PAPER

TRIMETHYLAMINE AS A FRESHNESS INDICATOR FOR SEAFOOD STORED IN ICE: ANALYSIS BY GC-FID OF FOUR SPECIES CAUGHT IN THE TYRRHENIAN SEA

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ABSTRACT

In seafood products, trimethylamine (TMA) is an indicator of the conservation status. It is almost absent in freshly caught samples, and its content increases during spoilage. In the present work, a new simple GC-FID method that uses a commercial capillary column, specifically designed, was applied. TMA was measured at increasing time intervals in four marine species caught in the Tyrrhenian Sea and stored in ice; 852 individuals were analyzed. An assessment of the maximum allowable time of storage in ice was made for each species. Existing guidelines for the level of trimethylamine are reviewed and discussed.

Keywords: Trimethylamine (TMA), seafood, freshness, shelf life, storage in ice, Gas Chromatography-Flame Ionization Detector (GC-FID)

1. INTRODUCTION

Fish, mollusks, crustaceans, and other marine species are among the most perishable food products (BOURIGUA *et al*., 2011; DIMOGIANOPOULOS and GRIGORAKIS, 2014; ŠTERNIŠA *et al*., 2016), and their spoilage leads to the formation of some substances that may cause intoxication when ingested (BIJI *et al*., 2016). Seafood spoilage leads also to the formation of low-molecular-weight volatile amines, which results in off-flavors. The main compound responsible for the typical smell of spoiled fish is trimethylamine (TMA). TMA is a good chemical marker of freshness (POPELKA *et al*., 2014): its concentration increases with spoilage by bacterial degradation of TMAO (trimethylamine N-oxide), an important osmoregulatory organic molecule that is commonly found in the muscle of marine fish (TREBERG and DRIEDZIC, 2002).
A bad conservation status of seafood represents a topic of safety concern. Ideally, seafood should be stored under conditions such that bacteria cannot grow at all: when preserved in ice, the product is safe only within a limited period. As such, an indicator of freshness may be very useful.
TMA is actually used as an indicator of seafood conservation status in ice (BOURIGUA *et al*., 2011; TONIOLO *et al*., 2014; BALIÑO-ZUAZO and BARRANCO, 2016). The various analytical techniques for the determination of TMA include colorimetric assays (PENA-PEREIRA *et al*., 2010), flow injection analyses (RUIZ-CAPILLAS and HORNER, 1999), biosensor analysis (BOURIGUA *et al*., 2011), capillary electrophoresis (TIMM and JØRGENSEN, 2002), and HPLC utilizing derivatization (BALIÑO-ZUAZO and BARRANCO, 2016). For gas chromatography, there are no simple instrumental methods available that allow a direct split/splitless injection into a capillary column. Packed columns were used for this purpose, especially in the past (VECIANA-NOGUES *et al*., 1996). Alternative methods use complex hyphenated techniques such as headspace-gas chromatography and solid phase microextraction-gas chromatography (KRZYMIEN and ELIAS, 1990; DEHAUT *et al*., 2016). In the present work, an innovative instrumental method was applied. Thanks to a capillary column specifically designed for the volatile amines and coated with a proprietary phase, simple instrumental analysis using gas chromatography with a flame ionization detector (GC-FID) was possible. This was done by injecting the liquid sample in split mode.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Trimethylamine hydrochloride (TMA·HCl) and *n*-propylamine hydrochloride (n-PA·HCl) were purchased from Sigma Aldrich® (St. Louis, MO, USA). Toluene, potassium hydroxide (KOH), and trichloroacetic acid (TCA) were from Carlo Erba Reagents® (Milan, Italy). Testmix CP0043 was from Varian® (Walnut Creek, CA, USA).
Standard amine solutions were prepared by dissolving TMA·HCl and n-PA·HCl in distilled water in order to obtain the desired concentrations expressed as free bases (TMA and n-PA).

2.2. Seafood samples

Red mullet (*Mullus barbatus*), European anchovy (*Engraulis encrasicolus*), and deep-water rose shrimp (*Parapenaeus longirostris*) were caught by professional fishermen during the months of June, July and September for the summer campaign and during the months of

February, March and April for the winter campaign. Atlantic mackerel (*Scomber scombrus*) was caught in the summer campaign only. For red mullet two different sizes were selected and analyzed in order to verify whether spoilage is influenced by this parameter. In some cases, the study had to take into account the amount of sample available at the moment. Fishing was done by trawling along the coast of the Tyrrhenian Sea, near Civitavecchia located in the region of Latium in Central Italy. The collected seafood samples were selected directly on board and separated by species and size, and then were placed in polystyrene boxes and covered with ice. The polystyrene boxes were stored in a refrigerated cell at 0-1°C until the day of the analyses that started on day 1 and continued on days 3, 6, 8, 10, and 13.
The size and number of the individuals collected are reported in Table 1.

Table 1. Size of the seafood species collected (minimum – maximum) and total number of individuals analysed.

	Weight (g)		Length (cm)		Number of individuals	
	Summer	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**
Red mullet, big size *(Mullus barbatus)*	52-276	23-99	16-27	13-20	17	42
Red mullet, small size *(Mullus barbatus)*	19-61	7-33	12-18	9-15	48	173
European anchovy *(Engraulis encrasicolus)*	10-18	9-23	12-14	12-16	120	138
Atlantic mackerel *(Scomber scombrus)*	51-135		19-26		25	
Deep-water rose shrimp *(Parapenaeus longirostris)*	8-25	6-24	10-16	10-14	109	180

2.3. Sample preparation

2.3.1 Filleting and homogenization

On the day of the analysis, a minimum of 2-4 and a maximum of 10-30 individuals of each species, based on the number available, were pooled. This was followed by gutting, skinning, and filleting. Subsequently, homogenization was carried out for 30 seconds at a low speed with a Waring blender (model 8010E, Waring® Products Division, New Hartford, CT, USA) and by using a previously cooled stainless-steel cup. The resulting homogenate was ready for TMA extraction.

2.3.2 TMA extraction

Analyses were performed in duplicate. Sample preparation followed the methods of PEREZ MARTIN *et al*. (1987) and VECIANA-NOGUES *et al*. (1996) with minor modifications.
Approximately 10 g of homogenized product was weighed into 250 mL plastic bottles. To the weighed sample, 40 mL of TCA 6% (w/w) solution was added, and then a second homogenization was carried out for 1 min at 11000 rpm with an Ultra Turrax homogenizer (Model T25B, IKA®, Staufen, Germany); the plastic bottle was kept immersed in ice to prevent any heating.

Subsequently, centrifugation was carried out for 10 min at 4°C (12000 rpm, 22214*g*) using a Beckman Coulter Avanti™ J25 ultracentrifuge. The supernatant was collected in a 100 mL volumetric flask by using a funnel with an inserted Whatman N° 2 filter paper 15 cm in diameter. The filter, residue pulp, and plastic bottle were washed with distilled water to ensure that all of the TMA extracted was quantitatively collected. Finally, the solution was brought to volume with distilled water. The solution (TCA extract) was transferred into 10 mL tubes and kept at -30°C until the day of gas chromatographic analysis.

2.3.3 Sample preparation for GC injection

On the day of the gas chromatographic analysis, the TCA extract from the previous step was left to thaw at room temperature. To 7 mL of the TCA extract in a glass tube, the internal standard (IS) n-PA·HCl was added. The added amount corresponded to a concentration of 19.04 mg/L of n-PA as free base. Subsequently, 2 mL of toluene and 10 mL of KOH 65% (w/v) solution were added, and the tube was shaken at 1500 rpm for 1 min by means of a vortex mixer (Heidolph®, Schwabach, Germany). One microliter of the toluenic upper layer was injected in GC-FID.

2.4. Instrumental analysis

The apparatus used was a 6890 Agilent gas chromatograph with a flame ionization detector (GC-FID), equipped with a CP-Volamine fused silica capillary column (60 m × 0.32mm I.D., 0.45 mm O.D., 5 μm film thickness, from Varian®). Helium as the carrier gas was used in constant flow mode at 0.9 mL/min.
The operating conditions were as follows. The initial oven temperature was 45°C, which was held for 10 min and then increased to 250°C at a rate of 20°C/min. This final temperature was maintained for 10 min. The FID temperature was 300°C, while the injector temperature was 270°C. Injections were made in split mode (5:1) with an injection volume of 1 μL. Fig. 1 shows two gas chromatograms for a shrimp sample and a standard solution of TMA.

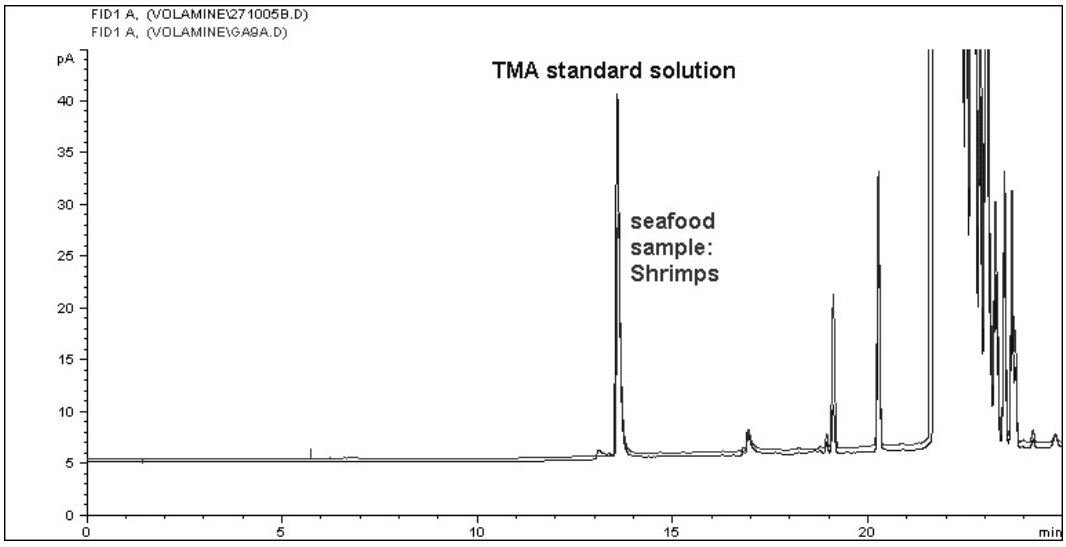

Figure 1. GC-FID chromatograms, peak of TMA. Standard solution of TMA and a shrimp sample. Chromatograms are overlaid.

2.5. Analytical quality control

Instrumental performance was investigated by using the Testmix CP0043 provided by Varian®. The composition of the Testmix was 0.1% of each component, including TMA, in isopropanol.
For the quantitative analysis of TMA, an appropriate calibration curve was constructed (Fig. 2).

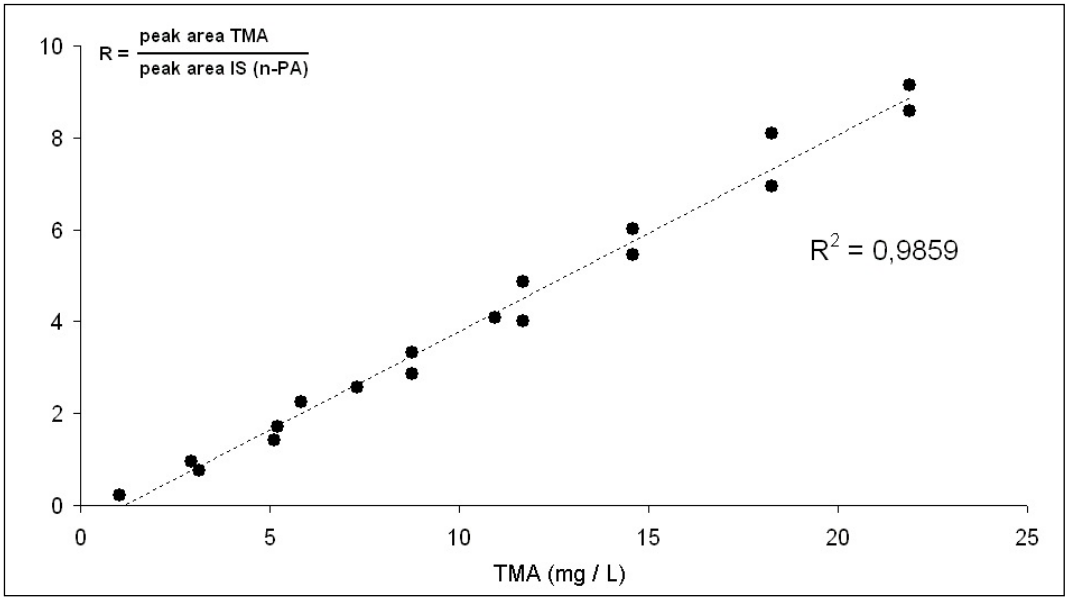

Figure 2. Calibration curve used to quantify TMA in the seafood samples.

Solutions of known composition for the points of the calibration curve were prepared in TCA by using pure TMA·HCl. Pure n-PA·HCl was used as IS. The real samples that were observed to have very high levels of TMA were again prepared and appropriately diluted before the GC injection to fall within the linear dynamic range of the calibration curve.
The limit of quantitation (LOQ) is the amount of TMA in the seafood sample that is easily quantifiable because of the good signal-to-noise ratio of the final chromatographic peak. The limit of detection (LOD) is the amount of TMA with a signal-to-noise ratio that is sufficient only for the detection of its presence without the possibility of a reliable integration. For the present method, we measured a LOQ of 3 mg of TMA per kilogram of seafood sample and a LOD of 2 mg/kg (corresponding to a LOQ of 0.07 mg N/100 g and to a LOD of 0.05 mg N/100 g when TMA is expressed in mg N/100 g of sample). Blanks and recovery measurements were carried out to ensure that no false positives nor false negatives were produced.
For the recoveries, the TCA extract (section 2.3.2) coming from a red mullet sample was spiked with TMA at three different concentration levels, as reported in Table 2. Recovery was 90.2±8.0%; this is similar to that reported by other researchers (PEREZ-MARTIN *et al*., 1987).

Table 2. TMA recovery measurements.

Sample		Measured concentration of TMA in the native TCA extract (mg/L)	Expected concentration of TMA in the spiked TCA extract (mg/L)	Measured concentration of TMA in the spiked TCA extract (mg/L)	Recovery (%)
Red mullet, big size winter campaign (T2)	No adding	2.11			
	Level 1, adding		3.20	2.70	84.4
	Level 2, adding		4.29	4.26	99.3
	Level 3, adding		5.37	4.66	86.8

After each addition of TMA, the extract was normally processed and injected in GC-FID.

3. RESULTS AND DISCUSSION

3.1. Guidelines for TMA concentration

TMA is considered a good indicator of fish spoilage when the product is preserved in ice; however, there is no regulation for TMA levels (MACÉ *et al*., 2012). The European Community Regulation cites that when the organoleptic examination reveals any doubt as to the freshness of the fishery products, samples may be taken and subjected to laboratory tests to determine the levels of TMA (Reg. CE No 854/2004); surprisingly, the Regulation itself does not provide any tolerance or reference value. There are, however, generally accepted values or values recommended by international organizations.
TMA is sometimes reported in milligrams of TMA per kilogram of fish; in other cases, it is reported in milligrams of TMA-N (milligrams of Nitrogen) per 100 g of fish, a situation that can be potentially confusing. For clarification, we report the equation for converting TMA into TMA-N and vice versa:

$$TMA\,(mg/kg) \times 0.0237288 \;=\; TMA-N\,(mg/100g) \qquad (1)$$

The Food and Agriculture Organization of the United Nations (FAO, 1988) reports that good-quality cold-water fish contains less than 63 mg/kg of TMA.
EL MARRAKCHI *et al*. (1990) investigated the freshness of marine fish (*Sardina pilchardus*) both by TMA determination and by sensory evaluations by two official veterinary inspectors. In their study the product was judged as fresh up to a TMA level of 50 mg/kg (1.19 mg/100g of TMA-N). This is in good agreement with what has been reported by FAO, and is the same as the maximum reference TMA content cited by the Italian Istituto Zooprofilattico Sperimentale (IZSUM, Ministry of Health) for the freshness of marine fish (HAOUET, 2001). VECIANA-NOGUES *et al*. (1996) reported that hake can be graded as excellent quality when its TMA level is lower than 42 mg/kg (1 mg/ 100 g of TMA-N).
We see that recommendations by national and international organizations, as well as experimental works of researchers, agree that the range 42-63 mg/kg of TMA (1-1.5 mg/100g of TMA-N) in marine fish is the limit below which the freshness status is at an optimum.

3.2. Deep-water rose shrimp

As Table 3 shows, shrimps exhibited a TMA content suggestive of a bad freshness state after three days of storage in ice (151.7 mg/kg in the summer campaign). We must emphasize that on day 3 the colour of the shrimps had in fact turned black from the original pink. It can be concluded that the shelf life of deep-water rose shrimp in ice is very limited when the product does not undergo to any conservative treatment, as is generally done (ZHANG *et al*., 2015). Another work (LAGHMARI and EL MARRAKCHI, 2005) confirms the short shelf life (3-5 days) of *P. longirostris* stored in ice.
As it can be seen for shrimps in Table 3 after many days of storage in ice the TMA content in some marine species could also stabilize or even decrease: this is a known phenomenon because the content of the precursor TMAO runs out, but that does not mean, of course, an improvement in the conservation status.

3.3. Red mullet

Red mullet (*M. barbatus*) stored in ice showed a status of good freshness until 8 days, when the TMA level remained constantly below 65 mg/kg for both sizes and campaigns studied (Table 3). It appears that even over 8 days the product in some cases is in an acceptable status of conservation. At long storage times in ice (i.e. 8 days and 10 days) the bigger size tends to release a lower quantity of TMA than the smaller size. This means that the larger size is more resistant to degradation than is the smaller one. It was already observed in other fish species a better resistance to degradation for bigger sizes (ORBAN *et al*., 2011).
A similar shelf life was obtained in a study in which sensory and microbiological analyses were carried out on red mullet (ÖZYURT *et al*., 2009).

3.4. Atlantic mackerel

Atlantic mackerel (*S. scombrus*) was caught in the summer campaign only. *S. scombrus* is a species of great commercial importance that is mainly distributed as a canned product. A bad conservation process of the product can lead to high levels of histamine in mackerel during storage, which may cause health problems for consumers (scombroid fish poisoning). This is due to the relatively high content of free histidine in this species, which during bad storage is converted to histamine (BENNOUR *et al*., 1991). Therefore an indicator of freshness is extremely useful.
From Table 3 it can be deduced that at 8 days in ice, the rejection status was reached being 170.2 mg/kg the TMA content; up to six days, the product can be considered in good state. A similar shelf life for mackerel stored in ice has been reported (BENNOUR *et al*., 1991). In the study by BENNOUR, the histamine concentration was also measured. It was concluded that even when mackerel is allowed to spoil in ice until it becomes unfit to eat (over eight days), the level of histamine does not rise much above 5 mg/100 g of flesh, the level established by the United States Food and Drug Administration as a guidance value (FDA, 2011).

3.5. European Anchovy

From the TMA content in Table 3 we can be deduce that the maximum allowable time of storage in ice for anchovies is 5-6 days in summer. In fact, the TMA concentration at day 6 is 68.1 mg/kg in the summer campaign.
This result is perfectly in agreement with a study on *E. encrasicolus* collected in the Mediterranean area during summer (PONS-SÁNCHEZ-CASCADO *et al*., 2006). Such

work performed microbiological and sensory assays on anchovies stored in ice. A team of eight panel members trained in fish freshness assessment developed the schemes proposed for raw anchovies, and the final judgement was that the limit of acceptability for anchovies is reached after 5 days of storage in ice. The mean size of the individuals was practically the same in the work of PONS-SÁNCHEZ-CASCADO *et al*. and in the present one.
The winter campaign seems to indicate a possible slightly longer time of storage in ice.

Table 3. Concentrations of TMA measured for different seafood species at different days of storage in ice.

Days of storage in ice	Species	TMA (mg/kg)		TMA-N (mg/100g)	
		Summer	Winter	Summer	Winter
1 day (T0)	Red mullet, big size	n.d.	n.d.	n.d.	n.d.
	Red mullet, small size	5.4±0.2	< 3	0.13±0.00	< 0.07
	European anchovy	28.8±2.6	16.2±0.3	0.68±0.06	0.38±0.01
	Atlantic mackerel	18.2±0.6		0.43±0.01	
	Deep-water rose shrimp	40.3±0.3	18.3±15.8	0.96±0.01	0.43±0.37
3 days (T1)	Red mullet, big size	11.2±0.2	< 3	0.27±0.00	< 0.07
	Red mullet, small size	7.8±0.1	5.2±0.4	0.18±0.00	0.12±0.01
	European anchovy	32.6±0.1	21.7±0.1	0.77±0.00	0.51±0.00
	Atlantic mackerel	48.1±1.6		1.14±0.04	
	Deep-water rose shrimp	151.7±2.3	76.8±4.8	3.60±0.06	1.82±0.11
6 days (T2)	Red mullet, big size	24.9±0.2	18.3±0.0	0.59±0.00	0.43±0.00
	Red mullet, small size	23.6±0.1	20.0±0.1	0.56±0.00	0.47±0.00
	European anchovy	68.1±0.7	49.6±0.0	1.62±0.02	1.18±0.00
	Atlantic mackerel	80.2±0.0		1.90±0.00	
	Deep-water rose shrimp	283.4±11.1	623.9±98.9	6.72±0.26	14.81±2.35
8 days (T3)	Red mullet, big size	33.8±1.8	24.5±6.8	0.80±0.04	0.58±0.16
	Red mullet, small size	46.6±0.0	64.8±1.1	1.10±0.00	1.54±0.03
	European anchovy	153.4±0.5	57.0±8.4	3.64±0.01	1.35±0.20
	Atlantic mackerel	170.2±0.3		4.04±0.01	
	Deep-water rose shrimp	548.4±8.6	1041.0±12.6	13.01±0.20	24.70±0.30
10 days (T4)	Red mullet, big size		98.4±0.8		2.33±0.02
	Red mullet, small size	46.3±0.1	142.5±14.0	1.10±0.00	3.38±0.33
	European anchovy	192.9±0.4	71.6±1.7	4.58±0.01	1.70±0.04
	Atlantic mackerel	187.3±0.6		4.44±0.02	
	Deep-water rose shrimp	638.6±1.1	679.2±40.3	15.15±0.03	16.12±0.96
13 days (T5)	Atlantic mackerel	261.6±2.2		6.21±0.05	
	Deep-water rose shrimp	1229.1±257.7	1089.0±87.2	29.16±6.11	25.84±2.07

Results are reported as mean±standard deviation (n = 2) and are expressed both in mg/kg of TMA and in mg/100g of TMA-N (see equation 1, section 3.1).
n.d. = not detected (below the LOD)

3.6. Comparison among species

Figures 3 and 4 plot the TMA content measured in all species as a function of the days of storage in ice. It is evident that shrimps constantly released a much higher amount of TMA when compared with fish species: this resulted in a much lower shelf life.

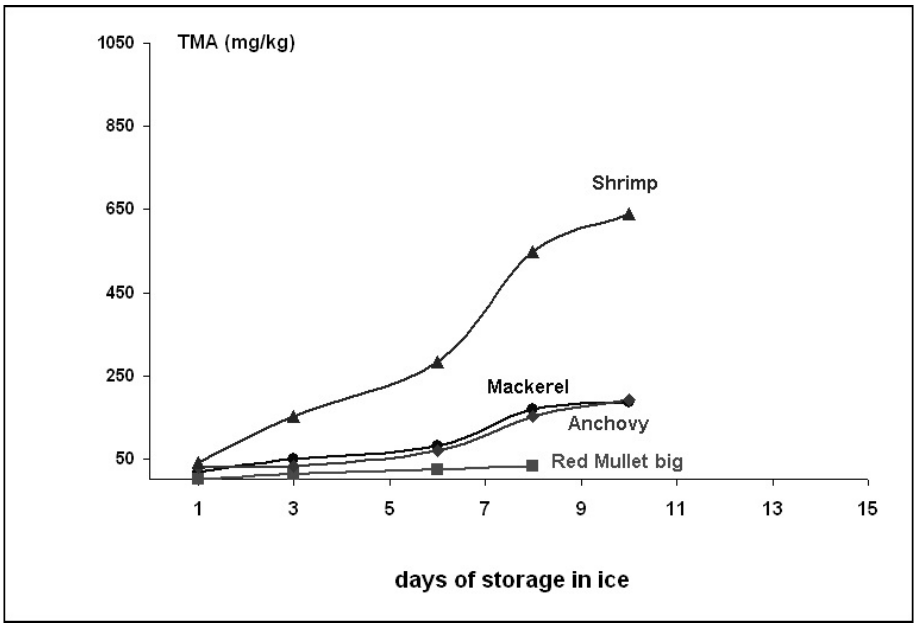

Figure 3. TMA content of the different species as a function of the days of storage in ice (summer campaign).

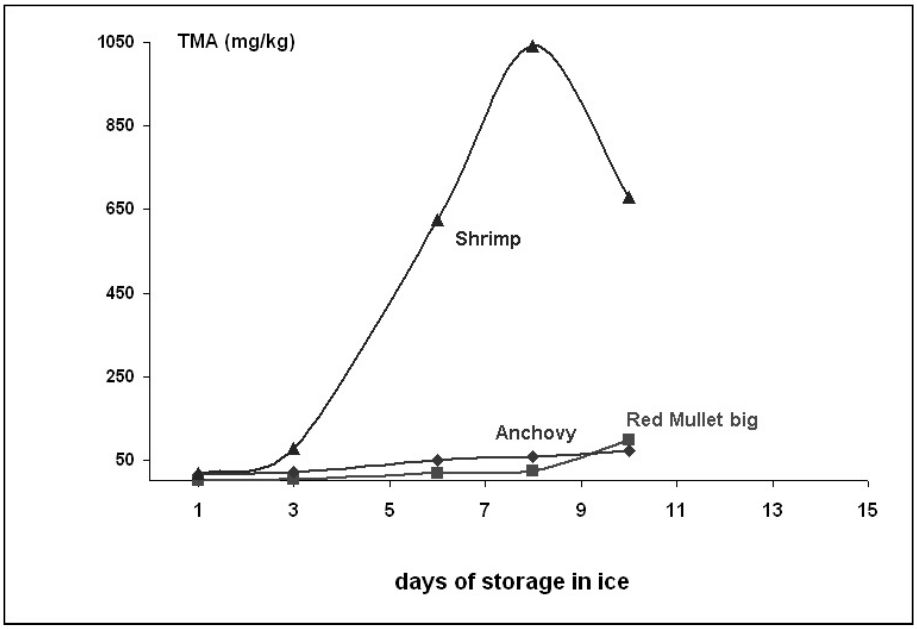

Figure 4. TMA content of the different species as a function of the days of storage in ice (winter campaign).

3.7. Method validation

In order to assess the reliability of the method presented here, a very different approach for evaluating the freshness status was applied. The species investigated for the TMA content (red mullet big size, red mullet small size, European anchovy, Atlantic mackerel) were also analyzed for their total volatile basic nitrogen (TVB-N) content, a widely accepted spoilage indicator that was established by the European Community (Comm. Reg. EC No 2074/2005). Measurements were carried out according to the EC official method (Decision 95/149/EC). They involved acid extraction followed by alkalinization, distillation, and titration.
Comm. Reg. EC No 2074/2005 stipulates that fish is unfit for human consumption when the TVB-N content is above 25-35 mg/100 g.
On the other hand, the value 1.5 mg/100g of TMA-N is the limit above which the fish begins to lose the optimum state of freshness (section 3.1).
We can easily see in Table 4 that the two indicators are in perfect agreement.

Table 4. Maximum allowable time of storage in ice as indicated both by TVB-N and TMA-N content (mg/100g) for the summer campaign.

		Day 1	Day 3	Day 6	Day 8	Day 10
TVB-N	Red mullet, big size	13.66±0.29	15.50±0.15	15.78±0.17	17.44±0.27	not analyzed
	Red mullet, small size	12.11±0.52	14.86±0.04	16.40±1.22	18.74±0.17	19.83±0.56
	European anchovy	14.49±0.54	16.80±0.08	23.04±0.81	30.43±0.14	37.97±0.97
	Atlantic mackerel	19.30±0.27	26.54±0.23	25.72±1.24	35.81[a]	57.94±1.04
TMA-N	Red mullet, big size	n.d.	0.27±0.00	0.59±0.00	0.80±0.04	not analyzed
	Red mullet, small size	0.13±0.00	0.18±0.00	0.56±0.00	1.10±0.00	1.10±0.00
	European anchovy	0.68±0.06	0.77±0.00	1.62±0.02	3.64±0.01	4.58±0.01
	Atlantic mackerel	0.43±0.01	1.14±0.04	1.90±0.00	4.04±0.01	4.44±0.02

[a]single measure
Results are reported as mean±standard deviation (n = 2). n.d. = not detected
Analyses of TVB-N were performed on another aliquot of the same homogenate that was processed for TMA

From the TVB-N content we can conclude that red mullet is in a good conservation status up to 8 days (10 days for the small size), since the concentration remains always below 20 mg/100 g. An identical situation is indicated by TMA, which never exceeded 1.10 mg/100 g in red mullets.
For European anchovy and Atlantic mackerel, the TVB-N level is acceptable up to day 6 (≤25 mg/100 g), and it begins to exceed 30-35 mg/100 g on day 8, when degradation starts.
The same is valid for the TMA content, which shows that on day 8 degradation is starting (3.64 and 4.04 mg/100 g).
From the above it may be concluded that TMA, as measured in the present study using the developed GC-FID method, is a reliable freshness indicator.

4. CONCLUSIONS

A quick and easy gas chromatographic method for the analysis of TMA in seafood was developed and validated. A capillary column and a classical split injection were used so greatly simplifying the procedure. It was applied to different seafood species caught in the Tyrrhenian Sea that had been stored in ice. Thanks to its simplicity, the method appears very suitable for routine controls.
By comparison with fish species, crustaceans such as shrimps exhibited a much greater release of TMA, which resulted in a much shorter shelf life (1-2 days).
Fish species maintained a good freshness state for almost a week, up to 8-10 days for red mullet, with the bigger sizes being more resistant to degradation than the smaller ones.
In the present study, the existing guidelines for the TMA content are reviewed and discussed. Not always there is full clarity on this topic in the literature. The generally accepted criterion for an "optimum freshness state" is a TMA content below 42-63 mg/kg (1.0-1.5 mg/100g of TMA-N). Observations made in the present research fully confirm this limit, at least for the marine species here investigated.

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REFERENCES

Bennour M., El Marrakchi A., Bouchritf N., Hamama A. and El Ouadaa M. 1991. Chemical and Microbiological Assessments of Mackerel (*Scomber scombrus*) Stored in Ice. J. Food Protect. 54:784. https://doi.org/10.4315/0362-028X-54.10.784

Biji K.B., Ravishankar C.N., Venkateswarlu R., Mohan C.O. and Srinivasa Gopal T.K. 2016. Biogenic amines in seafood: a review. J. Food Sci. Technol. 53:2210. https://link.springer.com/article/10.1007%2Fs13197-016-2224-x

Bourigua S., El Ichi S., Korri-Youssoufi H., Maaref A., Dzyadevych S. and Renault N.J. 2011. Electrochemical sensing of trimethylamine based on polypyrrole–flavin-containing monooxygenase (FMO3) and ferrocene as redox probe for evaluation of fish freshness. Biosens. Bioelectron. 28:105. https://doi.org/10.1016/j.bios.2011.07.005

Dehaut A., Duthen S., Grard T., Krzewinski F., N'Guessan A., Brisabois A. and Duflos G. 2016. Development of an SPME-GC-MS method for the specific quantification of dimethylamine and trimethylamine: use of a new ratio for the freshness monitoring of cod fillets. J. Sci. Food Agr. 96:3787.
http://onlinelibrary.wiley.com/doi/10.1002/jsfa.7570/abstract;jsessionid=2FB7B19E3285D71DFCD4FEED0A540691.f02t01

Decision 95/149/EC fixing the total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products and specifying the analysis methods to be used. Official Journal of the European Communities L 97/84

Dimogianopoulos D. and Grigorakis K. 2014. Nondestructive textural assessment of fish freshness: a stochastic model-based approach robust to fish size variations. J. Texture Stud. 45:274.
http://onlinelibrary.wiley.com/doi/10.1111/jtxs.12072/abstract

El Marrakchi A., Bennour M., Bouchriti N., Hamama A. and Tagafait H. 1990. Sensory, chemical, and microbiological assessments of moroccan sardines (*Sardina pilchardus*) stored in ice. J. Food Prot. 53:600. https://doi.org/10.4315/0362-028X-53.7.600

FAO, (1988). Food and Agriculture Organization of the United Nations, Fisheries Series No. 29. Huss H.H., Fresh fish quality and quality changes.

FDA, 2011. Food And Drug Administration (USA). Fish and Fishery Products Hazards and Controls Guidance, Fourth Edition - April 2011. http://www.fda.gov/FoodGuidances. Accessed July 2017.

Haouet, M.N. (2001). Conservazione ed alterazioni chimiche dei prodotti ittici. Webzine Sanità Pubblica Veterinaria, Numero 8-9. Ottobre 2001. Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche. http://www.spvet.it/arretrati/numero_8_9/ittici.html#2. Accessed July 2017.

Krzymien M.E. and Elias L. 1990. Feasibility Study on the Determination of Fish Freshness by Trimethylamine Headspace Analysis. J. Food Sci. 55:1228. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.1990.tb03903.x/abstract

Laghmari H. and EL Marrakchi A. 2005. Appréciation organoleptique et physico-chimique de la crevette rose *Parapenaeus longirostris* (Lucas, 1846) conservée sous glace et à température ambiante. Revue de Méd. Vét. 156:221.

Macé S., Cornet J., Chevalier F., Cardinal M., Pilet M.-F., Dousset X. and Joffraud J.-J. 2012. Characterisation of the spoilage microbiota in raw salmon (*Salmo salar*) steaks stored under vacuum or modified atmosphere packaging combining conventional methods and PCR-TTGE. Food Microbiol. 30:164. https://doi.org/10.1016/j.fm.2011.10.013

Orban E., Nevigato T., Di Lena G., Masci M., Casini I., Caproni R., Rampacci M. 2011. Total volatile basic nitrogen and trimethylamine nitrogen levels during ice storage of European hake (*Merluccius merluccius*): A seasonal and size differentiation. Food Chem. 128:679. https://doi.org/10.1016/j.foodchem.2011.03.086

Özyurt G., Kuley E., Özkütük S. and Özogul F. 2009. Sensory, microbiological and chemical assessment of the freshness of red mullet (*Mullus barbatus*) and goldband goatfish (*Upeneus moluccensis*) during storage in ice. Food Chem. 114:505. https://doi.org/10.1016/j.foodchem.2008.09.078

Pena-Pereira F., Lavilla I. and Bendicho C. 2010. Colorimetric assay for determination of trimethylamine-nitrogen (TMA-N) in fish by combining headspace-single-drop microextraction and microvolume UV–vis spectrophotometry. Food Chem. 119:402. https://doi.org/10.1016/j.foodchem.2009.07.038

Perez Martin R.I., Franco J.M., Molist P. and Gallardo J.M. 1987. Gas chromatographic method for the determination of volatile amines in seafood. Int. J. Food Sci. Tech. 22:509. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.1987.tb00516.x/abstract

Pons-Sánchez-Cascado S., Vidal-Carou M.C., Nunes M.L. and Veciana-Nogués M.T. 2006. Sensory analysis to assess the freshness of Mediterranean anchovies (*Engraulis encrasicholus*) stored in ice. Food Control 17:564. https://doi.org/10.1016/j.foodcont.2005.02.016

Popelka P., Nagy J., Pipová M., Marcinčák S. and Lenhardt L. 2014. Comparison of chemical, microbiological and histological changes in fresh, frozen and double frozen rainbow trout (*Oncorhynchus mykiss*). Acta Vet. Brno, 83:157. https://doi.org/10.2754/avb201483020157

Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union L 155/206

Regulation (EC) No 2074/2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. Official Journal of the European Union L 338/27

Ruiz-Capillas C. and Horner W.F.A. 1999. Determination of trimethylamine nitrogen and total volatile basic nitrogen in fresh fish by flow injection analysis. J. Sci. Food Agr. 79:1982. http://onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-0010(199911)79:14%3C1982::AID-JSFA459%3E3.0.CO;2-G/abstract

Sterniša M., Mraz J. and Možina S.S. 2016. Microbiological aspects of common carp (*Cyprinus carpio*) and its processing-relevance for final product quality: a review. Aquacult. Int. 24:1569.
https://link.springer.com/article/10.1007%2Fs10499-016-0051-8

Timm M. and Jørgensen B.M. 2002. Simultaneous determination of ammonia, dimethylamine, trimethylamine and trimethylamine-N-oxide in fish extracts by capillary electrophoresis with indirect UV-detection. Food Chem. 76:509. https://doi.org/10.1016/S0308-8146(01)00289-8

Toniolo R., Dossi N., Svigely R., Susmel S., Casella I.G. and Bontempelli G. 2014. Amperometric Sniffer for Volatile Amines Based on Paper-Supported Room Temperature Ionic Liquids Enabling Rapid Assessment of Fish Spoilage. Electroanal. 26:1966. http://onlinelibrary.wiley.com/doi/10.1002/elan.201400276/abstract

Treberg J.R. and Driedzic W.R. 2002. Elevated Levels of Trimethylamine Oxide in Deep-Sea Fish: Evidence for Synthesis and Intertissue Physiological Importance. J. Exp. Zool. 293:39. http://onlinelibrary.wiley.com/doi/10.1002/jez.10109/abstract

Veciana-Nogues M.T., Albala-Hurtado M.S., Izquierdo-Pulido M. and Vidal-Carou M.C. 1996. Validation of a gas-chromatographic method for volatile amine determination in fish samples. Food Chem. 57:569. https://doi.org/10.1016/S0308-8146(96)00031-3

Zhang B., Ma L.-k., Deng S.-g., Xie C. and Qiu X.-h. 2015. Shelf-life of pacific white shrimp (*Litopenaeus vannamei*) as affected by weakly acidic electrolyzed water ice-glazing and modified atmosphere packaging. Food Control 51:114. https://doi.org/10.1016/j.foodcont.2014.11.016

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