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Research Article

Validation of a ^{1}H-NMR Spectroscopy Quantitative Method to Quantify Trimethylamine Content and *K*-Index Value in Different Species of Fish

Alessandra Ciampa (ID)**, Luca Laghi** (ID)**, and Gianfranco Picone** (ID)

Dipartimento di Ingegneria Civile, Ambientale, del Territorio, Edile e di Chimica (DICATECh), Polytechnic University of Bari, via Orabona 4, Bari I-70125, Italy

Correspondence should be addressed to Gianfranco Picone; gianfranco.picone@unibo.it

The chemical analysis that is frequently employed for the evaluation of the freshness of fish includes (i) the quantification of trimethylamine (TMA) and (ii) the estimation of the *K*-Index, based on the ratio between the concentrations of adenosine triphosphate (ATP) and its breakdown products. TMA is quantified using a colorimetric reference method (AOAC), while the *K*-Index is usually determined by HPLC. The present work proposes a method for the above freshness biomarkers based on HR ^{1}H-NMR as an alternative method able to assess both indexes simultaneously on aqueous fish extracts. To validate the proposed ^{1}H-NMR method, a large set of validation checks has been addressed, such as accuracy, precision, specificity, limits of detection, linearity, and range of linearity and quantification according to EuroChem guidelines. The results show that the methodology satisfies all the validation requirements at the same level as the most frequently used methods, with the advantage of being faster and more repeatable, avoiding the use of solvents, such as toluene and formaldehyde, or dangerous reagents such as picric acid.

1. Introduction

Compared to most food products, seafood can be considered one of the most perishable products, with a short shelf life due to its biological origin, which is a mixture mainly composed of proteins, lipids, and carbohydrates dispersed in water. Besides these main classes of compounds, there are other minor components, some of which are involved in the spoilage process, and they comprise primarily free amino acids and volatile nitrogenous bases such as trimethylamine oxide (TMAO). Fish freshness, intended as the absence of negative characteristics due to enzymatic or bacterial activity, can be therefore considered the main quality parameter to be considered during commercialization. European Community legislation bases the assessment of fish freshness on methods combining sensory evaluations such as the color and smell of the gills or the characteristics of the skin. Those methods, the most known of which goes

under the name of the Quality Index Method or QIM [1], are considered to be fast, simple, and provide immediate quality information [2]. Unfortunately, all these methods depend on panels, which are inherently characterized by subjective responses and by the need for extensive and expensive training. These drawbacks have brought research into the development of nonsensorial methods, most of which are based on biochemical analysis. European regulation 854/2004 provided that, in cases of uncertainty in the determination of freshness by organoleptic investigations, trimethylamine (TMA) should be quantified because fishes accumulate trimethylamine oxide (TMAO) as osmoregulator, reduced to TMA during storage by the microorganisms' action [3]. Indeed, the concentration of TMA in fish flesh has been found to be closely related to organoleptic estimations [4], particularly in wild fishes, characterized by high levels of TMAO [5–7]. For farmed fishes, which accumulate lower levels of TMAO [8], the TMA quantification

as a freshness indicator performs more poorly, especially in the first days after catching, when the growth of the microorganisms is still in its lag phase. Generally, in fresh fish, the TMA quantity is in the 1–5 mg/100 g range [9]. An alternative to TMA quantification is offered by the *K*-Index, indicative of enzymatic autolytic mechanisms already active immediately after catching, as it is based on the catabolic activities which degrade adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and inosine 5′-monophosphate (IMP) to inosine (HxR) and hypoxanthine (Hx). *K*-Index [10] is defined as follows:

$$K - \text{Index} (\%) = \frac{\{[\text{HxR}] + [\text{Hx}]\}}{\{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{HxR}] + [\text{Hx}]\}} * 100. \tag{1}$$

K-Index as a freshness indicator was indeed successfully applied on farmed European sea bass (*Dicentrarchus labrax*) [11], on cultured gilthead sea breams (*Sparus aurata*) [12], and on a wide number of other fish species [8, 13, 14]. A *K*-Index of 20–30% has been set as the limit for consumption of raw fish, while a *K*-Index of 70–80% has been suggested as a limit for an Atlantic salmon of good quality [8].

For the determination of TMA in fish samples, several methods are described in the literature, requiring liquid [15] and gas chromatography [16], as well as other techniques like electronic nose [17, 18]. The AOAC official method [19, 20] requires a liquid-liquid extraction of TMA with toluene, followed by its reaction with picric acid to form a yellow complex that can be quantified from its absorbance at 410 nm. This is a cumbersome method, subject to multiple errors because involving several time-consuming steps [21].

The methods used for the quantification of ATP and its catabolic products for the evaluation of the *K*-Index are at present based only on high-performance liquid chromatography (HPLC). The one that can be taken as a reference because most often applied has been described by Ryder [22, 23], characterized by the use of a reverse-phase C18 column.

To identify a single technique able to quantify both TMA and the ATP catabolites, giving in this way a complete view of the fish freshness, Nuclear Magnetic Resonance (NMR) spectroscopy seems to be the appropriate candidate. Using a single spectroscopic measure, several compounds from a complex mixture can be observed and identified simultaneously [24]. Moreover, the latest improvements in probe design and electronic performance make the NMR technique stand out from a quantitative point of view, as described by the works of Maniara et al. [25] and Wells et al. [26]. In these works, it is accepted that the level of the major chemical ingredients of a sample can be determined with accuracy and precision of 0.5%, and impurities with a concentration of 0.1% or lower can be quantified. A previous paper by Heude et al. [27] reported on a rapid analytical method based on ^{1}H high-resolution magic angle spinning (HR-MAS) NMR spectroscopy for the rapid determination of the *K*-Index value and the trimethylamine nitrogen (TMA-N) content. However, the validation of a method based on NMR spectroscopy from an analytical point of view is still missing, as repeatability, recovery, linearity, and other parameters are required for quantitative evaluations. Thus, this paper wants to fill the gap by validating the method but focusing on HR ^{1}H-NMR that can simultaneously and rapidly determine both TMA and *K*-Index. The method requires only an acid extraction, without further filtration, derivatization, or other manipulations. Moreover, the method can be applied to any seafood, both raw and cooked.

2. Materials and Methods

2.1. Chemicals. All reagents were of analytical grade or the highest grade available and were purchased from Sigma-Aldrich, including picric acid (1% in H_2O), trichloroacetic acid (TCA) (6.1°N), trimethylamine-HCl (98%), formaldehyde (37.3%), potassium hydroxide, toluene (99.7%), sodium sulfate granular anhydrous, adenosine 5′-triphosphate (98%), adenosine 5′-diphosphate (98%), adenosine 5′-monophosphate (99%), inosine 5′-monophosphate (97%), inosine (99%), hypoxanthine (99.5%), potassium dihydrogen phosphate, potassium hydrogen phosphate anhydrous, ethanol (99.5%), hydrochloric acid (37%), milliQ demineralized water, deuterium oxide (99.9%), and 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt ((TSP), 98 atom % D).

2.2. Preparation of Standard Solution. A trimethylamine TMA standard solution (1.00 mg TMA/mL) was prepared by dissolving 0.6820 g TMA-HCl in 100 mL of distilled water and then stored at 4°C. Before dissolving, TMA-HCl had been dried overnight in a desiccant with silica gel and accurately weighted (±0.01). The TMA working solution (10.0 μg TMA/mL) was obtained by diluting TMA standard solution with 100 ml of distilled water. Nucleotides standard solution was obtained by mixing 0.0229 g of ATP, 0.0195 g of ADP, 0.0144 g of AMP, 0.0163 g of IMP, 0.0111 g of HxR, and 0.0056 g of Hx in 250 mL distilled water with a final concentration of 0.166 mM.

2.3. Fish Samples. According to Ciampa [28], in the first part of the research, European sea bass (*Dicentrarchus labrax*) and Atlantic mackerel (*Scomber scombrus*) purchased from local stores were used to set up the experimental work, verify the effectiveness of the TMA extraction method, and verify the correlation between HR ^{1}H-NMR outcome and standard fish quality determination methods. Once the experimental method was set up and the correlation verified, ^{1}H-NMR was used to evaluate the concentration of TMA and the *K*-Index value in wild fish samples, namely, bogue fish (*Boops boops*) and red mullet (*Mullus barbatus*) supplied by Magna Grecia Mare® Portus Veneris (Casotto ex Locamare, Banchina sud

del Porto Vecchio, 73030 Tricase Porto (LE)) and stored at 4°C for 11 days. In detail, after catching, fish samples were placed in a polystyrene box, covered with ice flakes, and, after unloading, immediately carried to the laboratory of the Campus of the Food Science - University of Bologna (Italy). In the laboratory, fishes were individually inserted into plastic pouches and placed again in a polystyrene box at 4°C. Sampling was performed at time 0 (T0), corresponding to the day of arrival at the laboratory, and then after 1 (T1), 2 (T2), 3 (T3), 4 (T4), 5 (T5), 6 (T6), 7 (T7), 8 (T8), 9 (T9), 10 (T10), and 11 (T11) days.

2.4. TMA Quantification by UV Analysis. A calibration curve was firstly obtained, by adding distilled water to 1.0, 1.5, 2.5, and 3.0 mL aliquots of the standard solution (10 μg/mL), up to a final volume of 4.0 mL.

To determine the unknown quantity of TMA in fish samples, the method of Dyer and Mounsey [29] as modified by Tozawa et al. [30] was followed according to the AOAC official method [20]. In detail, 25 g of fish muscle was blended with 50 ml of a 7.5% trichloroacetic acid (TCA) solution and filtered with a cellulose filter paper (Whatman No. 4, particle retention 20–25 μm). The filtrate was stored at −80°C until analysis. Aliquots between 0.1 mL and 4.0 mL of extract were brought to a final volume of 4.0 mL with distilled water. For the blank, 4.0 mL of distilled water was used. Just before analysis, 1 mL of 10% formaldehyde, 10 mL of toluene, and 3.0 mL of 25% KOH were added, in order, to the filtrate. The reaction mixture was shacked vigorously for 30 min at 30°C, and after the separation of the phases, 7 mL of the upper toluene solution was moved to a new large dryer tube containing approximately 0.3 to 0.4 g anhydrous Na_2SO_4 and gently shacked until the solution was clear. Five milliliters of this solution was added to 5 mL of picric acid (0.02% in toluene) and mixed by swirling gently. Absorbance was measured at 410 nm with a Shimadzu UV-1601 UV-VIS spectrometer (Shimadzu Corporation, Kyoto, Japan) [28].

2.5. Sample Preparation for HPLC Analysis. Aliquots of the TCA extract (10 mL) described in Section 2.4 were used also for the *K*-Index calculation. The extracts were neutralized to pH 7.0 with 1 M KOH and then filtered with a cellulose filter paper (Whatman No. 1, particle retention 11 μm) to remove insoluble potassium trichloroacetate [18]. Twenty μl of the neutralized solution was loaded onto a 25 cm × 4.6 mm·C18 stainless steel column (Beckman, Fullerton, CA) connected to an HPLC equipped with a Frac-900 fraction collector and UV monitor UPC-900 (Amersham Pharmacia Biotech, Milan, Italy). Elution was performed using 0.1 M phosphate buffer pH 7.0 (0.04 M KH_2PO_4 and 0.06 M K_2HPO_4) as the mobile phase with a flow rate of 1 mL/min, and the eluant was monitored at 254 nm. Nucleotide standards included a mixture of ATP, ADP, AMP, IMP, HxR, and Hx at 0.166 mM each.

2.6. Sample Preparation and ¹H-NMR Analysis. The pH of 1 mL of TCA extract was adjusted to 7.8 using 9 M KOH in a microfuge tube and centrifuged at 18,600 × g for 5 min to remove potassium trichloroacetate precipitate. 160 μL of a D_2O solution of TSP 6.25 mM was added to the supernatant as a ¹H-NMR chemical shift (spectra alignment) reference. All ¹H-NMR FIDs were recorded using a Varian Mercury AS/400 NMR spectrometer (Varian Instruments, Sunnyvale, CA) operating at 9.4 T, corresponding to 400.098 MHz ¹H Larmor frequency. Each sample was obtained from 256 transients collected into 32 K data points, covering a 16 ppm spectral width and requiring 31 min of measurement time. A recycle delay of 4 s, an acquisition time of 2.56 s, and a 60° pulse of 6.30 μs were set up. The saturation of the residual water signal was achieved by irradiating its frequency during the recycle delay at δ equal to 4.703 ppm. Each FID was processed with MestRe-C 4.9.8.0 (Mestreab Research SL, Spain) by manually adjusting the phase and the baseline and applying a line broadening factor of 0.5 Hz [28]. Peaks related to TMA (Figure 1) and nucleotides were assigned by comparing their chemical shift and multiplicity with literature [31, 32]. When more peaks due to different protons of the same molecule were identified, both were employed for the quantification.

2.7. Data Analysis. ANOVA was performed to test the significance of key effects during the instrumental analysis. In particular, the presence of any variability effects due to the operator both during sample preparation and during the acquisition of UV and NMR data and data processing has been verified. The SPSS 17.0 for Windows software package (SPSS, Inc., Chicago, IL) was used to address the statistical analysis.

3. Results and Discussion

3.1. Preliminary Evaluations of the TMA Quantification Methods. The instrumental linearity of the calibration curve needed for the TMA quantification by the UV method was checked by reporting the absorbance values versus the concentrations of TMA standard solutions. The regression showed good linearity, with a correlation coefficient by least-squares analysis of 0.9946. These results confirm a linear relationship between the TMA concentration and instrumental response in the range of 0–30 μg/ml. The instrumental accuracy was tested on three replicates per sample. Mean (x), relative standard deviation (RSD), and bias, obtained at four different concentration levels of TMA, are reported in Table 1 [28]. The RSDs obtained were always acceptable according to Horwitz's formula (RSDH) [33] and HorRat (Horwitz ratio) value [34].

[a]Mean of 3 measurements per point, obtained for 4 different aliquots trimethylamine standard solution. [b](mc-nc) × 100/nc, where mc is the measured amount and nc is the nominal amount. [c]Relative standard deviation. [d]Acceptable relative standard deviation according to Horwitz's formula for intra-laboratory studies. [e]Horrat = RSD measured/RSD Horwitz predicted. [f]Accuracy $= (p^2 + b^2)^{1/2}$, where p is precision (expressed as relative standard deviation, RSD) and b is the bias.

NMR is an intrinsically quantitative spectroscopic tool [35] because the area under the signal is directly proportional to the number of resonant nuclei (spins). Therefore, the quantification of TMA by ¹H-NMR spectroscopy did not rely
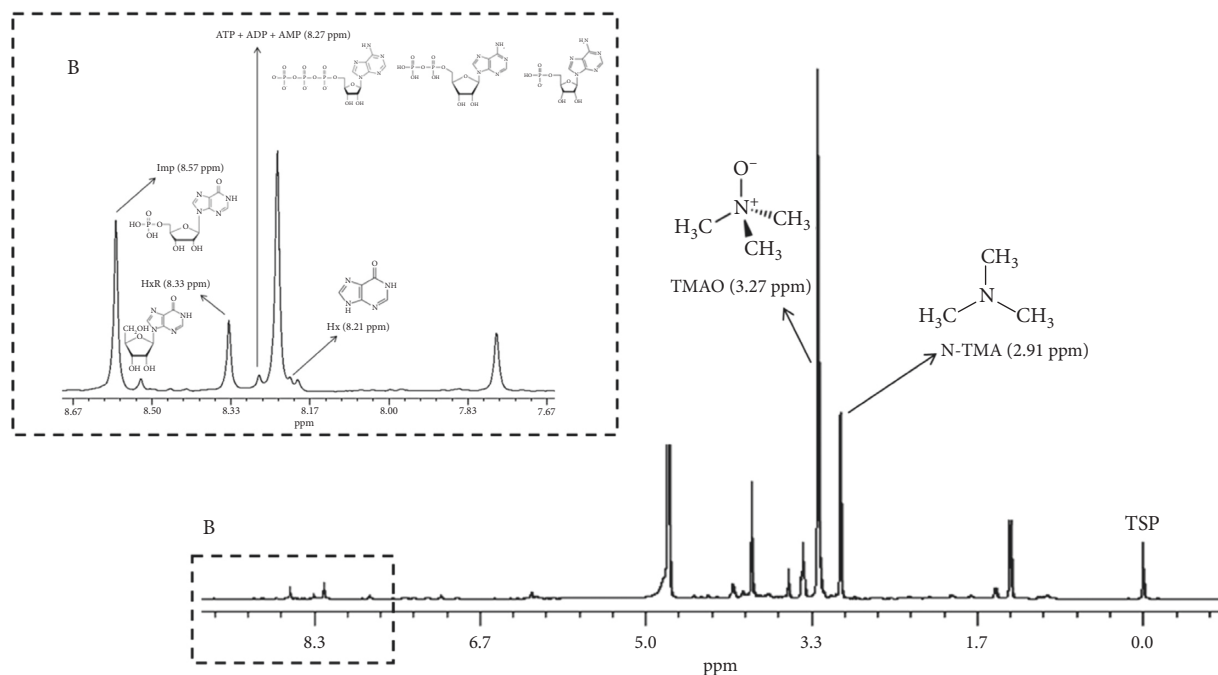
FIGURE 1: ^{1}H-NMR (400.13 MHz) spectrum of red mullet; (B) ATP, ADP, and AMP contribute to the formation of peak at 8.27 ppm, adn IMP, HxR, and Hx appear as a singlet, respectively, at 8.57 ppm, 8.33 ppm, and 8.21 ppm. Signals from TMAO and N-TMA are, respectively, at 3.27 and 2.91 ppm.

TABLE 1: Instrumental linearity.

TMA[a] (μg)	Bias[b] (%)	RSD[c] (%)	RSDH[d] (%)	HorRat[e]	Accuracy[f] (%)
10	−6.6	0.41	7.7	0.05	6.6
15	−0.2	3.55	7.1	0.50	3.6
25	0,1	5.46	6.6	0.83	5.5
30	0.2	3.41	6.4	0.53	3.4

on a calibration curve but was performed using an absolute method based on TPS as an internal standard compound.

3.2. Analytical Recovery and Repeatability of the TMA Quantification Methods.
To evaluate the accuracy and the extraction efficiency of the UV and ^{1}H-NMR-based methods, recovery experiments were performed on both European sea bass and Atlantic mackerel samples, in which 0.1 mg/100 g TMA was detected, spiked with three concentrations of standard TMA for a total of 4 samples for each fish species (Figure 2). The results presented in Table 2 are satisfactory for both methods, as recovery was close to 100% for all spiking levels [28].

[a]Mean of 6 measurements per point, obtained for 3 different trimethylamine standard solutions. [b]Recovery = ((C1−C2)/C3)* 100, where C1 is the analyte concentration, measured after the addition of standard solution. C2 is the analyte concentration, measured before the addition of standard solution. C3 is the concentration of the added standard solution. [c](mc-nc) × 100/nc, where mc is the measured concentration and nc is the nominal concentration. [d]Relative standard deviation. [e]Acceptable relative standard

deviation according to Horwitz's formula for intralaboratory studies. [f]Horrat = RSD measured/RSD Horwitz predicted. [g]Accuracy = $(p^2 + b^2)^{1/2}$, where p is precision (expressed as relative standard deviation, RSD) and b is the bias.

Before repeatability evaluation, an analysis of variance (ANOVA) was used to highlight potential fixed effects: analyst spectrometer, sample preparation, and ^{1}H-NMR spectrum processing. Generally, a good processing step determines the precision of integration and consequently the accuracy of quantification; it depends on the noise level of the spectrum, the line shape, quality of shimming and phasing, and baseline and drift corrections [36]. For a significance level $\alpha = 0.05$, the variance component is not statistically significant if its p-value is equal to or greater than 0.05 [25]. The data show that the variance component was not statistically significant for analyst (pUV = 0.345; pNMR = 0.885), for acquisition-instrumental (pUV = 0.345; pNMR = 0.123), for sample preparation (pUV = 0.062; pNMR = 0.123), and for ^{1}H-NMR processing (pNMR = 0.875) [28].

The repeatability of both analytical methods, expressed by RSD, was determined using 12 fish samples of red mullet ($n = 6$) and bogue fish ($n = 6$), with TMA concentrations ranging from 0.1 mg/100 g to 37 mg/100 g. The values of RSD reported in Table 2 express the precision of both analytical methods.

3.3. Limits of Detection (LOD) and Quantitation (LOQ).
Concerning TMA quantification by UV, LOD and LOQ were calculated according to Long and Winefordner [37] concerning the IUPAC method [38]. In the present work, an LOD of 0.3 mg/100 g and an LOQ of 0.9 mg/100 g were
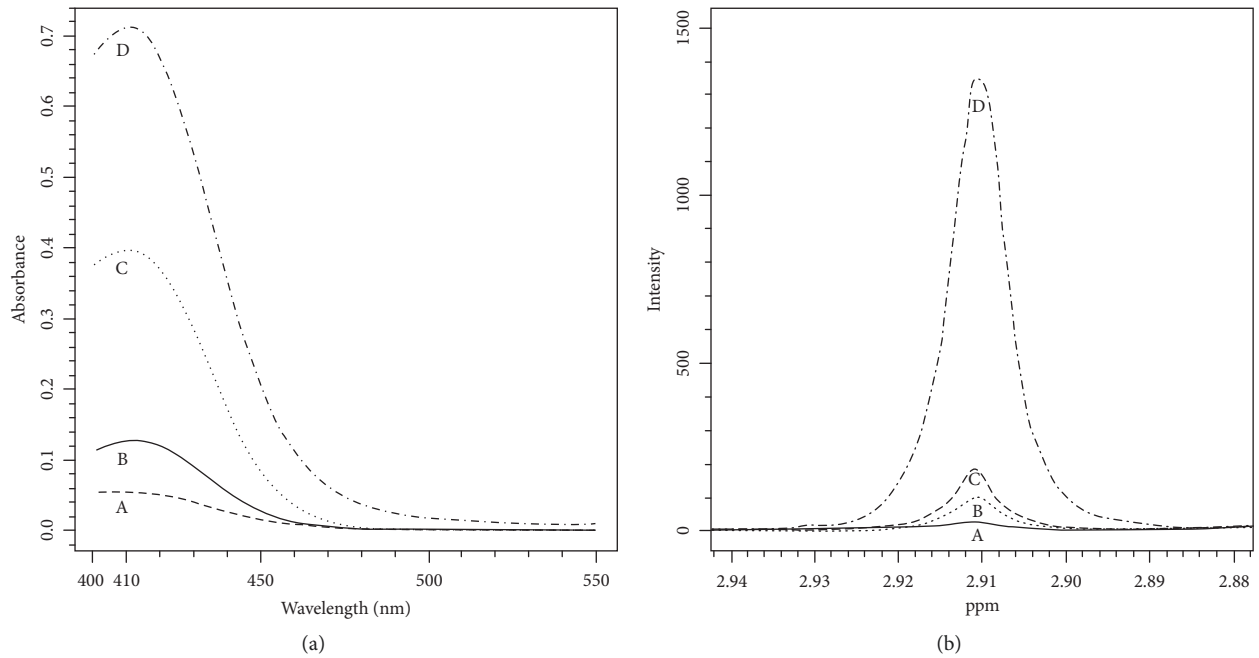
FIGURE 2: Detection of TMA by (a) UV method and (b) 1H-NMR method; (A) no spiked sample, (B) sample spiked with 200 μL of the standard solution, (C) sample spiked with 400 μL of the standard solution, and (D) sample spiked with 600 μL of the standard solution (1 mg/mL TMA).

TABLE 2: Recovery of TMA during extraction and accuracy of methods in spiked samples.

European sea bass (Dicentrarchus labrax)											
TMA[a] (μg/mL)	Recovery[b] (%)		Bias[c] (%)		RSD[d] (%)		RSDH[e] (%)		Horrat[f]		Accuracy[g] (%)
	UV	NMR	UV	NMR	UV	NMR	UV	NMR	UV	NMR	UV	NMR
20 (0.002)	100.9 (1.1)	99.4 (2.3)	0.9	-0.6	5.0	5.5	9.7	9.7	0.5	0.6	5.6	5.6
40 (0.003)	98.9(0.9)	100.3 (0.7)	−1.1	0.3	4.9	5.3	8.7	8.7	0.6	0.6	5.4	5.6
60 (0.003)	100.3 (0.5)	100.5(0.5)	0.3	0.5	4.9	5.2	8.2	8.2	0.7	0.6	5.5	5.5
Atlantic mackerel (Scomber scombrus)											
TMA[a] (μg/mL)	Recovery[b] (%)		Bias[c] (%)		RSD[d] (%)		RSDH[e] (%)		Horrat[f]		Accuracy[g] (%)
	UV	NMR	UV	NMR	UV	NMR	UV	NMR	UV	NMR	UV	NMR
20 (0.007)	96.6 (3.3)	98.1 (2.8)	−3.5	−1.9	5.3	5.6	9.7	9.7	0.5	0.6	6.4	5.9
40 (0.007)	98.8 (1.7)	101.1 (2.0)	1.2	1.1	5.5	5.7	8.7	8.7	0.6	0.7	5.6	5.8
60 (0.005)	99.8 (0.8)	99.3 (1.4)	0.2	0.7	5.7	5.4	8.2	8.2	0.7	0.7	5.6	5.5

obtained. Interestingly, from Table 3, it is possible to observe that most of the data with RSD (%) >5% from samples with the content of TMA not only lower than LOQ but also lower than LOD. In ^{1}H-NMR spectroscopy, the LOD and LOQ values depend on instrumental acquisition parameters, mainly the number of scans (NS), which influences the signal/noise (S/N) ratio and the receiver gain (RG) [25]. The LOD and LOQ values, measured on samples of fish at T0, in which TMA content was equal to 0.11 ± 0.029 mg/100 g, were calculated by using the following equations:

$$LOD = \frac{3C}{(S/N)},$$
$$LOQ = \frac{10C}{(S/N)}, \qquad (2)$$

where C is the TMA concentration and S/N is the signal/noise ratio. With N/S equal to 128 and RG equal to 6, an LOD of 0.1 mg/100 g and an LOQ of 0.4 mg/100 g were obtained.

TABLE 3: TMA content in red mullet and bogue fish.

	Red mullet (*Mullus barbatus*)							
# Fish sample	TMA (mg/100 g)		RSD (%)[a]		RSDH (%)[b]		Horrat[c]	
	UV	NMR	UV	NMR	UV	NMR	UV	NMR
1 (T1)	0.14 (0.04)	0.16 (0.02)	6.9	7.4	10.1	10.3	0.7	0.7
2 (T2)	0.29 (0.1)	0.27 (0.02)	3.4	4.3	9.2	9.2	0.4	0.5
3 (T4)	3.80 (0.5)	2.86 (0.2)	6.1	3.3	6.1	6.2	1.0	0.5
4 (T7)	18.87 (3.6)	11.95 (2.4)	3.6	3.8	4.8	5.2	0.7	0.7
5 (T9)	26.3 (3.9)	19.26(1.2)	4.0	1.2	4.7	4.8	0.9	0.3
6 (T11)	37.54 (1.8)	25.11 (1.1)	2.2	2.0	4.4	4.7	0.5	0.4
	Bogue fish *(Boops boops)*							
# Fish sample	TMA (mg/100 g)		RSD (%)[a]		RSDH (%)[b]		Horrat[c]	
	UV	NMR	UV	NMR	UV	NMR	UV	NMR
1 (T1)	0.45 (0.16)	0.33 (0.05)	9.2	6.6	8.7	9.2	1.0	0.7
2 (T2)	1.02 (0.07)	0.66 (0.07)	3.2	4.7	7.6	8.0	0.4	0.6
3 (T4)	6.6 (0.05)	6.8 (0.4)	1.3	2.7	5.7	5.7	0.2	0.5
4 (T7)	16.7 (1.3)	11.4 (0.9)	3.4	3.4	5.0	5.3	0.7	0.6
5 (T9)	17.7 (3.6)	12.8 (1.9)	2.7	3.0	5.2	5.2	0.5	0.6
6 (T11)	21.5 (4.3)	20.03 (4.3)	3.9	4.7	4.7	5.1	0.8	0.9

[a]Relative standard deviation. [b]Acceptable relative standard deviation according to Horwitz's formula for intralaboratory studies. [c]Horrat = RSD measured/ RSD Horwitz predicted.
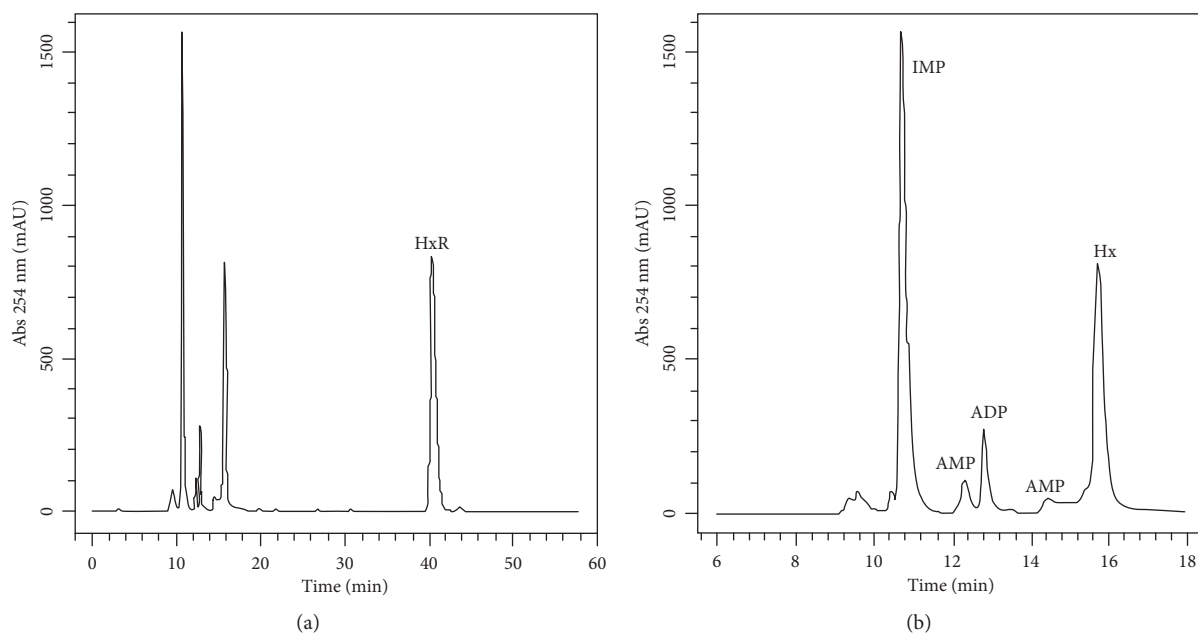

FIGURE 3: (a) Nucleotides standard solution chromatogram. Operating conditions: column Beckman C18; flow rate, 1.0 ml/min; mobile phase, 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen orthophosphate. (b) Chromatogram of nucleotides from red mullet sample.

3.4. Comparison of UV and ^{1}H-NMR Methods and Specificity. R^2 of the relationship between TMA values calculated by ^{1}H-NMR and UV methods was greater than 0.9, showing an excellent agreement between these two methods. Although a generally good correlation and data precision were found, in some cases, the concentrations of TMA evaluated by UV were higher than those from ^{1}H-NMR by about 41%. The over-estimation of the UV method is probably a consequence of undesired reactions of picric acid with nonvolatile amines, such as putrescine, cadaverine, tyramine, and histamine. This is in agreement with Dyer and Mounsey [29], who showed

that many amines, formed during the bacterial breakdown of protein during the spoilage of fish, react with the picric acid reagent. Gill and Thompson also reported that colorimetric TMA data obtained using the Dyer procedure were consistently 35% higher than the results obtained by HPLC [39].

3.5. Identification of Nucleotides' Peaks by HPLC and ^{1}H-NMR. The HPLC determination of nucleotides peaks from fish samples was done by comparing their chromatograms with those from a nucleotides standard solution (Figure 3).

TABLE 4: K-Index value for red mullet and bogue fish.

| # Fish sample | Red mullet (Mullus barbatus) | | | | | | | |
| | K-index (%) | | RSD (%)[a] | | RSDH (%)[b] | | Horrat[c] | |
	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR
1 (T1)	32.9 (2.9)	31.2 (1.0)	1.1	1.5	1.6	1.6	0.68	0.95
2 (T3)	53.1 (1.1)	50.0 (2.3)	0.05	0.5	1.5	1.5	0.34	0.034
3 (T4)	69.3 (1.2)	69.3 (1.5)	0.7	1.0	1.4	1.4	0.48	0.74
4 (T7)	83.5 (1.2)	83.2 (0.7)	0.4	0.4	1.4	1.4	0.32	0.30
5 (T10)	88.6 (1.1)	92.7 (2.0)	0.35	1.0	1.4	1.4	0.26	0.75
# Fish sample	Bogue fish (Boops boops)							
	K-index (%)		RSD (%)[a]		RSDH (%)[b]		Horrat[c]	
	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR
1 (T1)	23.3 (1.9)	21.4 (1.2)	2.34	0.43	1.7	1.7	1.4	0.25
2 (T5)	36.7 (1.4)	35.5 (1.3)	0.90	0.77	1.5	1.5	0.60	0.51
3 (T6)	43.4 (2.99)	42.1 (2.8)	1.9	1.3	1.5	1.5	1.3	0.87
4 (T9)	66.1 (2.5)	65.7 (3.4)	1.5	1.6	1.4	1.4	1.07	1.14
5 (T10)	68.8 (1.69)	74.4 (1.9)	1.1	0.4	1.4	1.4	0.75	0.29

[a]Relative standard deviation. [b]Acceptable relative standard deviation according to Horwitz's formula for intralaboratory studies. [c]Horrat = RSD measured/ RSD Horwitz predicted.

The RSD value < 2% calculated for the elution time demonstrates good reproducibility of the HPLC method. To determine the K-Index, each nucleotide's peak area was integrated. The variation of the K-Index during the spoilage is reported in Table 4. Focusing on ^{1}H-NMR, nucleotides assignment was performed by comparison with previously published data [31, 32, 40] and is represented in Figure 1.

The signals from hypoxanthine, inosine, and inosine monophosphate are all well separated, and the area of the peaks can be determined by integration. On the opposite, the peaks ascribed to ATP, ADP, and AMP are overlapped with each other and can therefore not be integrated separately. When estimating the K-Index, there is no need for separate quantification of these compounds since phosphorylated compounds all contribute to its calculation, and the total amounts of ADP, ATP, and AMP can be determined as a sum. The repeatability of these two analytical procedures in determining the K-Index value was calculated using 10 samples, 5 red mullet samples, and 5 bogue fish samples. RSD values are reported in Table 4.

As shown in Table 4, only in some cases do the values of K-Index by HPLC show an RSD > 1; this is probably due to the rapid postmortem dephosphorylation and deamination of adenine nucleotides (ATP, ADP, and AMP) throughout IMP by the autolytic process. Consequently, their detection, also after 24 hours postmortem, is characterized by a lower accuracy than that found for IMP, HxR, and Hx [28].

3.6. Comparison of HPLC and ^{1}H-NMR Methods for the K-Index Evaluation.
The correlations between the K-Index obtained by ^{1}H-NMR and HPLC are characterized by a high value of R^2 (0.97). Interestingly, the K-Index measured by the two methods tends to differ from 10 days of storage onwards, with NMR providing larger values. This finding could be explained by considering that the amount of catabolites of ATP after longer storage times is so large that the HPLC method loses linearity. Alternatively, after 10 days of spoilage, other metabolites (even derived from further

degradation of Hx) are formed, with signals overlapping with those from ATP catabolites. The initial levels of ATP and the level of its breakdown compounds are subjected to large inter- and intraspecies differences. In particular, the ATP level in the fish flesh depends on capture conditions, time after catching, sexual maturity, water temperature, and storage conditions [41]. In addition to these conditions, the degradation of ATP to AMP and/or IMP is very quick and consequently more difficult to detect during fish spoilage [28].

4. Conclusions

The comparison revealed a similar degree of accuracy between the data obtained from conventional analytical methods and HR ^{1}H-NMR. In particular, the ^{1}H-NMR has allowed both to observe a greater specificity in the measurement of TMA and a shorter acquisition time in the K-Index determination [28]. Another important advantage of ^{1}H-NMR analysis compared to UV-based method was the possibility of avoiding any pretreatment, thus reducing the use of toxic solvents and hazardous reagents such as toluene and picric acid in the determination of the TMA. Consequently, the use of the ^{1}H-NMR method has proved to be a smart strategy to provide both environmental and economic benefits, to be also potentially proposed in the future as a "Sustainable Analytical Procedure" (SAP). In addition, the spectroscopic method used in this work for identifying some metabolites in the evaluation of the quality and freshness of the fish can be seen as a further study that increases the chance of nuclear magnetic resonance to officialize its analytic methods as standard conventional methods in the seafood field. Currently, there is an increasing requirement for proper analytical methods capable of giving a complete picture of fish metabolism and assessing the nutritional quality of the product. These methods should be based on the analysis of a variety of metabolites and not only of a few specific classes of compounds. There are also some disadvantages related to NMR methods. For example, the cost of

the analysis is considerably higher since deuterated solvents are required, and NMR spectrometers are expensive and not widely used, especially outside research centers, while alternative UV spectrometers are widely used and compact. However, the potential of NMR with a benchtop spectrometer should be considered. Our work is carried out with an old generation 400 MHz, which gives results comparable to the latest generation bench instruments, much less expensive, with costs of the same order of magnitude as HPLC apparatus. Deuterated solvents are used in such low quantities (a few microliters) that their cost is not as impactful as the cost of an operator working with time-consuming procedures. Finally, NMR could provide quantities of many analytes simultaneously.

Data Availability

All data generated or analysed during this study are included within this published article.

Disclosure

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Gianfranco Picone contributed to conceptualization and supervision. Alessandra Ciampa and Gianfranco Picone contributed to methodology, validation, investigation, data curation, and original draft preparation. Gianfranco Picone and Luca Laghi reviewed and edited the paper. Alessandra Ciampa provided support through "Development of Methodologies for Fish Freshness Assessment Using Metabonomics Applications" Ph.D. thesis (Alma Mater Studiorum, University of Bologna, Italy).

Acknowledgments

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