid solution (Panreac); Tashiro indicator (Methylene Blue/Methyl Red, 1:2) (Sigma Aldrich), and 0.01 N HCl (Merck, Barcelona, Spain).

f) Reagents used for the determination of TMA-N by the GC method were toluene (Panreac) and 65% KOH (Panreac).

Determination of TMA-N and TVB-N by FIGD. Figure 1 shows the FIGD system for TMA-N and TVB-N determinations. A peristaltic pump (Ismatec IPC-8, Zürich, Switzerland) propelled the reagents, maintaining the flow rate at 1 mL/min. Sample extracts were introduced through a low-pressure injection valve (Rheodyne 5020) with a 100 μL sample loop. A laboratory-built gas diffusion cell was used, the channel dimensions being 240 mm × 1.5 mm × 0.2 mm. All manifold tubing used was made from Teflon 0.8 mm internal diameter (Omnifit, Barcelona, Spain), which also acted as the flow cell. The Teflon microporous membrane (Du Pont) was chemically inert and acid-resistant. Likewise, a laboratory-built spectrophotometer incorporating red light-emitting diodes ($\lambda = 635$ nm) was used as detector. Both the gas diffusion cell and the detector were provided by the Hull International Fisheries Institute (HIFI) of the University of Hull. Peak heights were recorded on a Hewlett-Packard 3396A series II integrator (Hewlett-Packard, Avondale, PA), and data were stored using a Hewlett-Packard 99114B disk unit.

For TMA-N determination, a 20% formaldehyde (FA) solution flow carried the injected extract through the mixing coil. FA was used to avoid the interference of primary and secondary amines, mainly ammonia, methylamine, and dimethylamine. Then, alkalization with 1 M NaOH was required to counterbalance the acidity of the injected solution and to turn the TMA-N ions (CH$_3$)$_3$NH$^+$ to TMA-N (CH$_3$)$_3$ gas, which goes through the permeable membrane rapidly and automatically. On the other side of the porous membrane, the arrival of TMA-N gas produces a pH increase and induces a color change of the BTB solution, which is quantified by the colorimetric detector. A calibration curve was made from standard solutions prepared with 7.5% TCA in the range of 0.035−0.7 mg of N/100 mL. Standard solutions were prepared by appropriate dilutions from trimethylamine hydrochloride in 7.5% TCA.

For TVB-N determination, the FA feed was disconnected because in this case all of the volatile amines had to be determined. The rest of the procedure was the same as for the TMA-N determination. The standard curve was made from ammonium chloride in 7.5% TCA in the range of 0.14−1.4 mg of N/100 mL.

Determination of TMA-N and TVB-N by Reference Methods. TMA-N was determined according to the current official procedure of the AOAC (*17*) based on the traditional Dyer colorimetric method. Briefly, the reference method used was as follows: 1 mL of sample extract and 3 mL of deionized water were placed in a test tube. For the calibration curve, 1, 2, 3, and 4 mL of a 0.01 mg/mL TMA-N standard solution were placed in different tubes and 3, 2, 1 and 0 mL of deionized water were added, respectively. Another tube containing 4 mL of deionized water was used as a blank. One milliliter of 20% FA, 10 mL of anhydrous toluene, and 3 mL of potassium
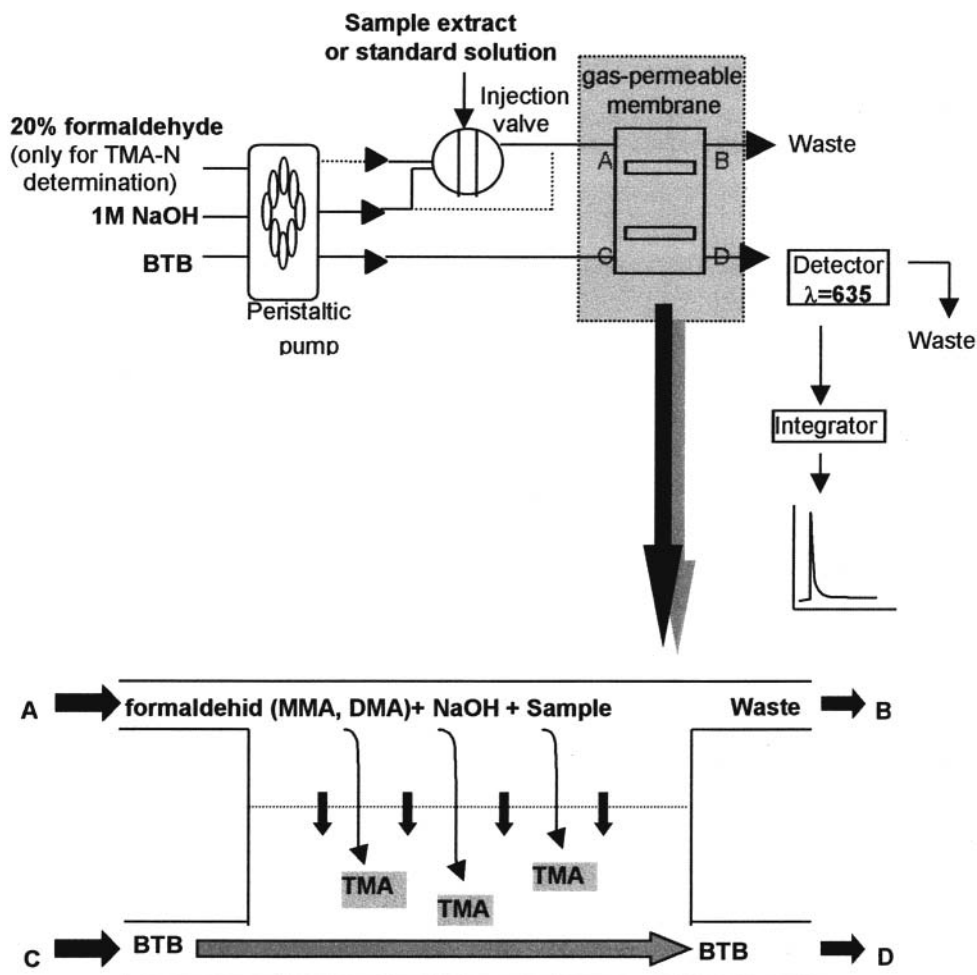
Figure 1. Flow injection system and gas diffusion cell scheme used for the TMA-N and TVB-N determinations.

carbonate solution were added to every tube. The tubes were stoppered and shaken vigorously. The toluene phase (∼8 mL) was then transferred into a tube containing 0.2 g of anhydrous sodium sulfate and shaken to obtain a dehydrated extract. Five milliliters of the water-free toluene extract was mixed with 5 mL of 0.02% picric acid solution in another tube. Absorbance was measured using a spectrophotometer (UV-160A, Shimazdu) at 410 nm wavelength. The amount of TMA-N in the samples was calculated from the optical densities by using the standard curve described.

The reference TVB-N determination followed the Official Method for the European Union (*26*). Semimicro steam distillation was performed using a Kjeltec System (1002 distilling unit, Tecator). Ten milliliters of sample extract was placed in the distillation tube together with 2 or 3 drops of phenolphthalein. Forty percent NaOH solution was added until the extract alkalized (until a pink color appeared). Two hundred and fifty milliliters of the distilled portion was collected in an Erlenmeyer flask containing 20 mL of 4% aqueous boric acid solution and 3 or 4 drops of Tashiro indicator. Basic solution was titrated with 0.01 N HCl. A standard curve was made from standard solutions in the range of 7–42 mg of N/100 mL prepared from a stock solution (140 mg of N/100 mL) of ammonium chloride in 7.5% trichloroacetic acid.

Determination of TMA-N by the GC Method. The GC procedure previously described and validated by Veciana-Nogués et al. (*20*) involves a first step of volatile amine extraction with 0.6 N perchloric acid, followed by alkalization with 65% (w/w) KOH, and subsequent toluene extraction. A Hewlett-Packard 5890 series II gas chromatograph equipped with a silcosteel packed column of 200 cm of 4% Carbowax 20M plus 0.8% KOH on Carbopack B 60/80 (Tecknocroma, Barcelona, Spain) and a flame ionization detector (FID) were used.

Statistical Analysis. Statistical analysis of data was carried out using the analytical procedures of SPSS 8.0 for Windows software (SPSS Inc., Chicago, IL). The precisions of different methods were compared by Levene's test. FIGD procedures and the corresponding reference methods were compared for recovery and precision with the Student *t* test. Cochran's test was used to find whether the recovery was independent of the analyte concentration in the samples. Finally, the nonparametric statistical method described by Passing-Bablok (*27*) was used to compare the data between methods.

RESULTS AND DISCUSSION

Trials were carried out to test the reliability of the TMA-N and TVB-N methods in terms of linearity, precision, recovery, and sensitivity limits in order to validate FIGD against the reference methods. The different methods were used on the same samples for purposes of comparison.

Linearity. The detector responses were linear for the ranges of TMA-N (0.35–7 mg of N/L) and TVB-N (1.4–14 mg of N/L) assayed. Least-squares analysis gave a correlation coefficient of $r > 0.99$ ($p < 0.001$) for both the reference and the FIGD methods when TMA-N and TVB-N were analyzed. In all cases, calibration curves for every set of samples were made and the coefficients of determination (r^2) were always >99.40%. The linearities of the different methods were compared by the analysis of variance of regression, with the results always satisfactory ($p < 0.001$).

Precision. Results of a within-day precision study are shown in Table 1. Eight determinations of TMA-N

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Table 1. Precision of Methods for TMA-N and TVB-N Determination in Hake (*M. merluccius*)

method	X (SD)[a]	RSD[b]	RSDH[c]
TMA-N, AOAC	2.36 (0.12)	4.12	5.35
TMA-N, FIGD	2.28 (0.05)	2.37	5.51
TVB-N, EU	21.55 (0.39)	1.81	4.01
TVB-N, FIGD	20.08 (0.29)	1.45	4.05

[a] Mean in mg of N/100 g and standard deviation. [b] Relative standard deviation (%). [c] Maximum relative standard deviation (%) according to Horwitz's criterion for intralaboratory studies.

and TVB-N were carried out on the same day and using the same reagents and apparatus. Relative standard deviations (RSDs) were always satisfactory according to Horwitz's criterion for intralaboratory studies (*28*). RSD values were slightly lower in FIGD procedures than in the corresponding reference methods, although the differences were not statistically significant ($p > 0.05$; Levene's test).

Recovery. Recovery was tested by standard addition using two addition levels, which are shown together with the results obtained in Table 2. According to the statistical analysis (Cochran's test), accuracy did not depend on the analyte content in any case. Moreover, in examining the mean recovery for each method, we checked with the Student's *t* test that there were no statistically significant differences between the mean recovery found and the theoretical value of 100% ($p < 0.50$). Mean recoveries ranged from 96.67 to 100.28%, and no statistical differences ($p > 0.05$) were found between recovery values of the reference and the FIGD procedures for both TMA-N and TVB-N determinations.

Sensitivity. The determination limit (DtL) was calculated according to the Long and Winefordner (*29*) criterion. Sensitivity limits were 0.01 and 0.6 mg/100 g of fish for TMA-N by FIGD and AOAC methods, respectively. DtLs for TVB-N were 0.14 and 5.6 mg/100 g of fish for FIGD and steam distillation, respectively. Therefore, FIGD methods were more sensitive than the reference methods, as they allowed quantification of ∼40−50-fold smaller amounts of TVB-N and TMA-N, respectively.

Specificity. The absence of interference from other volatile amines in the determination of TMA-N by FIGD was verified by means of the GC procedure, which permits differentiation between primary, secondary, and tertiary amines. Thus, the same samples were analyzed according to the two procedures, and the results were practically identical. As Figure 2 shows, the concordance between the values obtained from both methods was very satisfactory.

In addition, the GC method was used to confirm the high sensitivity of the TMA-N FIGD procedure. Thus, the minimum amounts of TMA-N detected were similar for FIGD and GC, which is known to be a highly sensitive method.

Comparison between Methods. After checking that the reliability of TMA-N and TVB-N determinations by FIGD was similar to the reliability of classic procedures, we also compared methods using the regression curves obtained by plotting TMA-N and TVB-N values provided by the reference method (*x*-axis) against those obtained by FIGD methodology (*y*-axis). The Passing−Bablok methodology (*27*) assumes that both methods show continuously distributed measurements and have a significant relationship between them. The estimated values for the intercept *a* and the slope *b* were tested against the null hypothesis $a = 0$ and $b = 1$. The

95% confidence interval (CI$_{95}$) of the intercept included the value of 0, meaning that there was no constant systematic error between the methods compared (Table 3). Very significant linear regressions ($p < 0.001$) were obtained in both cases, with $r > 0.995$ for TMA-N and $r > 0.928$ for TVB-N. Moreover, the CI$_{95}$ of the slope included the value of 1, and so the measurements between the compared methods were free of proportional systematic error. Therefore, values of TMA-N and TVB-N from FIGD are consistent with those obtained by reference methods ($p > 0.05$).

As well, the reliability of FIGD methodologies is as good as or even better than that of the classic procedures used for TMA-N and TVB-N determinations and provides high sensitivity for TMA-N, comparable to that obtained with GC procedures. FIGD methods offer, in comparison with classic procedures, noticeable advantages in time, cost, and simplicity of analysis. In addition, the semiautomatic FIGD technique can easily be integrated into routine on-line analysis and so meet the requirements of the seafood-processing industry. Additional advantages of the FIGD methodologies, especially over the official reference methods, are the suitability of the same equipment for the determination of both TMA-N and TVB-N and the small quantity of sample required to carry out the analyses.

Changes in TMA-N and TVB-N Contents during Hake Storage: Suitability of the *P* Ratio as Freshness Index. Table 4 shows the changes observed in TMA-N and TVB-N contents and in *P* ratio values during ice storage of Mediterranean hake. The initial TMA-N value (time 0) was <1 mg of N/100 g of fish and, according to the classic Castell criterion (*30*), led to the classification of the hake as excellent-quality grade. Assuming 5 mg of N/100 g as a limit of acceptability for hake (*31*), samples are acceptable until almost day 9 of ice storage. This is in agreement with the findings of Moral et al. (*32*), who reported a preservation time of 8 days for gutted hake stored in ice. The TMA-N level remained <7.75 mg of N/100 g after 11 days of storage, and from this point, a steep increase occurred, with a maximum value of ∼20 mg of N/100 g of fish after 14 days of storage. Thus, there was an induction phase in the first days of storage, in which the production of TMA-N was very slow. This TMA-N profile was similar to that reported by Pastoriza et al. (*33*) in iced hake fillets and is consistent with their bacterial origin (*5*). According to Ryder et al. (*2*), the few changes in the initial stages of hake storage in ice limit the suitability of TMA-N as a monitor of loss of freshness. Other authors also reported the suitability of TMA-N as a freshness index in fish (*34*). The limit set at 5 mg/100 g of fish seems to be appropriate as a freshness limit for hake because it corresponds to the first part of the exponential curve adjusted to the TMA-N production.

Initial values of TVB-N were near 10 mg of N/100 g and were consistent with values reported by other authors for fresh hake (*35*, *36*). TVB-N levels slightly increased throughout the first 11 days of storage, and then a sharp increase was observed, with values rapidly reaching >30 mg of N/100 g. The European Union guidelines consider 30−35 mg of N/100 g of fish as a limit of acceptability for hake, a value reached at almost 14 days. At this point, hake was clearly rejected because of its sensory characteristics. Therefore, between TMA-N and TVB-N, there is a great difference in the time required to reach the corresponding rejection limits.

Validation of TMA-N and TVB-N Determination by FIGD in Hake

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Table 2. Recovery of Methods for TMA-N and TVB-N Determination in Hake (*M. merluccius*)

method	initial content (mg/100 g)	recovery (%) level I[a]	recovery (%) level II[b]	recovery (%) mean (SD)
TMA-N, AOAC	10.80 (*0.14*)	101.53 (*7.64*)	100.19 (*2.65*)	100.86 (*5.28*)
TMA-N, FIGD	9.86 (*0.11*)	98.53 (*1.48*)	98.36 (*2.04*)	98.49 (*1.72*)
TVB-N, EU	23.78 (*0.21*)	100.77 (*0.78*)	97.71 (*1.42*)	99.24 (*3.05*)
TVB-N, FIGD	20.52 (*0.17*)	96.00 (*3.89*)	97.34 (*3.34*)	96.67 (*2.83*)

[a] 2 mg of N/100 g for TMA-N and 20 mg of N/100 g for TVB-N. [b] 10 mg of N/100 g for TMA-N and 40 mg of N/100 g for TVB-N.

r = 0,994

p < 0,001
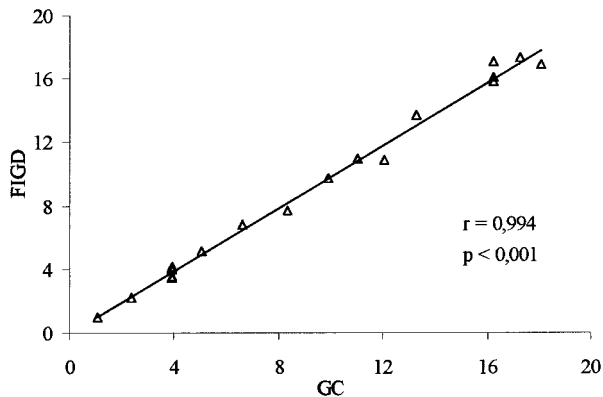
Figure 2. Correlation obtained between the FIGD and GC methods for TMA-N determination.

Table 3. Statistical Comparison of FIGD and Reference Methods for TMA-N and TVB-N Determination in Hake (*M. merluccius*)

	FIGD/EU method	FIGD/AOAC method	FIGD/GC
$y = a + bx$	$y = -3.81 + 1.03x$	$y = -0.01 + 0.95x$	$y = -0.22 + 1.03x$
r	0.928 ($p < 0.001$)	0.995 ($p < 0.001$)	0.994 ($p < 0.001$)
$a \pm SE_a t$*	−11.54 to 1.62	−0.13 to 0.05	−0.39 to 1.01
$b \pm SE_b t$*	0.63–1.38	0.88–1.01	0.89–1.02

*95% confidence interval; SE, standard error.

Table 4. TMA-N, TVB-N, and *P* Ratio Values throughout Ice Storage of Hake (*M. merluccius*)

days	TMA-N[a] (mg/100 g)	TVB-N[a] (mg/100 g)	*P* ratio (%)
0	0.17 (*0.02*)	9.87 (*0.08*)	0.72 (*0.26*)
1	0.77 (*0.09*)	9.07 (*0.22*)	8.48 (*0.94*)
2	1.10 (*0.18*)	10.20 (*0.67*)	10.44 (*1.03*)
3	1.45 (*0.21*)	7.74 (*0.44*)	18.86 (*3.28*)
5	2.43 (*0.22*)	7.63 (*0.09*)	31.90 (*2.98*)
7	4.08 (*0.12*)	13.21 (*2.82*)	31.96 (*6.02*)
9	5.37 (*0.19*)	11.45 (*0.28*)	46.96 (*2.76*)
11	7.75 (*0.54*)	11.58 (*0.48*)	66.93 (*4.13*)
14	19.83 (*0.50*)	30.56 (*0.23*)	64.89 (*1.60*)
16	21.57 (*1.01*)	33.08 (*0.38*)	65.23 (*3.14*)
r[b]	0.926 ($p < 0.001$)	0.830 ($p < 0.001$)	0.967 ($p < 0.001$)
b[c]	1.308 (0.87–1.74)	1.391 (0.63–2.15)	4.350 (3.42–5.27)

[a] Mean (*standard deviation*). [b] Correlation coefficient (signification level). [c] Slope (confidence interval 95%).

Although the correlation between TVB-N and the time of ice storage was statistically significant ($r > 0.832$, $p < 0.003$), it is noticeable that it was worse than the correlation between TMA-N and days of ice storage. The clear TVB-N increase occurred after 11 days of storage, near the time required (14 days) to reach the rejection limit on the basis of TVB-N levels and clearly surpassing the time required to reach the TMA-N limit. This behavior in the TVB-N profile was also reported by Civera et al. (*13*), Morales et al. (*37*), and Perez-Villareal et al. (*38*) for chilled hake. Thus, in this case, results corroborate previous findings and suggest that indi-

vidual TVB-N values are better as an indicator of fish spoilage than of fish freshness.

The *P* ratio increased gradually throughout hake ice storage, from an initial average value of $0.72 \pm 0.26\%$ to a final value of $65.23 \pm 3.14\%$. In comparison with individual TMA-N and TVB-N values, the increase in the *P* ratio was earlier and more gradual, especially in the first days of storage. There was also, in this case, an increase in *P* ratio values until day 11, and then the values remained constant until the end of the storage. The regression between time of ice storage and *P* ratio values was higher than the regressions with TMA-N and TVB-N. Moreover, the slope of the *P* ratio/time regression was also higher, which is an additional benefit of the *P* ratio index. According to TMA-N limits, hake could be rated as excellent-quality freshness when the *P* ratio was <10%, which would correspond to a TMA-N level of <1 mg/100 g of fish. A systematic rejection threshold could be established if this ratio was ~40%, which would correspond to TMA-N values >5 mg of N/100 g of fish.

In conclusion, the *P* ratio value is more suitable than individual TMA-N or TVB-N values as an indicator of the degree of freshness. This conclusion is supported by the higher slope of the *P* ratio/time of storage relationship. Another argument for using the *P* ratio instead of TMA-N as a measure of freshness is that it changes more strongly during the early storage period, when the concept of freshness is most relevant. This confirms the findings of Malle et al. (*15*) that *P* ratio values seem to be less affected by the various factors acting on both the TMA-N and TVB-N contents. However, more studies are needed to confirm the suitability of the *P* ratio limits here proposed. In particular, sensory tests are needed to correlate chemical data with organoleptical scores.

ACKNOWLEDGMENT

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