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Analytical Methods

Colorimetric assay for determination of trimethylamine-nitrogen (TMA-N) in fish by combining headspace-single-drop microextraction and microvolume UV–vis spectrophotometry

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ABSTRACT

In this work, headspace-single-drop microextraction has been used in combination with microvolume UV–vis spectrophotometry to enhance the determination of trimethylamine-nitrogen (TMA-N) in fish samples. TMA-N is often used for monitoring fish freshness, but due to the low analytical sensitivity usually achieved, its determination must be performed after certain time of storage in some fish species. The proposed methodology, based on the extraction and subsequent complexation of volatile TMA-N onto a picric acid-containing xylene microdrop exposed to the headspace, involves an important improvement of sensitivity (detection limit 6×10^{-4} mg TMA-N per 100 g of fish), a miniaturization of the AOAC Official Method (971.14) and a simple approach for routine labs. This method is well suited to determination of TMA-N in different species of frozen and fresh fish samples from markets and to study the evolution of TMA-N concentration in farmed turbot at the earliest stages of deterioration.

1. Introduction

Freshness is the most important factor for fish quality evaluation. Traditionally, fish quality evaluation has been based on organoleptic tests, which are characterised by their subjectivity, even for experienced and well-trained personnel (Baixas-Nogueras, Bover-Cid, Veciana-Nogués, & Vidal-Carou, 2002). Different chemical parameters can also be used for this purpose, TMA-N being of widespread use. Concentration of TMA-N in fish has been found to be closely related to organoleptic estimations (Baixas-Nogueras et al., 2002), being an objective quality indicator for freshness determination in fish samples. TMA-N generation is attributed to the gradual conversion of trimethylamine oxide (TMA-O) by bacterial or enzymatic reduction.

The AOAC Official Method 971.14 for determination of TMA-N in fish samples (AOAC Official Method 971.14, 2000, chap. 35), based on Dyer's method (Dyer, 1945), encompasses a liquid–liquid extraction of TMA-N with toluene and its subsequent reaction with picric acid reagent to form a yellow complex. Due to the low analytical sensitivity achieved with this method, TMA-N determination in fish must be performed after certain time of *post-mortem* storage and as a result, it is applicable only to certain fish species. Thus, in teleosts, flat fish and pelagic species the amount of TMA-O precursor is very low and then the usefulness of determination of TMA-N is limited (Ruiz-Capillas & Horner, 1999). Moreover, this method involves several time-consuming steps as well as the use of large amounts of hazardous reagents. An overestimation of the total TMA-N values has been reported with this method (Ruiz-Capillas, Gillyon, & Horner, 2000).

Several methods have been described so far for the determination of TMA-N in fish samples including: flow injection/gas diffusion systems with spectrophotometric (García-Garrido & Luque de Castro, 1997; Sadok, Uglow, & Haswell, 1996), potenciometric (Adhoum et al., 2003) or Fourier transform-infrared spectroscopy detection (Armenta, Coelho, Roda, Garrigues, & de la Guardia, 2006); capillary electrophoresis with indirect UV-detection (Lista, Arce, Ríos, & Valcárcel, 2001; Timm & Jørgensen, 2002); gas chromatography with flame ionisation detector (FID) (Li et al., 2004; Veciana-Nogues, Albala-Hurtado, Izquierdo-Pulido, & Vidal-Carou, 1996), nitrogen–phosphorus detector (NPD) (Béné, Hayman, Reynard, Luisier, & Villettaz, 2001) or mass spectrometry (MS) detection (Chan et al., 2006). In the last years, headspace-solid-phase microextraction (HS-SPME) has also been used in combination with gas chromatographic systems (Béné et al., 2001; Chan et al., 2006; Li et al., 2004). Most of the mentioned approaches lack the necessary sensitivity to tackle TMA-N determinations at low level (i.e. below 0.01 mg TMA-N in 100 g mass sample). The fish industry is increasingly demanding sensitive analytical methodologies that are simple, rapid and low cost for TMA-N determination, especially when teleost, flat and pelagic fishes must be analyzed.

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Miniaturization has become an important trend in both sample preparation and instrumental analysis (Ríos, Escarpa, González, & Crevillén, 2006).

Single-drop microextraction (SDME), introduced by Jeannot and Cantwell (1997), is a miniaturized sample preparation technique where the extractant phase to aqueous sample ratio is greatly reduced. SDME, in its both headspace (HS-SDME) and immersed modes (Direct-SDME), has been shown to be a simple, inexpensive, fast, effective and virtually solvent-free sample preconcentration technique (Psillakis & Kalogerakis, 2002). HS-SDME, introduced by Theis, Waldack, Hansen, and Jeannot (2001), is considered the most appropriate mode of microextraction for volatile or semi-volatile compounds, since mass transfer of analytes in the headspace is faster than in the aqueous phase and potential interferences due to non-volatile compounds and high molecular weight species are not extracted in the drop placed in the headspace.

Currently, there is an increasing interest in reducing the sample volume to the microscale level in many fields such as food, environment, clinical, etc. Microsample quantitation is nowadays possible owing to the introduction of microvolume cuvettes and capillaries, as well as microvolume UV–vis spectrophotometers, which uses surface tension as a cuvetteless sample retention system (http://www.nanodrop.com). Although microvolume systems are being used in molecular biology, biochemistry and microbiology, these systems could also be useful in analytical chemistry, among other applications, in combination with sample preparation techniques such as SDME (Jeannot & Cantwell, 1997), hollow fibre liquid-phase microextraction (Pedersen-Bjergaard & Rasmussen, 1999) and/or dispersive liquid–liquid microextraction (Rezaee et al., 2006), where a few microlitres of extractant phase are used to extract and preconcentrate target analytes. Recently, the authors have reported the first analytical application of microvolume UV–vis spectrophotometry based on surface tension-shaped drop and combined with single-drop microextraction for determination of iodine (Pena-Pereira, Lavilla, & Bendicho, 2008).

The main goal of the present work is to increase the sensitivity, miniaturize, simplify and improve the rapidity of the AOAC Official Method for TMA-N determination in fish samples by hyphenation of HS-SDME with microvolume UV–vis spectrophotometry. The preconcentration process prior to the spectrophotometric measurement is based on the extraction and subsequent complexation of TMA-N onto a microdrop of xylene containing picric acid. The proposed methodology was applied to analysis of different species of teleost fish, including flat and pelagic fishes. This work includes a study about the TMA-N generation in turbot (flat fish) at the earliest stages of deterioration.

2. Materials and methods

2.1. Fish samples

Eight teleost fishes typically consumed in Spain were purchased from one fish market to study TMA-N levels in sold fish as fresh: Atlantic horse mackerel (*Trachurus trachurus*), European hake (*Merluccius merluccius*), gilthead seabream (*Sparus aurata*), mackerel (*Scomber scombrus*), megrim (*Lepidorhombus whiffiagonis*), pouting (*Trisopterus luscus*), sardine (*Sardina pilchardus*) and sole (*Solea solea*). Megrim and sole are as well flat fishes.

Commercial frozen teleost fish was purchased from a local supermarket: Southern hake (*Merluccius australis*), cod (*Gadus morhua*) and tuna (*Thunnus* spp). Recovery studies were carried out with frozen fish samples to validate the methodology.

A study about the TMA-N generation in turbot (*Psetta maxima*) at the earliest stages of deterioration was made. Turbot represents a teleost flat fish species of remarkable commercial value with low content of TMA-O. Farmed turbot specimens were obtained from Loitamar, S.A. (Moaña, Spain), a floating fish farm in the ocean, so they are considered as semi-wild turbots. Farmed fish guarantee the freshness and allows us the determination of TMA-N from the first moment of storage, i.e. at very low concentrations. Turbot specimens were sacrificed in the farm by immersion in flake ice and immediately transported in the same refrigerated system to our laboratory. The average weights, lengths and widths of turbot specimens were 326.4 ± 24.6 g, 32.1 ± 1.6 cm and 21.9 ± 1.2 cm, respectively. The turbots were neither heated nor gutted. They were stored in ice at a fish-to ice ratio of 1:2 (w/w) and stored at 4 °C. The ice mixtures were renewed when required.

2.2. Chemicals

All chemicals used were of analytical-reagent grade. Deionised water obtained from a Milli-Q water purifier (Millipore, Molsheim, France) was used throughout. Trimethylamine, dimethylamine and monomethylamine as hydrochorides, and ammonium chloride were obtained from Merck (Darmstadt, Germany). Toluene (Panreac, Barcelona, Spain), xylene (Fluka, Buchs, Switzerland), 1-octanol (Merck, Darmstadt, Germany), n-heptane (Fluka, Buchs, Switzerland) and N,N′-dimethylformamide (Merck, Darmstadt, Germany) were tried as solvents for the extracting drop. Picric acid (Probus, Badalona, Spain) was used to form a coloured complex with TMA-N in the drop. Sodium hydroxide was obtained from Panreac (Barcelona, Spain). Sodium chloride (Merck, Darmstadt, Germany) was used to favour the mass transfer of TMA-N to the headspace. Formaldehyde (39% mass/volume) (Prolabo, Paris, France) was employed to avoid interferences from primary and secondary amines. Trichloroacetic acid (Probus, Badalona, Spain) was used to extract TMA-N from the fish muscle.

Stock standard solutions of trimethylamine, dimethylamine and monomethylamine (as hydrochlorides) containing 1000 mg/L (as *N*) were prepared by dissolving the corresponding amounts in 0.5 M HCl. Ammonium chloride standard solution containing 1000 mg/L (as *N*) was prepared in water.

2.3. Apparatus

A Nanodrop® (Wilmington, USA) Model ND-1000 Spectrophotometer was used. The spectrophotometer is equipped with a xenon flash lamp and a 2048-element linear silicon CCD array detector. The optical path length is 1 mm. The sample droplet is held in place by surface tension when it is slightly compressed between the drop-supporting surface (pedestal) and the sample arm. Sample pedestals are made of stainless steel and quartz fibre. The spectrum measurement is performed with two optical fibres installed in the pedestal (emitting light of a xenon lamp) and the sample arm (spectrometer with linear CCD array). Absorption peak measurements were carried out at 410 nm.

Headspace-single-drop microextraction was performed with a commercially available 10-μL syringe containing a guided-PTFE plunger (Hamilton model 1701 RN, 10 AL). TMA-N generation was carried out in a 40 mL amber-vial with a silicone rubber septum.

2.4. Procedure for TMA-N determination in fish samples

Extraction of TMA-N from fish samples was performed according to the AOAC procedure by homogenising 10 g of fish muscles with 20 mL of 7.5% (mass/volume) aqueous trichloroacetic acid (TCA) solution and subsequent centrifugation of the homogenate at 3000 rpm for 15 min. The supernatant liquid was placed in a volumetric flask and enough TCA at 7.5% (mass/volume) concentration was added to make up to 25 mL.

A 2 mL volume of the fish extract was placed into a 40 mL-amber vial containing a stirring bar together with 5 mL of a salted (30% mass/volume NaCl) aqueous solution and 1 mL of formaldehyde (39% mass/volume). Then 2 mL of NaOH (10 M) were injected trough the septum. A 3 μL xylene drop containing 0.015% mass/volume of picric acid was then formed at the needle tip of a microsyringe and exposed to the headspace of the sample stirred at 1100 rpm for 3.5 min. The extractant solution must be refrigerated into a water–ice bath before extraction to extend the microextraction time and, therefore, enhance the extraction efficiency of TMA-N. Finally, the remaining drop was retracted back into the microsyringe and placed on the pedestal of the Nanodrop® spectrophotometer to obtain the corresponding analytical signal.

3. Results and discussion

3.1. Headspace-single-drop microextraction of TMA-N

In order to develop a new HS-SDME methodology for the determination of trimethylamine in fish samples, several parameters, such as the extractant phase, picric acid content in the drop, microextraction time, sample volume, NaOH concentration in the sample, stirring rate of the sample solution and ionic strength of the sample were exhaustively investigated.

3.1.1. Optimization of the extractant phase composition and microextraction time

Selection of the most suitable extractant phase is one of the main variables to be controlled when SDME is employed. In the present work, a drop of an organic solvent containing picric acid is proposed to perform simultaneously the extraction of TMA-N from the headspace and the formation of its yellow complex. As it has been previously mentioned, picric acid and toluene are used in the Dyer Picrate method and AOAC method (AOAC, 2000; Dyer, 1945, chap. 35) to determine TMA-N. In this case, organic solvents must satisfy two preliminary requirements, namely, to dissolve picric acid and to display low blank values at the wavelength of measurement (410 nm). Toluene, xylene, N,N′-dimethylformamide, N-heptane or 1-octanol containing picric acid were tested. Toluene and xylene dissolved picric acid perfectly, and in turn, they yielded low blank values. Picric acid showed a very low solubility in n-heptane. The rest of solvents that are able to dissolve picric acid, i.e., 1-octanol and N,N′-dimethylformamide, showed very high blank values. Therefore, toluene and xylene were preselected as possible extractant phases.

The effect of picric acid concentration in the drop using both toluene and xylene as extractant solvents was studied in the range 0.005–0.030% mass/volume. As can be seen in Fig. 1, absorbance increased with increasing picric acid concentration up to *ca.* 0.015% mass/volume. From this value, no further improvements in absorbance were achieved. Moreover, worse blank values were observed when the picric acid content in the drop was increased from this value, so 0.015% mass/volume picric acid was used for all the subsequent extractions. This concentration is similar to that used in the AOAC Method.

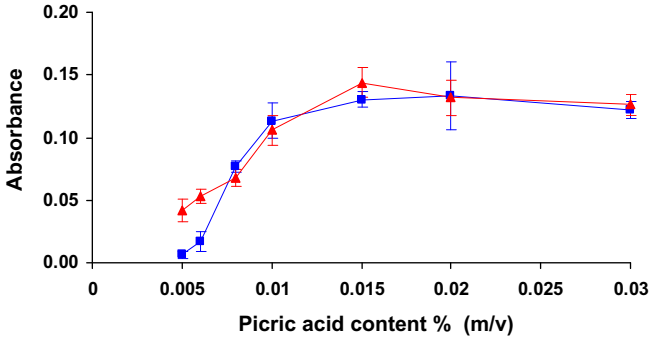

Fig. 1. Effect of the picric acid content in xylene (■) and toluene (▲) on the extraction of TMA-N.

Extractant phases employed in HS-SDME should have a high boiling point and low vapour pressure in order to reduce the risk of evaporation. Toluene and xylene show relatively high vapour pressure values at room temperature, which involves a limitation on the use of larger microextraction times. Nevertheless, given that vapour pressure is a function of temperature, a decrease in drop temperature would make the use of these solvents feasible for longer microextraction times. On the basis of these considerations, microextraction curves were performed by using 3 μL of the two-extractant solutions (i.e. xylene and toluene containing picric acid) stored in vials at room temperature or refrigerated into a water–ice bath before microextraction. The study was performed until a microextraction time corresponding to a final drop volume of 1.8 μL of xylene or toluene, since lower drop volumes could give rise to anomalous results when the liquid sample column is not completely formed during a measurement in the Nanodrop® spectrophotometer. Results are shown in Fig. 2. According to the curves, the extraction efficiency increases on increasing the microextraction time with both extractant phases. Moreover, overlapped microextraction curves were obtained when the extraction of TMA-N was carried out with organic microdrops at room temperature and refrigerated into a water–ice bath before the extraction. Xylene proved more appropriate than toluene to perform the extraction of TMA-N from the headspace, since its lower vapour pressure facilitates the use of longer microextraction times (up to 4 min), with the subsequent increase in the extraction efficiency of the analyte. Thus, on the basis of the results shown in Fig. 2, a picric acid-containing xylene drop was selected as extractant phase, while a short time of 3.5 min was selected to avoid events of drop evaporation.

The proposed methodology involves a drastic decrease in organic solvent volume employed (i.e., 3 μL) as well as hazardous residues generated in comparison with the classic method (AOAC 971.14), where a 10 mL volume of toluene is needed. Moreover, three consecutive steps are needed to carry out the determination by the classic method (extraction, dry with Na_2SO_4 and complex formation), whereas in the proposed method the extraction and complexation are performed simultaneously, and the dry step is not needed. Overall, this brings about an important time reduction on the determination of TMA-N in fish.

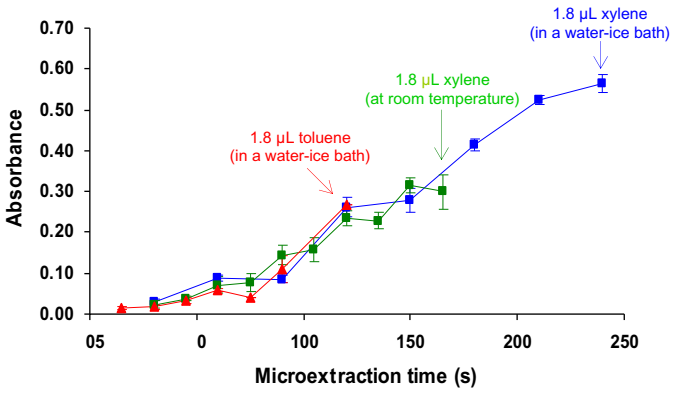

Fig. 2. Effect of the microextraction time on TMA-N absorbance by using xylene at room temperature (■) or refrigerated into a water–ice bath before the extraction (■) and toluene refrigerated into a water–ice bath before the extraction (▲). The study was performed up to a final drop volume of 1.8 μL of xylene or toluene.

3.1.2. NaOH concentration in the sample

Sodium hydroxide is used to convert the trimethylammonium present in the fish extract into volatile TMA-N. The effect of sodium hydroxide concentration on the signal obtained was studied in the range 0.01–5 M. Absorbance increased with NaOH concentration up to 2 M, after which no further increase in absorbance occurred. Thus, a NaOH concentration of 2 M was selected.

3.1.3. Sample volume

The effect of sample volume was studied in the range of 2–15 mL. A slight improvement in the analytical signal was observed in the studied range. This effect is attributed to the decreased headspace volume as the sample volume increases, which gives rise to an increased trimethylamine concentration in the headspace. A 10 mL sample volume was selected as a compromise between sensitivity and sample consumption.

3.1.4. Stirring rate

Sample stirring reduces the time required to reach the equilibrium between the sample solution and the headspace by enhancing the diffusion of the analytes towards the microdrop. Furthermore, convection is induced in the headspace by the stirring of the aqueous phase. The effect of the stirring rate was studied in the range of 100–1600 rpm. The analytical signal increased with increasing stirring rate up to 1100 rpm. Higher stirring rates caused a decrease in absorbance, probably because of spattering of the solution. Thus, 1100 rpm was selected as optimum stirring rate.

3.1.5. Addition of salt

Addition of salt to the solution increases the ionic strength and affects the solubility of analytes. The influence of ionic strength of the sample solution on the extraction efficiency was studied by addition of sodium chloride in the range of 0–18% mass/volume. The presence of salt enhanced extraction efficiency for TMA-N up to 15% mass/volume. Therefore, other measurements were carried out at a final NaCl concentration of 15% mass/volume.

3.2. Interference study

Primary and secondary amines, mainly ammonia, monomethylamine (MMA), and dimethylamine (DMA), are considered typical positive interferences on the determination of TMA-N in fish sample, since they can react with picric acid to form the corresponding yellow picrate. The interference effect of these volatile amines can be suppressed by addition of formaldehyde to the fish extract, which promotes the formation of non-volatile compounds without reacting with tertiary amines. Hence, the effect of ammonia, MMA and DMA was studied.

An interference effect was considered to be significant if a variation of more than ±5% in the measurement was observed. Addition of formaldehyde at a final content of 4% mass/volume was performed to minimise interferences owing to volatile amines on the determination of TMA-N in fish samples. Results showed that ammonia, MMA and DMA could be tolerated up to 100 mg/L, 10 mg/L and 5 mg/L when a final content of 4% mass/volume of formaldehyde was employed, which is the concentration of formaldehyde used in the AOAC Official Method to avoid these interferences (AOAC, 2000, chap. 35).

Unlike the AOAC Official Method, the proposed methodology is free from non-volatile interferences, given that the extraction process occurs in the headspace above the sample. Moreover, the matrix separation achieved with HS-SDME also makes the present method applicable to coloured samples such as fish sauces, while the AOAC Official Method overestimates the TMA-N content owing to the natural colour (Ruiz-Capillas et al., 2000).

3.3. Analytical figures of merit

Under the optimised conditions, limit of detection (LOD), repeatability and working range were determined. The equation for the linear range of the calibration curve of TMA-N was: Abs = 1885 [TMA-N] − 0.007, where the TMA-N concentration was expressed in mg/100 mL; the regression coefficient was $r = 0.9981$. The calibration curve was linear at least up to 3×10^{-4} mg/100 mL of TMA-N. The LOD, calculated as $3\sigma/m$ (σ being the standard

Table 1
Comparison of the proposed method with other developed methods to determine TMA-N in fish samples.

Analytical technique	LOD (mg/100 g)	LOD (mg/100 mL)	Linear range (mg/100 mL)	Estimated assay time (min)[a]	RSD (%)	Reference
LLE-UV–vis (AOAC Official Method 971.14)			0.8–4	20	2	Pons-Sánchez-Cascado, Izquierdo-Pulido, Mariné-Font, Veciana-Nogués, and Vidal-Carou (2000)
FI-pervaporation-UV–vis	8	0.16	0.2–3.0	7.5	4.27	García-Garrido and Luque de Castro (1997)
FI-gas-diffusion-UV–vis	0.053	8.4×10^{-3}	Up to 0.28	2	1.15	Sadok et al. (1996)
FI-gas-diffusion-potentiometry		5×10^{-3}	0.1–1.0	3	1.20	Adhoum et al. (2003)
VP-FT-IR-DTGS[b]		0.06	8.2–38.2	2	0.6	Armenta et al. (2006)
VP-FT-IR-MCT[c]		0.03	0.66–2.64	2	0.2	Armenta et al. (2006)
FI-TMA-N biosensor with FMO3[d]			1.4–70	3	4.39	Mitsubayashi et al. (2004)
CFS-CE-indirect UV (hydrodinamic injection)[e]		0.049	0.05–1.0	15	4	Lista et al. (2001)
CFS-CE-indirect UV (electrokinetic injection)[e]			2×10^{-3}–0.15	15	7	Lista et al. (2001)
CE-indirect UV		0.056	0.03–7	8		Timm and Jørgensen (2002)
LLE-GC-FID		0.038[g]	0.2–22	30	5.93	Veciana-Nogues et al. (1996)
HS-SPME-GC-FID[f]		2.589×10^{-3}	0.05–8	40	5.1	Li et al. (2004)
HS-SPME-GC–MS[f]		0.015	0.05–1	30	6.2	Chan et al. (2006)
HS-SDME-μ volume-UV–vis	6×10^{-4}	2.4×10^{-5}	5×10^{-5}–3×10^{-4}	4	5	This work

[a] Sample pre-treatment time is not included.
[b] VP-FT-IR-DTGS: Fourier transform-IR vapour phase with a temperature stabilised deuterated triglycine sulphate detector.
[c] VP-FT-IR-MCT: Fourier transform-IR vapour phase with a liquid nitrogen refrigerated mercury–cadmium–telluride (MCT) detector.
[d] FMO3: flavin-containing monooxygenase type 3.
[e] CFS: continuous flow system.
[f] HS-SPME: headspace-solid-phase microextraction.
[g] Determination limit.

deviation of 10 blank measurements and m the slope of the calibration line) was 2.4×10^{-5} mg/100 mL for TMA-N, i.e., 6×10^{-4} mg TMA-N per 100 g of fish. HS-SDME combined with microvolume UV–vis yielded an increase in sensitivity of at least two orders of magnitude compared to the AOAC Official Method and other alternative methods (Table 1).

The repeatability, expressed as relative standard deviation (RSD), was evaluated by extracting seven consecutive aqueous samples spiked at 10^{-4} mg/100 mL TMA-N level. The repeatability of the method was 5%.

Compared with other existing methods, the methodology proposed here is characterised by its high sensitivity and selectivity, being inexpensive, rapid and simple, with a negligible solvent consumption. Owing to the low LOD achieved, the present method is also proposed for a better knowledge of TMA-N levels in fish specimens from the first instant of spoilage, especially for flat and pelagic fishes, which are known to contain the lowest levels of precursor (TMA-O) (Huss, 1995).

3.4. Determination of TMA-N in fish samples

Recovery studies were made to evaluate the accuracy of the proposed methodology by adding known amounts of trimethylamine (at two different levels) to the fish extract. Three different frozen fish samples were employed. The corresponding results are shown in Table 2. Mean recoveries for TMA-N were between 97.1% and 103.2%, with a relative standard deviation (RSD) lower than 5% ($n = 6$). No significant differences occurred between the mean recovery found and the theoretical value of 100% since $t_{exp} < t_{crit}$ ($p = 0.05$).

The proposed method was also applied to the determination of TMA-N in the tissues of eight fresh fish samples purchased from a local fish market. The results obtained are summarised in Table 3. TMA-N content in the analyzed fish samples was less than 1 mg/100 g, the maximum allowance for fish graded as excellent (Castell, Geenough, Rodgers, & Macfarland, 1958), which indicates the quality of fish purchased on a fish market. These low levels of TMA-N were determined without problems in all cases with the proposed methodology.

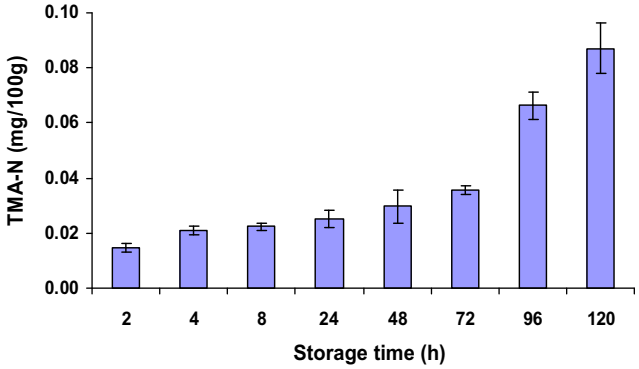

Fig. 3. TMA-N generated during turbot storage in flake ice.

Finally, farmed turbot (*P. maxima*) was selected to demonstrate the suitability of the proposed method on the monitoring of TMA-N evolution during the first hours after *post-mortem* storage of turbot specimens in flake ice. The evolution of TMA-N concentration in farmed turbot during a 120-h storage period in flake ice is shown in Fig. 3. Results were obtained with six replicate determinations. The generation of TMA-N was observed in turbot specimens even 2 h after been sacrificed (0.0147 ± 0.0017 mg TMA-N/100 g). An increase on the level of TMA-N was observed from this initial value up to a final value of 0.087 ± 0.009 mg TMA-N/100 g after 120 h of storage. The increase was more pronounced from a 48 h storage time.

4. Conclusions

HS-SDME has been employed along with microvolume UV–vis spectrophotometry for TMA-N determination in fish samples. A picric acid-containing xylene microdrop exposed to the headspace

Table 2
Recovery studies for the determination of TMA-N in different frozen fish samples by using the proposed method.

Sample	Added TMA-N (mg/100 g)	Found TMA-N (mg/100 g)[a]	Recovery (%)
Southern hake (*Merluccius australis*)	–	0.054 ± 0.003	–
	0.025	0.082 ± 0.003	103.2 ± 3.3
	0.050	0.102 ± 0.004	98.0 ± 4.2
Cod (*Gadus morhua*)	–	0.31 ± 0.03	–
	0.025	0.328 ± 0.014	97.1 ± 4.1
	0.050	0.369 ± 0.020	101.7 ± 5.4
Tuna (*Thunnus* spp)	–	0.319 ± 0.007	–
	0.025	0.344 ± 0.016	100.1 ± 4.8
	0.050	0.362 ± 0.007	98.1 ± 1.8

[a] Average value ± standard deviation ($N = 6$).

Table 3
Determination of TMA-N in different fish samples using the present method.

Sample	Environment	Average weight (g)	Average length (cm)	TMA-N (mg/100 g)[a]
Atlantic horse mackerel (*Trachurus trachurus*)	Pelagic	45	17	0.061 ± 0.005
European hake (*Merlucius merlucius*)	Demersal	505	41	0.84 ± 0.08
Gilthead seabream (*Sparus aurata*)	Demersal	470	27	0.38 ± 0.03
Mackerel (*Scomber scombrus*)	Pelagic	245	30	0.382 ± 0.012
Megrim (*Lepidorhombus whiffiagonis*)	Bathydemersal (flat)	100	24	0.150 ± 0.004
Pouting (*Trisopterus luscus*)	Benthopelagic	160	26	0.030 ± 0.004
Sardine (*Sardina pilchardus*)	Pelagic	105	19	0.401 ± 0.008
Sole (*Solea solea*)	Demersal (flat)	145	29	0.40 ± 0.03

[a] Average value ± standard deviation ($N = 6$).

of a closed vial was employed as extractant phase of TMA-N, involving a miniaturization of the AOAC Official Method, which gives rise to a considerable improvement of sensitivity and rapidity. The low limit of detection of the proposed method, together with a high freedom from interferences, allows the determination of TMA-N within a short time in different fish samples, including fish species that contain lower levels of precursor (TMA-O), from the first moment of storage.

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