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Evolution of Eight Biogenic Amines in Raw and Preserved Mackerel (*Scomber scombrus*) Fillets Monitored by UHPLC-PDA

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Abstract: The presence of biogenic amines (BAs) in seafood can pose a health risk to consumers, as they have been linked to adverse reactions such as histamine poisoning. Although the only biogenic amine for which maximum limits have been set is histamine, it is also important to regulate the presence of other amines associated with certain adverse effects. In this study, the official method for determining histamine was slightly modified and adapted for a UHPLC-PDA system and applied to analyze raw and preserved mackerel fillet samples. The evolution of biogenic amines during the storage period under refrigerated conditions revealed that, within two days, the limit for the content of histidine of 100 mg/kg was exceeded in raw fillets, while the histidine content in preserved mackerel (in oil and marinated) remained more stable. The thawing phase, whether in the fridge or at room temperature, did not significantly affect the BA content. Additionally, three different cooking methods (steaming, oven-baking, and boiling) significantly decrease the levels of BAs in highly contaminated raw mackerel fillets.

Keywords: biogenic amines; mackerel; histamine; UHPLC-PDA

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1. Introduction

Biogenic amines (BAs) are organic nitrogenous compounds found in various foods, including fish, produced as a result of amino acid decarboxylation. The proliferation of BAs in foods depends on the presence of amino acid precursors, bacteria capable of decarboxylating them, and favorable environmental conditions for bacterial growth and activity. The most common BAs include histamine (HIS), tyramine (TYR), putrescine (PUT), cadaverine (CAD), β-phenylethylamine (PHE), tryptamine (TRP), spermine (SPM), and spermidine (SPD). The types and concentrations of BAs vary depending on the bacterial types present and are strongly influenced by intrinsic food factors such as water activity, pH, constituents, and natural microflora, as well as extrinsic factors like storage time and temperature, which impact bacterial growth [1–3].

The presence of BAs in food is a significant concern for public health and food safety. Determining the toxicity of BAs is challenging because it relies on the efficiency of the detoxification process in the intestinal tract and the presence of other BAs. HIS and TYR are especially concerning among these amines, as they have the potential to cause serious foodborne illnesses such as respiratory, digestive, cardiac, and neurological diseases and have psychoactive, vasoactive, and hypertensive effects [4,5].

HIS formation in seafood is primarily associated with the enzymatic decarboxylation of the amino acid histidine, a process facilitated by specific bacterial species. This phenomenon is especially prevalent in scombroid fish species, such as mackerel, tuna, and bonito, which naturally contain high levels of free histidine. The risk of HIS formation is exacerbated by improper handling and storage conditions, which promote the growth of HIS-producing bacteria [1–3,6].

In the past year (from June 2023 to June 2024, https://food.ec.europa.eu/safety/rasff_en, accessed on 18 June 2024), the European Commission has published 29 notifications in

the Rapid Alert System for Food and Feed (RASFF) regarding the presence of HIS in fish and fish products worldwide. Out of these notifications, 15 were related to tuna products, 7 were related to mackerel products, 4 were related to anchovy products, and 3 were related to sardines.

Mackerel (*Scomber scombrus*) is a fish belonging to the family Scombridae (blue fish) that primarily inhabits the cold and temperate waters of the Mediterranean Sea and the coasts of the eastern Atlantic Ocean, stretching from Morocco to Norway. It primarily feeds on zooplankton and small fish and is a significant source of omega-3 fatty acids, high-quality proteins, phosphorus (P), potassium (K), and magnesium (Mg), offering numerous health benefits that make it a valuable addition to a balanced diet [7,8]. However, mackerel is prone to rapid spoilage, making it susceptible to HIS formation, which can lead to a type of food poisoning known as "scombroid poisoning". This reaction resembles an allergy-like response, with symptoms such as sweating, facial flushing, headaches, dizziness, nausea, abdominal cramps, and palpitations. In some cases, individuals with pre-existing conditions may experience cardiac and respiratory complications [9].

Currently, the only biogenic amine for which maximum limits have been set in the European Union (EU) and the United States is HIS, due to its toxicological effects in fresh, frozen, and processed foods [10]. The EU has established that, considering a sampling plan of nine samples from one lot, the average HIS concentration must be less than 100 mg/kg. Two samples may have a value greater than 100 mg/kg but less than 200 mg/kg. No sample may have a value greater than or equal to 200 mg/kg [11]. These maximum limits are doubled for fishery products that have undergone enzymatic maturation treatment in brine. For fish sauce, only one sample is taken, and the HIS content must not exceed 400 mg/kg [12]. Based on data collected from numerous outbreaks, the US Food and Drug Administration (FDA) set the stricter acceptable HIS level of 50 mg/kg for scombroid-like fishes [6,13].

It is recognized that HIS alone rarely causes toxicity, but the co-presence of other amines such as PUT and CAD can potentiate HIS toxicity by inhibiting metabolizing enzymes [14,15]. TYR, PHE, and PUT are vasoactive amines that can increase blood pressure, leading to heart failure or brain hemorrhaging [16]. Therefore, it is also important to regulate the presence of other amines that are associated with certain adverse effects.

The monitoring of selected BAs in seafood serves two purposes: as an indicator of decomposition and to prevent potential toxicity on human health [3,4,6]. The Biogenic Amine Index (BAI), developed by Mietz and Karmas in 1981 [17], has been widely used to monitor the freshness of seafood products [18]. It is based on increased levels of PUT, CAD and HIS along with a corresponding decrease in SPD and SPM.

There are various analytical methods for quantifying BAs in foods, among which liquid chromatographic separation methods are most commonly used due to their selectivity and sensitivity [3]. Derivatization is essential for ultraviolet (UV) and fluorescence detectors (FLD) [19]. Two reference methods commonly used for official controls are the Codex-approved HPLC method with ion exchange and FLD (AOAC 977.13) and the EU-approved HPLC method [20] with a reversed-phase column (C18) based on pre-column derivatization with dansyl chloride followed by UV detection at 254 nm.

Based on these premises, it is important to have methods available to investigate the presence of a wide range of BAs. Therefore, the main objective of this work was to explore the possibility of expanding the application of the EN ISO 19343:2017 [20] method for determining HIS in fish and fish products to include a larger number of BAs using UHPLC coupled with a photodiode array detector (PAD) for the final analytical determination. Additionally, the proposed method was used to study the evolution of eight BAs in both raw and preserved mackerel fillets, in oil or marinated, which may undergo longer storage periods at the deli counter of many supermarkets and then in the consumer's refrigerator. The effects of different cooking approaches on a highly contaminated sample and the evolution of BAs under drastic storage conditions were also investigated.

2. Materials and Method

2.1. Reagents and Standards

Acetone, acetonitrile and toluene, all of HPLC grade, were purchased from Merck (Darmstadt, Germany). 1,7-diaminoheptane (7.7 mg/mL) used as an internal standard (IS), perchloric acid 72%, dansyl chloride (10% in acetone), L-proline, and sodium carbonate decahydrate were purchased from Sigma-Aldrich (Milan, Italy). Ultrapure water was obtained with a Milli-Q purification system (Millipore, Bedford, MA, USA). The standard biogenic amine mixture in water (1 mg/mL each) consisted of TRP chlorohydrate (99.0%), PHE chlorohydrate (99.0%), PUT dichlorohydrate (99.0%), CAD dichlorohydrate (99.0%), HIS dichlorohydrate (99.0%), TYR chlorohydrate (99.0%), SPD trichlorohydrate (99.0%), and SPM tetrachlorohydrate (99.0%), all from Supelco (Bellefonte, PA, USA).

2.2. Samples

Raw and preserved mackerel fillets were collected from different supermarkets in northeastern Italy, specifically Veneto and Friuli Venezia Giulia. To achieve the goals of this study, specific storage conditions were used that exceeded the general recommendation of consuming fresh fish within one day.

Raw mackerel (RM) fillets (from the Atlantic Sea) were bought from the fish counter of a supermarket at two different times. Following the purchase, a first set of 4 fillets (RM-0, RM-2, RM-4 and RM-6) were stored for different time periods (0, 2, 4, and 6 days) in a refrigerator at 4 °C. Another set of samples consisted of 5 whole mackerels (RM-A, RM-B, RM-C, RM-D, RM-E), each of which was divided into two halves. After 5 days of refrigerated storage, one half was kept raw as a control, and the other half was cooked, as indicated in Table 1. After refrigeration and/or cooking, the skin was removed, the sample was finely chopped with a knife, and then stored at −18 °C until analysis.

Table 1. Sampling plan for raw and preserved mackerel samples.

Raw Mackerel			Preserved Mackerel	
Sample Code	**Storage at 4 °C (Days)**	**Cooking**	**Sample Code**	**Type of Preservation**
RM-0	0	No	PM-1 *	In oil
RM-2	2	No	PM-2 *	In oil
RM-4	4	No	PM-3	In oil
RM-6	6	No	PM-4	In oil
RM-A	0	No	PM-5	In oil
RM-B	4	No	PM-6	In oil
RM-C (a)	4	No	PM-7	In oil
RM-C (b)	4	Steamed for 25 min	PM-8 *	Marinated
RM-D (a)	4	No	PM-9	Marinated
RM-D (b)	4	Oven-baked at 160 °C for 20 min		
RM-E (a)	4	No		
RM-E (b)	4	Boiled for 10 min in 500 mL of water		

* Samples whose BA content was monitored after 1, 2, 3 and 5 days at 4 °C.

Preserved mackerel (PM) fillet samples (7 in oil and 2 marinated) were purchased from the deli counter of various supermarkets. Three samples (with an asterisk in Table 1), two preserved in oil (PM-1 and PM-2) and one marinated (PM-8), were chosen for monitoring the BA profile over time. Sampling was conducted at regular intervals (0, 1, 2, 3 and 5 days) and the obtained sub-samples were stored at −18 °C until analysis.

2.3. Sample Preparation

Sample preparation followed the guidelines of Reg. CE 2073/2005 [11] and Malle et al., 1996 [21]. Briefly, 5 g of the homogenized sample was weighed into a centrifuge tube

kept at 4 °C. Then, 10 mL of a 0.2 M perchloric acid solution and 100 µL of the internal standard solution were added. After homogenization (using an UltraTurrax, Ika-Werk, Staufen, Germany) and centrifugation (12,000 rpm for 5 min) at 4 °C, an aliquot of the obtained extract was derivatized.

Derivatization involved adding 100 µL of the standard solution or sample extract to 300 µL of a saturated sodium carbonate decahydrate solution and 400 µL of dansyl chloride (7.5 mg/mL in acetone). After 10 min in a thermostatic bath at 60 °C with magnetic stirring in the dark, 100 µL of L-proline (100 mg/L) was added to eliminate the excess of the derivatizing agent. Following 1 min of shaking and being left to rest for 15 min in the dark, the sample extract was mixed with 500 µL of toluene, cooled at −18 °C for 30 min, and the unfrozen organic phase containing the derivatized biogenic amines was collected. This phase was then evaporated under a nitrogen flow, added to 200 µL of acetonitrile, and injected into the UHPLC-PAD system.

2.4. UHPLC-PDA Analysis

The analytical determination of BAs was conducted using a UHPLC Nexera (Shimadzu, Kyoto, Japan) equipped with a gradient pump (LC-30AD), an autosampler (SIL-30AC), and a photodiode array (PDA) detector (SPD-M20A). The column used was an InfinityLab Poroshell 120 SB-C18 (100 mm × 4.6 mm × 2.7 µm, Agilent Technolgies, Santa Clara, CA, USA) thermostatted at 30 °C. The mobile phases consisted of acetonitrile and water at a flow rate of 0.45 mL/min. The gradient elution program started with 60% acetonitrile, increased to 75% in 6 min (held isocratically for 2 min), and then reached 95% in 5 min (held isocratically for 7 min) before returning to the initial conditions. The injection volume was 10 µL and the PDA detector was set at 253–254 nm.

The concentration of BAs was calculated by determining the peak area ratio of each individual amine to the internal standard and then correcting for the appropriate response factor. In accordance with the limit of detection (LOD) established by the Joint Research Center (JRC) [22] for HIS in mackerel, only values exceeding 3 mg/kg for all BAs were reported. Data were acquired and elaborated using LabSolution v.2.7 (Shimadzu, Kyoto, Japan). BAs in mackerel samples were identified based on the retention time via comparison with standard solutions.

3. Results and Discussion

Based the objective of this work, which was to evaluate the impact of different (sometimes extreme) storage and cooking conditions to which a mackerel fillet, raw or preserved, may be subjected before consumption on eight biogenic amines, in the first part of this study, the HPLC method was optimized and the performance of the method was evaluated.

3.1. Method Optimization and Performances

The reference method EN ISO 19343:2017 allows for the separation of HIS from other BAs in fish and fishery products. This method involves extraction with perchloric acid (0.2 M) in the presence of 1,7-diaminoheptane as an internal standard, derivatization using dansyl chloride (10 min at 60 °C), removal of the excess of the derivatizing agent with a proline solution, extraction of the derivative in toluene, and HPLC separation with UV detection at 254 nm. The chromatographic run was conducted on a Kromasil C18 reverse-phase column (250 mm × 4.6 mm × 5 µm) at 25 °C using a water/acetonitrile gradient (starting at 40/60 and finishing at 5/95, *v*/*v*) with a flow rate of 1 mL/min. After 15 min of separation, the chromatogram displayed the peaks of HIS and the internal standard [21].

For this study, the reference method was slightly modified by adjusting the chromatographic conditions for the UHPLC system and utilizing a Poroshell 120 SB-C18 column (100 mm × 4.6 mm × 2.7 µm). The column was maintained at a temperature of 30 °C and the flow rate was set at 450 µL/min. HIS eluted at around 9.8 min and the total run lasted 22 min. Figure 1 shows the chromatograms of standard solutions containing the eight principal BAs at a concentration of approximately 10 and 100 mg/kg each.

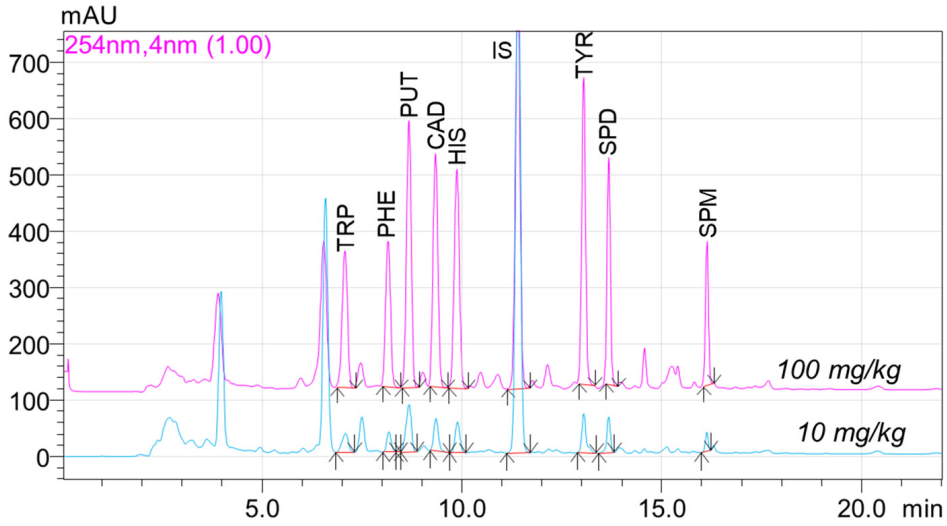

Figure 1. UHPLC-PDA (254 nm) chromatograms of standard solutions containing tryptamine (TRP), 2-phenilethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), 1,7-diaminoheptane (IS), tyramine (TYR), spermidine (SPD), and spermine (SPM) at approximately 10 and 100 mg/kg each.

Although the duration of the UHPLC run is longer (22 min) than the chromato-graphic run using the CEE method (15 min), as shown in Figure 1, the use of UHPLC, under the proposed conditions, allowed the optimal separation of all target amines. Additionally, the lower flow rate (compared to the reference HPLC method) enabled a significant reduction in organic solvent consumption.

Linearity was assessed by injecting, in triplicate after derivatization, various mixtures of BA standards at different concentrations, corresponding to levels found in real samples, approximately 5, 10, 50, 100, 200, 400, and 800 mg/kg. Since the PDA signal was saturated at the highest concentration, a range of 5–400 mg/kg was considered. The equations of the curves and the coefficients of correlation (R^2) are reported in Table 2. The matrix effect was evaluated using a spike recovery approach at two different concentrations and was found to be negligible.

Repeatability and recovery tests were conducted on mackerel fillets in oil with a very low biogenic amine content (Table 2). Repeatability tests involved analyzing six different aliquots of the same sample of mackerel in oil individually. Additionally, two more series of six aliquots each were fortified with approximately 100 and 400 mg/kg of each amine, and then analyzed using HPLC-PDA. The recoveries were calculated by determining the ratio between the quantity of BAs found in the spiked sample, considering the contamination of the initial mackerel, and the final concentration of the added standard.

The unspiked mackerel in oil sample had contamination levels of individual amines ranging from 5.3 to 27.6 mg/kg, with coefficients of variation (n = 6) ranging from 7.3% to 13.4%. The amines found in the highest quantities were SPD (27.6 mg/kg), PUT (12.8 mg/kg) and TYR (10.8 mg/kg). The lowest concentration was found for SPM (5.3 mg/kg) followed by HIS (6.7 mg/kg).

When the sample was fortified with approximately 100 mg/kg of each amine, the coefficients of variation ranged from 1.3% to 7.7%, with recoveries ranging from 59% (TRP) to 103% (TYR). At higher fortification levels, around 400 mg/kg, the coefficients of variation were between 3.2% and 10.8%, with recoveries ranging from 60% (TRP) to 123% (TYR).

For both fortification levels, the lowest recoveries were obtained for TRP (less than 60%) and SPM (between 62% and 89%), while the highest value (123%) was obtained for PUT in the sample spiked at around 500 mg/kg.

HIS consistently showed recoveries of around 94–96%, which is better than what was obtained for mackerel matrices (66–73%) in the ring trial conducted for the validation of the EN ISO 19343 method [23] using the calibration curve obtained in tuna.

Table 2. Linearity line equations and coefficients of determination (R^2) of BAs in solvents in the range of 5–400 mg/kg; repeatability (n = 6) and recoveries related to the content before (as-is) and after (spiked) the addition of two different levels (approximately 100 and 400 mg/kg) in a mackerel in oil sample. SD: standard deviation; CV: coefficient of variation.

Biogenic Amine	Unspiked Sample (n = 6)				Spiked Sample (n = 6)			
	Linearity Line Equation Curve	R^2	Mean ± SD (mg/kg)	CV (%)	Added Amount (mg/kg)	Mean ± SD (mg/kg)	CV (%)	Recovery (%)
TRP	y = 20,933x + 208,180	0.99	8.6 ± 0.7	8.5	100.6	68 ± 4	6.1	59
					402.2	263 ± 28	10.8	60
PHE	y = 24,570x + 173,017	0.99	7.1 ± 0.9	13.4	104.3	114 ± 6	5.3	102
					417.1	396 ± 26	6.4	93
PUT	y = 56,280x − 593,781	0.97	12.8 ± 1.2	9.2	123.0	136 ± 4	3.0	100
					492.2	616 ± 46	7.4	123
CAD	y = 46,731x − 393,655	0.98	9.6 ± 0.8	8.3	96.0	98 ± 3	3.7	92
					384.0	389 ± 14	3.6	99
HIS	y = 38,726x − 185,144	0.99	6.7 ±0.6	9.0	102.2	103 ± 1	1.3	94
					408.6	401 ± 15	3.7	96
TYR	y = 51,457x − 783,042	0.97	10.8 ± 1.3	12.2	102.0	116 ± 8	7.1	103
					407.8	407 ± 13	3.2	97
SPD	y = 47,107x − 604,957	0.97	27.6 ± 2.0	7.3	105.8	132 ± 3	2.2	99
					423.4	495 ± 36	7.3	110
SPM	y = 43,299x − 522,088	0.98	5.3 ± 0.6	12.1	105.8	71 ± 6	7.7	62
					423.0	381 ± 29	7.6	89

3.2. Effect of Storage under Refrigerated Conditions

3.2.1. Raw Mackerel

Table 3 illustrates the changes in BA content in raw mackerel fillets, measured in mg/kg, when stored under refrigerated conditions starting from the time of purchase (RM-0) and continuing for 2 (RM-2), 4 (RM-4), and 6 (RM-6) days. Figure 2 depicts the UHPLC-PDA chromatograms of the samples after different storage times at 4 °C and a standard mixture, where the dotted lines facilitate the identification of the eight BAs investigated.

Table 3. BAs (mg/kg) in raw mackerel fillets stored under refrigerated conditions (4 °C) at the time of purchase (RM-0) and after 2 (RM-2), 4 (RM-4) and 6 (RM-6) days. BAI: Biogenic Amine Index.

Biogenic Amine	RM-0	RM-2	RM-4	RM-6
Tryptamine	<3	<3	<3	<3
Phenylethylamine	<3	<3	<3	<3
Putrescine	15	23	52	63
Cadaverine	31	49	102	172
Histamine	55	71	286	728
Tyramine	<3	<3	23	27
Spermidine	11	8.8	6.9	5.8
Spermine	<3	<3	<3	<3
BAI	*8*	*14*	*54*	*123*

During the initial 2 days of storage, the HIS content increased from 55 mg/kg to 71 mg/kg, remaining below the EU regulation limit [11]. By day 4 (RM-4), HIS content surged to 286 mg/kg, a fivefold increase from RM-0. After 6 days (RM-6) of refrigerated storage, HIS content reached 728 mg/kg, while that of PUT and CAD increased by roughly four and five times, reaching 63 and 172 mg/kg, respectively. Additionally, TYR levels, initially <3 mg/kg at RM-0, rose to 27 mg/kg. The levels of TRP, PHE and SPM remained insignificant throughout the storage period. SPD content, on the other hand, decreased from 11 to 5.8 mg/kg. The freshness of the fish was evaluated using the Biogenic Amine Index

(BAI) calculated as (putrescine + cadaverine + histamine)/(spermidine + spermine + 1), where the biogenic amines in the numerator are indicators of microbial spoilage, while SPM and SPD naturally occur in living cells and their presence can counterbalance the negative impact of the spoilage indicators. The addition of 1 in the denominator helps to prevent division by zero and stabilizes the index [17]. BAI values close to or below 1 indicate minimal spoilage, values between 1 and 5 suggest some degree of spoilage, while values above 5 indicate significant spoilage. The progressive increase in BAI during storage reflected a decline in quality [24], which is not detectable by monitoring HIS alone.

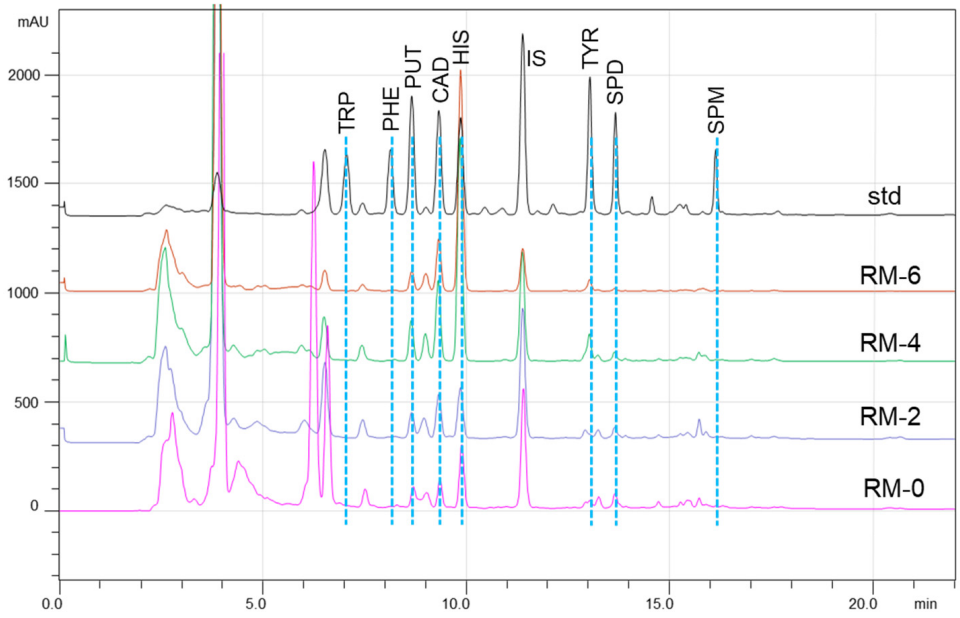

Figure 2. UHPLC-PDA chromatograms of raw mackerel (RM) fillets stored at 4 °C for up to 6 days, along with a BA standard (std) mixture. The dotted lines indicate the amine correspondence between standards and samples.

These data differ from the findings of Jiang et al. (2012) [25]. Storage at 4 °C slows down microbial proliferation compared to storage at 25 °C, as well as histamine production, and it took 8 days to exceed 50 mg/kg. It must be emphasized, however, that sample preparation was carried out under aseptic conditions, while normal household care was specifically used in this study.

The data from this study are more similar to those reported by He et al. (2020) [26], showing that after 3 to 4 days of storage at 4 °C, the HIS content increased from approximately 25 to 125 mg/kg, reaching approximately 625 mg/kg after 6 days. In fact, it is not always possible to control BA production through temperature alone, since some bacteria produce biogenic amines at temperatures below 5 °C [27,28].

3.2.2. Preserved Mackerel Fillets

Table 4 displays the contamination levels of seven samples of mackerel fillets in oil (from PM-1 to PM-7) and two samples (PM-8 and PM-9) of marinated mackerel fillets, all purchased at various deli counters in supermarkets.

The levels of HIS in the samples of mackerel in oil (between <3.0 and 6.0 mg/kg) were well below the legal limit. Very low levels of PHE were also found, with a maximum value of 7.5 mg/kg. PUT and CAD were present at levels between <3.0 and 13 mg/kg and <3.0 and 12 mg/kg, respectively. SPD (<3.0–32 mg/kg) and, to a lesser extent, SPM (<3.0–15 mg/kg) also showed varying but moderate levels.

On the other hand, TYR showed highly variable levels, ranging from <3.0 to 95.0 mg/kg. TYR is one of the most relevant vasoactive amines in food, particularly in cheese, and can induce increased blood pressure (hypertension). Furthermore, a syn-

ergistic cytotoxic effect of HIS and TYR in an in vitro model of human intestinal cells has been documented [4]. PUT, CAD, SPD, and SPM appear to be less hazardous than HIS, TYR and PHE. They are not toxic themselves and do not have direct adverse effects, but they inhibit enzymes that detoxify histamine and tyramine, resulting in potentiated toxic reactions [4].

Table 4. BAs (expressed in mg/kg) in samples of preserved mackerel (PM) samples.

BA	PM-1	PM-2	PM-3	PM-4	PM-5	PM-6	PM-7	PM-8	PM-9
TRP	<3.0	<3.0	6.6	9.3	5.1	4.2	9.1	<3.0	<3.0
PHE	<3.0	<3.0	<3.0	<3.0	6.1	5.3	7.5	<3.0	<3.0
PUT	3.9	7.1	5.9	5.6	7.7	4.1	13	3.0	<3.0
CAD	9.2	<3.0	7.3	4.9	12	6.2	9.6	27	<3.0
HIS	<3.0	<3.0	<3.0	<3.0	5.1	<3.0	6.0	<3.0	<3.0
TYR	11.3	<3.0	4.3	21	63	95	12	<3.0	<3.0
SPD	3.9	32	<3.0	5.6	8.9	6.4	28	7.2	<3.0
SPM	8.4	6.9	<3.0	11	11	15	5.0	<3.0	<3.0
BAI	***3***	***0***	***13***	***1***	***1***	***0***	***1***	***4***	***0***

Marinated mackerel fillets, with the exception of CAD in sample PM-8 (27 mg/kg), exhibited lower levels of amines (often less than 3 mg/kg).

For most samples, the BAI indicates acceptable products from a freshness perspective, with values lower than 5, while sample PM-3 could be classified as an acceptable product but with initial signs of spoilage.

The relatively low levels of BAs in canned mackerel were also documented by Weremfo et al. (2020) [29]. They found a maximum concentration of HIS and TYR, which are amines with toxicological effects, of approximately 64 mg/kg and 27 mg/kg, respectively.

Table 5 illustrates the impact of storage time under refrigerated conditions (4 °C), starting from the time of purchase (time 0), on the concentration (mg/kg) of BAs in two different mackerel fillets in oil (PM-1 and PM-2) and one marinated fillet (PM-8). The products were stored in the same resealable plastic tray provided at the time of purchase to mimic home storage, and they were not immersed in oil, contrary to the recommended histamine prevention guidelines [30].

Table 5. Content of BAs (expressed in mg/kg) in mackerel fillets in oil monitored during a refrigerated storage period of 5 days.

BA	PM-1					PM-2					PM-8				
	Time 0	1 Day	2 Days	3 Days	5 Days	Time 0	1 Day	2 Days	3 Days	5 Days	Time 0	1 Day	2 Days	3 Days	5 Days
TRP	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PHE	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PUT	3.9	3.2	3.8	3.7	<3.0	7.1	7.2	6.8	8.6	8.7	3.0	<3.0	3.1	<3.0	<3.0
CAD	9.2	8.9	6.8	9.8	9.1	<3.0	<3.0	<3.0	<3.0	<3.0	27	17	5.2	41	7.1
HIS	<3.0	<3.0	<3.0	4.3	4.5	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
TYR	11.3	9.9	21.3	73.5	57.8	<3.0	<3.0	<3.0	<3.0	7.7	<3.0	<3.0	<3.0	<3.0	<3.0
SPD	3.9	3.2	4.2	5.9	6.1	32	26	29	27	29	7.2	11	10	11	11
SPM	8.4	7.3	4.8	3.0	<3.0	6.9	5.7	6.6	6.0	5.8	<3.0	<3.0	<3.0	<3.0	<3.0
BAI	***3***	***2***	***1***	***1***	***1***	***0***	***0***	***0***	***0***	***0***	***4***	***2***	***1***	***3***	***1***

At time 0, sample PM-1 had an HIS level of less than 3 mg/kg, which remained unchanged for 2 days before slightly increasing, but staying below 5 mg/kg. PUT (3.9 mg/kg) and CAD (9.2 mg/kg) showed no significant changes during the refrigerated storage period. The content of TYR, initially at levels of 11.3 mg/kg, significantly increased during storage at 4 °C, reaching levels of approximately 60–70 mg/kg after 3–5 days. BAI increases after 1 to 3 days of storage, mainly due to the increase in the content of CAD and HIS and the decrease in the content of SPM.

In sample PM-2, TRP, PHE, CAD, and HIS content remained unchanged at levels below 3.0 mg/kg, influencing the very low level of the BAI. TYR content increased after 5 days of storage, reaching 7.7 mg/kg, and PUT content increased from 7.1 to 8.7 mg/kg. Additionally, the levels of SPD and SPM slightly decreased, from 32 to 29 mg/kg and from 6.9 to 5.8 mg/kg, respectively.

No significant changes were observed in the biogenic amine profile of marinated mackerel. There is considerable variability in the CAD content, but this does not correlate with storage time. A slight increase in the SPM content was also observed, increasing from 7.2 mg/kg to 11 mg/kg after the first day and then remained constant.

The marinated products' stability was also documented in the literature. Marinated fish products, including horse mackerel and chub mackerel, stored under refrigerated conditions reached the limit of sensory acceptance after 75 days of storage and exceeded a content of HIS of 50 mg/kg after 90 days. In all of the marinated samples, the TYR and CAD levels were beneath the detection limit (<2 mg/kg) [31].

3.3. Thawing and Cooking Effect

Raw mackerel fillets (RM-A) were left for 4 days under refrigerated storage conditions (RM-B) in order to increase the level of HIS and evaluate the effect of cooking. Additionally, the RM-A sample was subjected to thawing at room temperature (24 °C) and in the fridge (4 °C) to assess if this phase could be critical in the formation of BAs. The RM-A sample was therefore frozen and allowed to thaw for one and a half hours at room temperature (RT), wrapped in aluminum foil (as it was frozen), and stored for approximately 4 h in the fridge. After thawing, the raw fillets were analyzed, and no significant differences were observed compared to the starting sample. Only the content of CAD slightly increased from 5.8 mg/kg to 6.3 and 6.8 mg/kg after thawing at RT and in the fridge, respectively, indicating a correlation with the time of storage rather than with the temperature of storage.

After 4 days of storage, the level of HIS increased from <3.0 mg/kg (RM-A) to 1317 mg/kg (RM-B), exceeding the recommended value of 100 mg/kg by over 13 times. The other BAs also increased during the 4-day storage period in the fridge. The PHE level went from being undetectable to 35 mg/kg, the level of CAD increased by a factor of about 40 to reach 255 mg/kg, and the TYR content reached 62 mg/kg. The TRP and SPE content remained <3 mg/kg, while that of PUT had a small increment of about four times. In contrast, the content of SPD decreased from 32 to 8.3 mg/kg.

The effect of various cooking methods on high levels of HIS contamination (generated by prolonged refrigerated storage) was tested by subjecting three mackerel half fillets from the same batch to different cooking procedures (Table 6). The data obtained were then compared with those from the corresponding half fillet used as controls. The three cooking methods included steaming, baking, and boiling, as described in Section 2.2 and Table 1. Cooking times were chosen based on recipes that indicated different time/temperature combinations with different cooking methods. Cooking resulted in a decrease in the product weight of 13%, 8%, and 21% for steaming, oven-baking, and boiling, respectively.

Since BAs are thermostable compounds, heat treatments during cooking are not expected to eliminate them [16]. However, Yoon et al. (2017) [32] reported a significant decrease in TRP, PHE, PUT, and CAD concentration in Doenjang (a traditional fermented soybean product from Korea) after 10 min of roasting. Shalaby (2000) [33] observed a complete transfer of BAs from legumes into boiling water, with only a slight decrease for sprouted legumes.

As shown in Figure 3, HIS was the most abundant BA, followed by CAD and TYR. Except for HIS in the oven-baked sample, all cases reported a reduction in contamination after cooking. The highest reduction in HIS was recorded after boiling (34%), probably due to its dissolution in boiling water. Boiling also resulted in the highest percentage reduction for CAD (35%) and TYR (38%), compared to other cooking methods. The increase in HIS concentration observed after oven-backing (+23%) is in agreement with data reported by Kim et al. (2021) [34], who found that due to the chemical decarboxylation of amino acids,

the total BA content in mackerel increased up to 190% when the roasting temperature was increased from 150 to 250 °C for 15 min.

Table 6. BAs' (expressed in mg/kg) evolution in mackerel after thawing and cooking. RM-A: raw mackerel fillets at the time of purchase; RM-B: raw mackerel fillets (RM-A) left for 4 days under refrigerated storage conditions (4 °C); (a) raw mackerel half fillet left for 4 days at 4 °C used as a control; (b) cooked mackerel half fillet; RT: room temperature.

BA			RM-A Thawing		Steaming		Oven-Backing		Boiling	
	RM-A	RM-B	RT	4 °C	RM-C (a)	RM-C (b)	RM-D (a)	RM-D (b)	RM-E (a)	RM-E (b)
TRP	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PHE	<3.0	35	<3.0	<3.0	32	13	43	6.9	21	14
PUT	3.5	16	3.3	3.7	14	12	17	12	11	15
CAD	5.8	225	6.3	6.8	192	172	342	297	281	231
HIS	<3.0	1317	<3.0	<3.0	1417	1426	1038	1384	1336	1115
TYR	<3.0	62	<3.0	<3.0	85	65	71	49	66	52
SPD	32	8.3	29	32	8.3	5.8	13	13.9	8.8	13
SPM	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	4.0	3.2	3.2	3.7
BAI	***0***	***168***	***0***	***0***	***175***	***235***	***76***	***93***	***125***	***76***

The data in this table are expressed based on the product as consumed, without adjustments for weight loss. Figure 3, on the other hand, presents data normalized for the weight loss recorded after cooking.

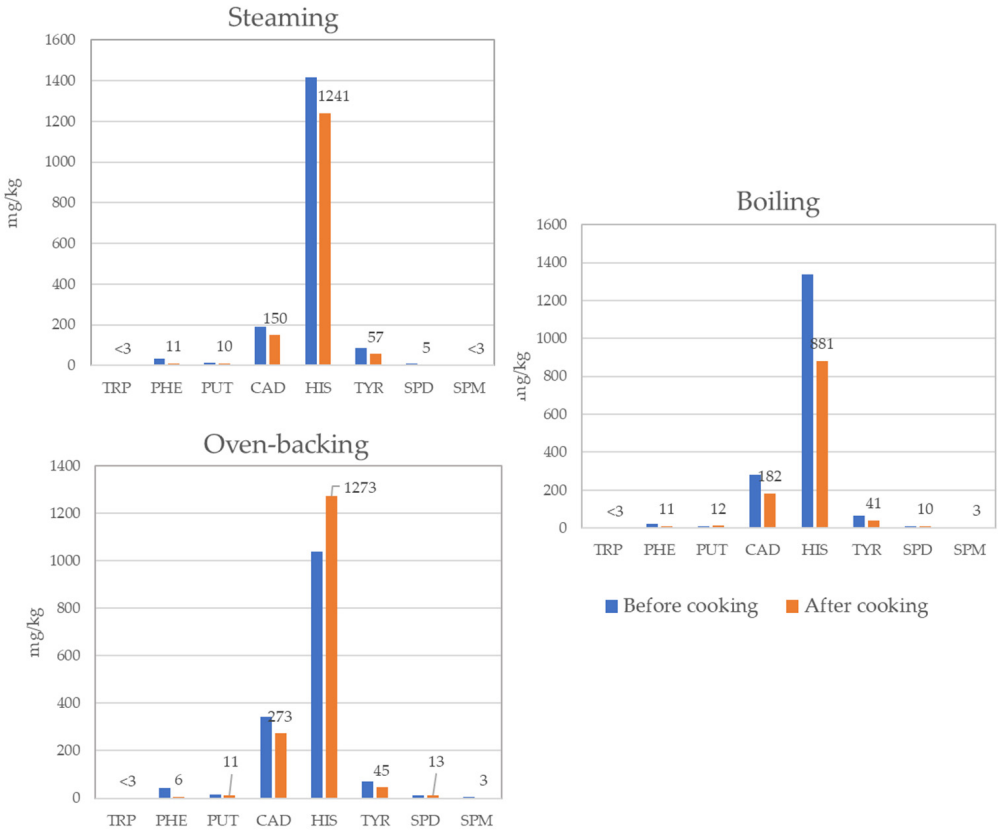

Figure 3. Normalized BA contents (mg/kg) before and after cooking.

4. Conclusions

Due to the importance of detecting BAs for both safety and freshness characteristics, it would be useful to have a method and legal limits not only for HIS but also for the other BAs. The method proposed by EU regulation using HPLC-UV, after slight modifications

to adapt it to a UHPLC instrument, is suitable for the separation of eight BAs and their analysis in mackerel samples. This also allowed for all the parameters necessary to calculate the BAI, an index of fish freshness.

The evolution of the BA content in mackerel fillets was monitored under refrigerated storage conditions for different periods. The results confirm that storage for more than one day can lead to a high HIS content, often accompanied by the presence of TYR, PUT and CAD, which can increase HIS toxicity by inhibiting intestinal metabolizing enzymes. On the contrary, preserved mackerel samples (in oil and marinated) showed more stability, with no significant increase in amine content during a 5-day storage period, despite not being stored as recommended by the guidelines.

The thawing phase does not to significantly influence the increase in the content of biogenic amines, whether conducted at room temperature or in a refrigerator. What seemed to have the most impact was not the thawing temperature, but the thawing time. Finally, three different cooking methods (steaming, oven-baking and boiling) were tested to investigate their possible influence on particularly high amine levels. BAs do not undergo volatilization or thermal degradation during cooking, but more polar amines can dissolve in water, causing a remarkable decrease, especially in boiled mackerel fillets. On the other hand, the higher temperature reached with oven baking (160 °C) seems to cause an increase in HIS content due to the chemical decarboxylation of histidine. These preliminary results, obtained from single trials, should be confirmed by analyzing an adequate number of replicates.

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