Evaluation of Biogenic Amine and Free Fatty Acid Profiles During the Manufacturing Process of Traditional Dry-Cured Tuna

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Abstract

The aim of this study was to investigate the changes in the formation of biogenic amines and free fatty acids occurring dur- ing the manufacturing process of a traditional dry-cured product based on yellowfin tuna, *Thunnus albacares* (protected geographical indication—*Mojama*) and how the different processing stages could affect food safety aspects. The biogenic amines profile was determined by HPLC–DAD, following the official methodology, and free fatty acids were quantified by GC–MS. Histamine levels found in all stages of the manufacturing process did not exceed the maximum limits established in the European Commission (100–200 mg/kg) and US Food and Drug Administration (50 mg/kg) regulations. Other bio- genic amines, such as cadaverine and putrescine, were detected at low level or below the limit of detection. Yellowfin tuna filets could be classified as lean fish flesh, presenting 1.18% fat on average. An increment in the free fatty acid fraction was evidenced along the manufacturing process, ranging from 10.37% of the total lipids in fresh loins to 16.88% in the dry-cured filet product. The results indicated that the traditional manufacturing process of *mojama*, consisting of salting and drying tuna loins kept at a controlled temperature, promoted a moderate lipolysis phenomenon, and the formation of free fatty acids with high proportions of unsaturated fatty acids, likely arising from the lipolysis of muscle phospholipids.

Keywords *Thunnus albacares* · *Mojama* · Food safety · Histamine · Free fatty acids · DHA

Introduction

The increasing interest of consumers in food quality nowa- days has led to them choosing more new fish products, considered to possess high nutritional value and the ability to positively influence human health (Mercogliano et al., 2013). In this context, fresh tuna and its derived products are important around the world due to their high market demand. Regarding its nutritional value, tuna represents a good source of high-quality protein (rich in essential amino acids), micro- and macro-nutrients, and high-quality lipids, including relevant amounts of unsaturated fatty acids and fat-soluble vitamins (Esteves & Anibal, 2019).

Mojama is a traditional tuna product manufactured in several Mediterranean regions of the Iberian Peninsula (Andalusia, Murcia, Alicante, and Valencia). It is produced following a traditional process, mainly involving salting and drying (known as *curing*) fresh tuna loins (Esteves & Aníbal, 2019). Drying is one of the earliest known methods for preserving food devised by humans and it is currently the most common operation in the seafood industry (Arason et al., 2014). During the *mojama* manufacturing process, drying is preceded by a salting stage, aimed at decreasing the availability of water. Thus, the reduction of the water activity inhibits microbial growth and several enzyme-induced chemical reactions (Roseiro et al., 2017). This method can ensure the safety of food processing conditions during the whole production and sales chain, besides having a great impact on the organoleptic characteristics of the final product. Dry-cured products have a combination of flavor, texture, color, and nutritional quality traits that are unique and highly valued by consumers. For these reasons, Spanish *mojama* is considered to be a delicacy (Esteves & Aníbal, 2019). The traditional process and the value of the dry-cured tuna were reflected between 2015 and 2016 with the registra- tion of two Protected Geographical Indications (PGI) within the European Union's quality system, namely "Mojama de Barbate" (Cádiz, Spain) Regulation (EU) 2016/199 modified by Regulation (EU) 2020/1326, and "Mojama de Isla Cristina" (Huelva, Spain) Regulation (EU) 2015/2110, modified by Regulation (EU) 2020/913, in the Andalusia region.

Despite the high nutritional value, it is important to maintain the proper storage conditions, since the consumption of spoiled tuna or its derived products has been related to food safety issues, such as the conceivable presence of biogenic amines. These compounds are organic nitrogenous bases of low molecular weight, resulting from the decarboxyla- tion of amino acids or by amination and transamination of aldehydes and ketones (EFSA, 2011). Particularly, histamine (HIS) is formed by enzymatic decarboxylation of free histidine. Large amounts of this amino acid can be found free in the muscle of fish belonging to the *Scombridae* family, such as tuna or bonito (Ordóñez et al., 2016). Hence, when histidine is heavily decarboxylated to HIS, the consumption of fish products can cause a foodborne intoxication known as "scombroid fish poisoning", which is related to differ- ent symptoms, such as headache, palpitations, urticaria, and vomiting (Ishimaru et al., 2019). The US Food and Drug Administration (FDA) established a regulation fixing a maximum level for HIS of 50 mg/kg in scombroids and related families as an indication of its potential health risk (FDA, 2011). Similarly, the European Commission established maximum levels of 100–200 mg/kg of histamine in

fresh fish; that is, in the sampling selection of batches for every 9 samples randomly collected, none of them can exceed the histamine concentration of 200 mg/kg, and a maximum of 2 of the selected samples can have a histamine concentration between 100 and 200 mg/kg (Regulation (EU) No. 2073/2005). The other biogenic amines that can be formed and found in food products are not defined in any regulations at present.

Beyond the regulation issues, we need to clarify the health-related issues observed in the literature. In general, the studies focused on determining the tyramine (TYR) levels of toxic concentration in humans obtained very different data, which highlights the difficulty for the legislative regulation of this amine. According to Askar and Treptow (1986), a TYR dose of 10–80 mg can cause toxic inflammation, and more than 100 mg can induce migraine. However, Nout (1994) suggested that the maximum allowable level of this amine in foods should be in the range of 100–800 mg/ kg. A report from the European Food Safety Authority (EFSA) concluded that there is still insufficient information to establish the no observed adverse effect level (NOAEL) in humans. Thus, no adverse effects are observed in healthy individuals at concentrations of 600 mg (EFSA, 2011). Likewise, the same authors demonstrated lower toxicological properties for putrescine (PUT) and cadaverine (CAD). However, it has been demonstrated that these compounds can interact with detoxifying enzymes and enhance the toxicological effects of HIS or TYR (Ordoñez et al., 2016). The total content of biogenic amines in dry-cured fish products (not regulated) is affected by several external factors such as the manufacturing process and preservation. Thus, an inadequate control of some parameters during the drying/maturation processes, such as temperature, pH, or salt content, could favor the accumulation of free amino acids in the tissues by enzymatic decarboxylation, and therefore stimulate the formation of unsafe levels of biogenic amines (Mercogliano et al., 2013).

As mentioned above, fish is a good source of high-quality lipids, including relevant amounts of unsaturated fatty acids and fat-soluble vitamins. With regard to fatty acids (FAs) composition, tuna flesh from different species is known to contain large amounts of eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, the most representative long-chain fatty acids (FAs) of the n-3 series (Roy et al., 2010). The consumption of these FAs through the diet has been associated with beneficial effects for consumers, mainly related to the prevention of cardiovascular, inflammatory and autoimmune diseases, and the promotion of a correct neural development (Cejas et al., 2004). A high unsaturation degree of FAs in the lipid portion is known to affect the rate of lipolysis and oxidation reactions occurring during the manufacturing processes of food products (Belitz et al., 2009). Lipolysis phenomena are known to cause the release of free fatty acids (FFAs) in food matrices, mainly affecting the phospholipid fraction of tissues. This is due to the high unsaturation of FAs bonded in phospholipid molecules. Lipolysis has been observed during the salting and drying steps in transformed products of animal origin (Toldrá, 1998), and it is considered to influence the organoleptic properties of the final product (Xu et al., 2008). Lipolysis mechanisms and the formation of FFAs have been widely studied in dry-cured meat products, but in a lesser extent in dry-cured fish products.

Therefore, the aim of this study was to evaluate the effect of the *mojama* production process on the formation of two groups of compounds related to food safety, namely biogenic amines and free fatty acids. In this sense, we studied the different stages of the *mojama* production process (initial fresh tuna, salting, salt-washing and drying steps) in order to (a) evaluate the content of BAs throughout the process to detect possible critical points of formation and/or accumulation and (b) study for the first time the formation of FFAs that could have a great impact on the final organoleptic proper- ties of the *mojama*.

Materials and methods

Chemicals and reagents

Toluene, perchloric acid, LC–MS grade acetonitrile and methanol, LC–MS deionized water, sodium carbonate (purity ≥ 99%), and potassium carbonate (purity ≥ 99%) were obtained from Panreac (Barcelona, Spain). Chloroform, hexane, acetyl chloride, butylhydroxytoluene (BHT), and sodium chloride (purity ≥ 99%) were purchased by Merck KGaA (Darmstadt, Germany). Spermidine (SPD), putrescine (PUT), histamine (HIS), methylamine (MET), cadaverine (CAD), tryptamine (TRP), tyramine (TYR) and spermine (SPM), dansyl chloride, L-proline, and 1,7-diaminoheptane were supplied by Sigma-Aldrich (Darmstadt, Germany). Analytical–grade standards of fatty acids: FAME37 (CRM47885), cis-11-vaccenic acid methyl ester (CRM46904), cis-7,10,13,16,19-docosapentenoic acid methyl ester (CRM47563), cis-4,7,10,13,16,19-docosahexaenoic acid methyl ester (05832), and nonadecanoic acid (N5252) were supplied by Merck KGaA (Darmstadt, Germany).

Mojama processing

Dry-cured tuna "*mojama*" were produced from fresh yellowfin tuna fish (*Thunnus albacares*-YFT), weighting between 40 and 60 kg and captured in FAO 34 area (Eastern Atlantic Ocean) during the year 2020. Processing was carried out according to the traditional process of two Spanish Protected Geographical Indications (PGIs) (Fig. 1). The period of storage in the companies of tunas selected for processing was 3 months at − 20 °C.

The process begins with the head being cut off and the tuna being eviscerated. Fresh tunas are washed with drinking water and then the tuna is cut into four loins: the upper two (black) and the lower two (white), removing the skin and bones. These four loins are subsequently cut into strips of a thickness no more than 5 cm and are categorized depending on their fat level (class extra or primera). This cutting process is known as "*ronqueo*" and is performed manually by specialized people. During the salting phase, the strips of tuna loins are covered in sea salt for a period of between 18 and 50 h depending on different factors, such as the size and thickness of the strips, their fat con- tent and the moisture of the product. This standardized processing step is needed to promote water and fat losses in the tissues and, consequently, to increase the stability of the product. Then, during the salt washing phase the strips of tuna loins are washed to remove the

excess of salt in two successive phases. During the first one, the strips are given a washing to remove any salt clinging to them. Subsequently, they are placed in baths of cold water and left to rest placed in containers filled with water to eliminate the salt on the surface for 7–9 h. This phase is performed to achieve the optimum level of salt concentration. At the end of the step, the strips of loins are compressed forcing an additional fat and water losses. Once pressing is completed, in the drying phase the tuna strips are dried on a horizontal surface for at least 2 days and finally are hung for 18 ± 3 days. The conditions of temperature and humidity will be between 16 to 17 °C and 60 to 70%, respectively. The dry-cured product was vacuum-packaged in transparent plastic bags, allowing *mojama* to maintain its physical and chemical characteristics and to increase the shelf life (6 months) at refrigeration temperatures (5 ± 1 °C).

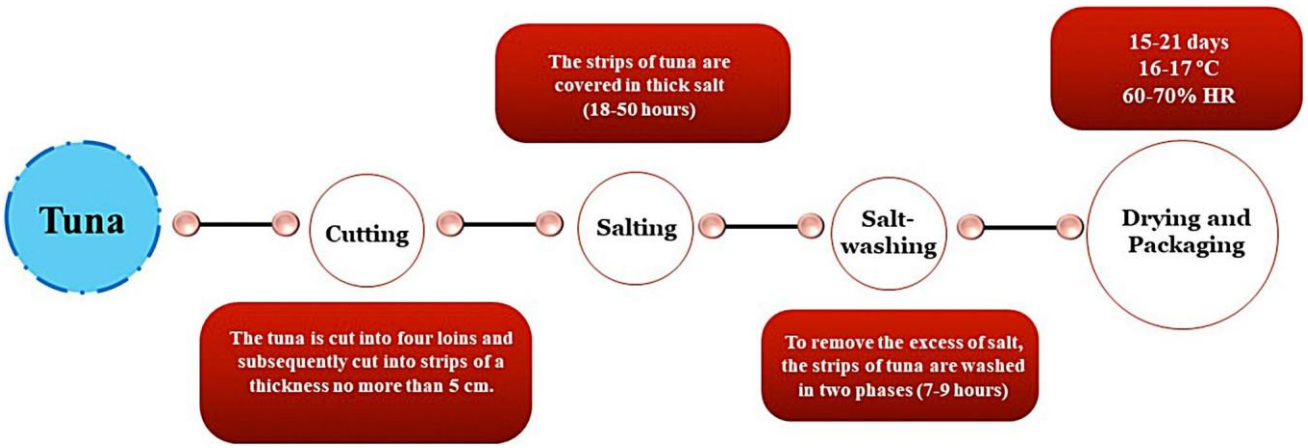

Fig. 1 Flow chart for dry-cured tuna production

Sampling

The research was carried out sampling *mojama* (dry-cured tuna) samples at different stages of manufacturing obtained by processing 45 fresh yellowfin tuna (*Thunnus albacares*- YFT) weighting between 40 and 60 kg and captured in FAO 34 area (Eastern Atlantic Ocean) from May to November 2020. The 45 tuna samples were distributed as follows: 11 from each of the companies numbered 1, 2, and 3 and 12 more provided by the company number 4. *Mojama* processing and curing conditions were the same in the four companies involved and following the traditional process of the Spanish Protected Geographical Indications (PGIs), as summarized in Fig. 1. The strips of fresh tuna (after cutting) classified as class *Extra*, were labeled to have full traceability information during the processing. 500 g were sampled for each tuna and sample steps. The samples obtained after the fresh tuna, salting, and salt-washing steps were stored on ice during the sampling day and their transportation to the laboratory, while the samples collected for the final product (*mojama*) were vacuum-packed and kept at refrigeration temperatures (5 ± 1 °C) following the commercial procedure until their arrival at the laboratory. Then, the samples were stored at − 20 °C until their analysis.

Determination of Biogenic Amines

The determination of biogenic amines was performed following the official reference method in Europe for histamine testing in fish and seafood required by the Regulation (EU) No. 2073/2005. Briefly, the tuna samples (5 ± 0.1 g) were added to the centrifuge tube with 10 mL of 0.2 M perchloric acid and 100 µL of 1,7-diaminoheptane (internal standard, 6.4 mg/L), and everything was mixed in an ice bath using an Ultraturrax® (Stauten, Germany). When the mixture was homogenized, the samples were centrifuged at 15,000 rpm at 4 °C for 10 min. For derivatization, 100 µL of supernatant was transferred into an Eppendorf tube and mixed with 300 µL of saturated Na_2CO_3 solution and 400 µL of dansyl chloride solution (7.5 mg/mL). The tube was vortexed and incubated for 5 min at 60 °C in a stirred water bath (Unitronic Reciprocating Shaking Bath, model 6,032,011, J.P. Selecta, Barcelona, Spain). After cooling the tube, 100 µL of L-proline solution (0.1 mg/L) was added to neutralize the excess of dansyl chloride and it was placed in the dark for 15 min. Later, 500 µL of toluene were added and the tubes were vortexed. The organic phase, containing derivatized histamine, was recovered by freezing the aqueous phase (− 80 °C for a minimum of 15 min). The isolated organic phase was concentrated by drying using a Speedvac concentrator (Eppendorf, Hamburg, Germany) and the dry residue was resuspended in acetonitrile:water (60:40, v/v). The bio- genic amines profile was analyzed using a liquid chromato- graph HPLC system (Perkin Elmer, Waltham, MA, USA) with a diode-array detection (DAD) detector set at 254 nm following the EC regulation. The separation was performed on a reverse-phase column Luna C18 (5 µm, 250 × 4.6 mm) equipped with a pre-column C18, 4.0 × 3.0 mm, from Analytical Phenomenex (Torrance, CA, USA). The biogenic amines were quantified using the calibration curve for each individual standard. All measurements were performed in duplicate. The intra- and inter-day precisions were studied with quality control samples and the data was calculated as relative standard deviation (RSD). The intra-day samples were measured in five replicates in 1 day, and inter-day variation was measured on 5 separate days (Supplementary Table 3).

Determination of Free Fatty Acids (FFAs)

Lipid Extraction and Lipid Fractions Separation

Total lipids were extracted from tuna samples according to the method of Folch et al. (1957) using a mixture of $CHCl_3$:MeOH (2:1, v/v) on 3.0 ± 0.2 g of minced sample. Total lipid content was calculated gravimetrically. The total lipid extract was used for lipid fractions separation by a solid phase extraction (SPE) method, using Sep-Pak Aminopropyl (NH_2) cartridges (6 mL bed capacity) (Waters Corporation, Milford, MA, USA). The cartridges were previously activated with 8 mL of hexane to prevent complete drying. 15 mg of total lipids were dried and re-dissolved in 1 mL of hexane:$CHCl_3$:MeOH (95:3:2, v/v) as solvent. The extract was then charged on the SPE columns. After the discard of the neutral lipid fraction, free fatty acids (FFAs) were eluted using 5 mL of a diethyl ether:acetic acid (98:2, v/v) mixture. The flow rate was adjusted to approximately 1 mL/ min. The FFAs fraction was collected in a screw-cap glass tube (10 mL), dried under vacuum and recovered in 1 mL of toluene containing nonadecanoic acid (C19:0) as internal standard (250 µg/mL).

Derivatization of Free Fatty Acids

Methyl esters of free fatty acids (FFAMEs) were prepared using an acid catalyst according to Christie (1993). Briefly, 1% methanolic hydrogen chloride (2 mL) was added to each tube and then they were sealed and heated at 50 °C overnight. The day after, the FFAMEs were extracted with 2 × 2 mL of n-hexane with butylated hydroxytoluene (BHT) at 0.01% as antioxidant. A total of 5 mL of 5% NaCl solution were added to each sample to clean the extract from any other adduct formed during the esterification reaction and 2 mL of a 1 M potassium carbonate solution were used to buffer the acidity of the solution. The solution containing the extracted FFAMEs was dried, re-dissolved in 1 mL of hexane, put into an amber glass vial and stored at − 80 °C until the analysis by GC–MS.

FFAMEs Analysis by GC–MS

FFAMEs analysis was performed on a CP-3800 gas–chromatograph (Varian, Inc., Walnut Creek, CA, USA) fitted with an automatic sampler (COMBI PAL) and a 4000MS mass spectrometer (Varian, Inc., Walnut Creek, CA, USA). The extract (1 µL) was injected into the gas-chromatograph in split mode (split ratio 1:10) with the injection port set at 225 °C. The carrier gas was helium, with a flow rate of 1.0 mL/min at a pressure of 12.5 psi at the injection port. A CP-Sil88 fused silica capillary column (60 m × 0.25 mm I.D., 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used to separate the FFAMEs. The oven temperature program was the following: from 100 °C to 180 °C at 3 °C/min, then from 180 to 225 °C at 2.5 °C/ min and this last temperature was maintained for 2 min. FFAMEs were identified relative to known external stand- ards (FAME37 mixture—CRM47885, cis-11-vaccenic acid methyl ester -CRM46904, and cis-7,10,13,16,19-Docosapentenoic acid methyl ester—CRM47563) supplied by Supelco (Sigma Aldrich, San Luis, MO, USA). The mass detector operated in electron ionization (EI) mode with an emission current of 70 µAmps in the ion trap. The full scan range of the MS was set to m/z 45–350, with a scanning rate of 0.22 s/scan. Data were acquired by MS Data Review (Varian, Inc., Walnut Creek, CA, USA) and then the chromatograms were analyzed by Xcalibur™ software by Thermo Fisher Scientific (Waltham, MA, USA). The calibration curve was built preparing reference stock solutions of target analytes (the mixture and the individual standards) in hexane and stored in a vial under nitrogen at − 80 °C. Calibration point solutions were prepared diluting the stock solution in hexane, and in a concentration range from 0.5–1.5 to 275–825 g/mL. An exception was docosahexaenoic acid methyl ester (22:6n-3 ME), which was injected alone in the range of concentrations from 2.5 g/mL to 1.2 mg/mL due to its high concentration. Nonadecanoic acid methyl ester was added as internal standard to each point of the calibration curve at the same concentration (250 µg/mL). The intra- and inter-day precisions were studied with quality control samples and the data was calculated as relative standard deviation (RSD) (Supplementary Table 3). The intra-day samples were measured in five replicates in 1 day, and inter- day variation was measured on 5 separate days.

Statistical Analysis

A univariate statistical analysis was performed using R software (v. 3.6.3, R Core Team, Vienna, Austria). A one–way ANOVA followed by a comparison of means according to the Tukey's honestly significant

difference (HSD) post–hoc tests were applied to identify the differences among the different tuna samples and the factor studied (manufacturing process). The level of significance was established at $p < 0.05$.

Results and Discussion

Biogenic Amines Analysis

The biogenic amine content was evaluated for the different steps of the manufacturing process of Spanish *mojama.* In addition, the content of total and individual biogenic amines in the black and white muscles of the tuna was studied. No statistical differences ($p > 0.05$) were found for all the para- meters studied between the two types of muscle (Supplementary Table 1).

Table 1 shows the data for the different BAs analyzed in this study. Three of them, TRYP, PHE, and PUT, were not detected in any of the samples from the different steps of the manufacturing process, as it was stated in literature (Sánchez-Parra et al., 2022; Xu et al., 2023). Regarding CAD, an increase after the last step in the final product (6.71 ± 0.40 mg/kg) was observed. CAD is formed prior to HIS and/or accumulated as a faster rate during storage in tuna (Rossi et al., 2002). Our results are in agreement with the levels found in the literature by Roseiro et al. (2017) (4.62–19.97 mg/kg) in *muxama* tuna samples elaborated with salt contents (< 5% and 5–10%, respectively) close to those included in the manufacturing process for the IGPs. CAD has been proposed as a suitable marker of the decom- position for fish and fish products (Prester, 2011). However, no threshold levels have been established for this amine in food because, at present, there are no data about the dose–response effects in humans (EFSA, 2011). A concentration below the detection limits is evidence of both good storage preservation methods of the fresh tuna samples and proper handling during the manufacturing process.

The average concentration of MET in fresh tuna was4.91 ± 0.15 mg/kg. We observed slight increases during the four steps of the manufacturing, reaching a concentration of 6.24 ± 0.12 mg/kg after the drying stage. However, those increases were not statistically significant ($p > 0.05$). This molecule is a volatile amine that can be formed in small levels from non-nitrogen compounds such as aldehyde and ketones due to the process of reductive amination. Among others, MET plays an important role in the off-flavor taste of fish (Jeewantha & Abeyrathne, 2015).

Table 1 Means ($n = 45$), standard errors of the means (std.error) and ranges (min–max) of biogenic amines (mg/kg) found for different stages of *mojama* production process

Biogenic amines		Stages				
		Fresh tuna	Salting	Salt-washing	Drying	*p*-value
Tryptamine (TRYP)	mean ± std.error	n.d	n.d	n.d	n.d	
	min–max					
Phenylethylamine (PHE)	mean ± std.error	n.d	n.d	n.d	n.d	
	min–max					
Putrescine (PUT)	mean ± std.error	n.d	n.d	n.d	n.d	
	min–max					
Cadaverine (CAD)	mean ± std.error	n.d	n.d	n.d	6.71 ± 0.40 a	***
	min–max				n.d. – 27.34	
Methylamine (MET)	mean ± std.error	4.91 ± 0.15	5.24 ± 0.18	5.98 ± 0.20	6.24 ± 0.12	ns
	min–max	1.91 – 7.90	n.d. – 13.72	n.d. – 13.93	n.d. – 16.02	
Tyramine (TYR)	mean ± std.error	5.84 ± 0.17 a	3.16 ± 0.29 b	1.84 ± 0.04 c	1.40 ± 0.14 c	***
	min–max	n.d. – 15.14	1.18 – 4.36	0.70 – 3.15	n.d. – 2.43	
Histamine (HIS)	mean ± std.error	0.51 ± 0.03 b	0.47 ± 0.02 b	1.90 ± 0.11 a	1.08 ± 0.11 ab	**
	min–max	n.d. – 3.88	n.d. – 0.51	n.d. – 16.75	n.d. – 5.97	
Spermidine (SPD)	mean ± std.error	36.05 ± 2.97 a	34.79 ± 1.86 a	27.04 ± 1.43 ab	16.39 ± 1.21 b	***
	min–max	n.d. – 186.13	23.41 – 152.16	4.17 – 149.01	2.21 – 135.36	
Spermine (SPM)	mean ± std.error	16.22 ± 0.72 a	15.58 ± 0.96 a	12.34 ± 0.49 ab	9.37 ± 0.59 b	***
	min–max	n.d. – 24.05	11.94 – 43.16	n.d. – 24.34	n.d. – 32.19	
Total amines	**mean ± std.error**	**63.53 ± 4.28 a**	**59.24 ± 3.14 a**	**49.10 ± 2.75 b**	**41.19 ± 1.81 b**	***
	min–max	**11.34 – 121.37**	**48.29 – 99.78**	**14.59 – 135.48**	**14.02 – 93.22**	

Values with different letters are significantly different (*ns* non-significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) *n.d* not detected

The average value found in the fresh tuna for TYR was 5.84 ± 0.17 mg/kg (n.d.–15.14 mg/kg). This result agrees with those reported in literature for fresh tuna samples by Santos et al. (2019) (7.66 mg/kg). We found a significant decrease ($p < 0.001$) in the TYR concentration after the salting step (54.11%, Table 1). Several authors reported that this could be explained by changes in the physico-chemical properties of tuna during the salting step, such as a decrease in water activity and antimicrobial activity, increased water retention capacity and a decrease in the activity of some enzymes (Roseiro et al., 2017; Ruiz- Capillas & Herrero, 2019). These changes make the salting step an essential stage during the manufacturing process to avoid the formation and/or accumulation of BAs. Possibly the decrease of TYR in this stage was due to transfer of compounds to the drained water by the salt concentration. Moreover, the TYR content decreased during the salt-washing step (1.84 ± 0.04 mg/kg), which can be explained by the transfer of different components such as water, salt (NaCl), and some nitrogenous compounds including biogenic amines (Arason et al., 2014). Finally, the TYR concentration from the mojama samples and those from the previous step (salt-washing) were statistically similar ($p > 0.05$). The results were lower than those found by Roseiro et al. (2017) (102.99 to 155.82 mg/kg) and by Saaid et al. (2009), who analyzed thirteen different dry-cured fish products (n.d. to 369.4 mg/kg). Despite the mentioned values, all the results were far below the concentrations (> 1000 mg/kg) that could cause toxicological effects in humans (EFSA, 2011).

HIS is the most important BA in tuna, this being a specie with high concentrations of the amino acid histidine. As shown in Table 1, the HIS levels remained stable during the first two steps of the *mojama* production process (0.51 ± 0.03 mg/kg and 0.47 ± 0.02 mg/kg, respectively). This result agrees with those described in literature, where the concentrations of histamine in fresh fish were below 1 mg/kg (Ordóñez & Callejón, 2019; Sánchez-Parra et al., 2022). HIS is formed in raw fish by bacterial formation, mainly histidine decarboxylase activity. Thus, histidine existing in tuna muscles can be transformed in HIS under inadequate storage conditions, such as high temperatures, where this reaction is accelerated (Mercogliano et al., 2013). After the cutting step, no increase of histamine was observed (0.47 ± 0.02 mg/kg). This can be attributed to the addition of salt and the decrease in a_W. Studies support the idea that the use of 3–6% of salt during the salting step, a range commonly used in the preparation of *mojama,* could inhibit the growth of histamine-forming bacteria (Flick & Granata, 2004). Moreover, some authors reported that during the salt- ing of fish, the HIS content can diffuse from the flesh into the brine (Hernandez-Herrero et al., 2002). After the salt-washing of the loins, a significant increase of HIS was observed ($p > 0.01$; Table 1), indicating a possible proliferation of microorganisms in this stage that promote the increase in the concentration of BAs. Frequent changes of the washing water could be necessary. Nevertheless, the mean HIS level was 1.90 ± 0.11 mg/kg, ranging from n.d. to 16.75 mg/kg. Finally, after the drying step, the mean histamine concentration in the tuna loins was 1.08 ± 0.11 mg/kg, a no significant differences were found comparing to the previous step. The HIS levels found in the samples during the different stages were far below the thresholds established by the European Regulation (100–200 mg/kg) and the US Food and Drug Administration (50 mg/kg). This fact is mainly due to high quality raw starting material and the adequate processing conditions that control microbial growth and therefore avoid the formation of HIS.

Regarding polyamines, namely SPD and SPM, the mean concentrations found in the fresh tuna

samples were 36.05 ± 2.97 and 16.22 ± 0.72 mg/kg, respectively. These polyamines have been identified previously in fresh fish, mainly because they are naturally present in live fish, playing an important role in their metabolism and cellular growth (Du et al., 2002). The content of both polyamines decreased significantly after the drying stage to obtain the final product (16.39 ± 1.21 and 9.37 ± 0.59 mg/kg for SPD and SPM, respectively). Thus, the formation of SPD and SPM in fish is not related to bacterial spoilage (Veciana-Nogués et al., 1997). In general, they do not pose a serious risk to human health and their content in all processing steps were low.

In general, the individual BA contents found during the manufacturing process of *mojama* were never a cause for concern. The levels of HIS in every stage were low and far below the regulated values established by the European Commission and the FDA. Furthermore, the remaining BAs did not show any increase during the salting step, rather they decreased significantly with the drying process. These findings suggest the importance of the use of good hygiene practices and good manufacturing practices by the companies from PGIs "Mojama de Barbate" and "Mojama de Isla Cristina", in such a way as to prevent microorganisms from spreading during the handling of raw tuna, and consequently, accumulated during the processing and storage of the final dry-cured tuna product.

Free Fatty Acids Analysis

According to the classification described by Stansby (1976), *Thunnus albacares* (YFT) can be ranked as a low-fat species (fat content < 2%). Effectively, we found a total lipid content of 1.18% in YFT fresh loins after cutting (Table 2). In this sense, it is interesting to highlight that YFT analyzed in this study appeared to be leaner than other tuna species, such as bigeye tuna *(Thunnus obesus),* another species commonly used in the Iberian Peninsula for the production of the dry-cured tuna muscle products, which was reported to reach average lipid contents of 4.47% *(*Roseiro et al. (2017). However, the bigeye tuna is not included in the specifications of *mojama* PGIs "Mojama de Barbate" *and* "Mojama de Isla Cristina", which indicate that only the species *Thunnus albacares* and *Thunnus thynnus* are allowed in the manufacturing process. A low lipid content could be considered very important since fish-meat characterized by a low-fat content is less susceptible to oxidation. The final dry-cured tuna product (*mojama*) contained a concentration of total lipids significantly higher (1.84%) (Table 2) than the samples from the previous steps (1.18% at fresh tuna, 1.02% at salting, 1.25% at salt-washing). Such an increment could be related to a concentration effect related to the manufacturing process, resulting from the water loss in the tuna loins during the drying stage.

Another phenomenon observed in this study was a varability of the total lipid content among fresh tuna loins from different fish during the sampling period (0.36–2.50%). Similar results were previously observed, where a significant variation among loins obtained from different fish was reported (Roseiro et al., 2017). This is an expected phenomenon, since fat distribution—and therefore FA compo- sition—in fish flesh is very variable and strictly dependent on the species (Passi et al., 2002), and other physiological or external factors, such as age, season of capture, location, environmental conditions, and diet composition (Özogul & Özogul, 2007).

In this study, we evaluated the amount of free fatty acids (FFAs) at different stages of *mojama* production, considered as indicators of the extent of lipolysis in transformed products during the

manufacturing process. No statistical differences ($p > 0.05$) between black and white tuna muscles were found in the content of free fatty acids (Supplementary Table 2). The FFAs fraction accounted for 10.06–10.37% of total lipids during the first steps of manufacturing, namely fresh tuna, salting, and salt-washing of the tuna loins (Table 2). The amount of FFAs measured in fresh loins analyzed in this study was comparable to the levels detected by Sprague et al. (2012) in the fresh muscle of *Thunnus thynnus* brood stock, accounting for 9.9 ± 0.9% of total lipids. Successively, we observed a significant increase for the mean content of FFAs ($p < 0.001$), which reached 16.88% of total lipid, at the end of the dry-curing process (Table 2). As expected, the increase observed in the total amount of FFAs between the salt-washing and drying stages was the result of an equal contribution from all the classes of FAs detected: saturated (SFAs), branched-chain (BCFAs), mono- unsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids.

As shown in Table 2, SFAs represented 40–50% of the total FFAs, with palmitic acid (16:0) being the most abundant and showing a significant increase from the first steps of processing, fresh tuna (202.19 ± 13.26 μg/kg), salting (189.75 ± 11.22 μg/kg) and salt-washing (206.05 ± 14.87 μg/ kg), to the end of the process (495.8 ± 39.26 μg/kg in *mojama*). Then, MUFAs represented 18–23% of total FFAs, with oleic acid (18:1n-9) and palmitoleic acid (16:1n-7) being the most abundant. Both these fatty acids showed a 3.5-fold increase during the manufacturing process of *mojama,* starting from a concentration of 17.50 ± 1.62 μg/ kg for palmitoleic acid and 68.71 ± 3.46 μg/kg for oleic acid in fresh tuna and reaching values of 58.81 ± 4.53 μg/kg and 246.27 ± 26.37 μg/kg in *mojama*, respectively. Furthermore, among PUFAs, which represented 25–37% of total FFAs, EPA (eicosapentaenoic acid, 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3) were the most abundant, both showing a significant increase ($p < 0.001$) during fresh tuna trans- formation to *mojama.* Particularly, EPA increased from the range 13.71–23.23 μg/kg during the first steps (fresh tuna to salt-washing) to 56.64 ± 3.26 μg/kg in *mojama;* meanwhile, DHA increased from the range 85.07–170.71 μg/kg during the first steps to 415.71 ± 22.40 μg/kg in *mojama.*

Finally, even BCFAs, which represented a minor proportion (0.70–0.92%) of total FFAs, showed a significant increment ($p < 0.001$) between the first steps of processing (5.43 ± 0.40 μg/kg, 5.38 ± 0.39 μg/kg, 6.10 ± 0.52 μg/kg) and the packaging stage (12.80 ± 0.60 μg/kg), similarly to the other classes of FFAs detected. Total fatty acids composition of fish flesh is highly dependent on the diet of the fish. The YFT is an opportunistic predator, feeding on a great variety of preys such as cephalopods, crustaceans and other fish (Potier et al., 2004). Marine environment is a complex system, comprising a wide variability of feeding sources for those that are at the top of the food web, like carnivore fish.

Table 2 Changes in free fatty acids profile (µg/g sample) during the *mojama* production process (*n* = 45). Data are presented as mean ± standard error.

	Stages				
	Fresh tuna	**Salting**	**Salt-washing**	**Drying**	**p-value**
Total Lipid content (%)	1.18 ± 0.08 b	1.02 ± 0.07 b	1.25 ± 0.09 ab	1.84 ± 0.10 a	**
FFAs proportion on total lipids (%)	10.37 ± 0.44 b	10.14 ± 0.21 b	10.06 ± 0.28 b	16.88 ± 0.37 a	***
FFAs profile (µg/g sample)					
14:0	10.28 ± 0.85 b	11.71 ± 1.12 b	14.93 ± 0.86 b	42.24 ± 2.65 a	***
15:0	4.82 ± 0.44 b	6.30 ± 0.57 b	6.37 ± 0.61 b	16.56 ± 0.90 a	***
16:0	202.19 ± 13.26 b	189.75 ± 11.22 b	206.05 ± 14.87 b	495.79 ± 39.26 a	***
17:0	7.61 ± 0.75 b	7.47 ± 0.63 b	7.59 ± 0.89 b	27.13 ± 1.40 a	***
18:0	76.83 ± 3.72 b	74.80 ± 3.66 b	81.43 ± 3.90 b	182.71 ± 13.36 a	***
20:0	3.32 ± 0.23	4.39 ± 0.41	8.24 ± 0.50	8.38 ± 0.55	ns
22:0	3.24 ± 0.35 b	3.80 ± 0.31 b	4.31 ± 0.39 b	7.83 ± 0.68 a	***
Total SFAs	**308.29 ± 19.14 b**	**298.23 ± 17.81 b**	**328.91 ± 18.98 b**	**780.64 ± 33.27 a**	***
16:1n-7	17.50 ± 1.62 b	17.96 ± 1.81 b	20.34 ± 1.80 b	58.81 ± 4.53 a	***
16:1n-11	2.06 ± 0.20 b	2.07 ± 0.20 b	2.24 ± 0.25 b	5.96 ± 0.49 a	***
17:1	4.35 ± 0.30 b	4.14 ± 0.30 b	4.65 ± 0.46 b	14.41 ± 1.47 a	***
18:1n-9	68.71 ± 3.46 b	76.54 ± 7.93 b	78.50 ± 8.26 b	246.27 ± 26.37 a	***
18:1n-7	10.43 ± 0.96 b	10.74 ± 1.15 b	12.89 ± 1.19 b	34.29 ± 4.00 a	***
20:1n-9	6.06 ± 0.45 b	7.36 ± 0.85 b	6.67 ± 0.78 b	22.82 ± 1.13 a	***
22:1n-9	4.56 ± 0.40 b	5.38 ± 0.42 b	5.72 ± 0.56 b	10.25 ± 1.05 a	***
24:1n-9	9.07 ± 0.63 b	8.52 ± 0.47 b	9.80 ± 0.79 b	26.12 ± 1.56 a	***
Total MUFAs	**122.74 ± 11.29 b**	**132.71 ± 13.54 b**	**140.80 ± 14.12 b**	**418.91 ± 30.33 a**	***
18:2n-6	8.79 ± 0.73 b	8.00 ± 0.64 b	9.63 ± 0.96 b	22.83 ± 1.58 a	***
18:4n-3*	3.13 ± 0.30 b	2.65 ± 0.25 b	3.36 ± 0.43 b	7.66 ± 0.78 a	***
20:2n-6	2.51 ± 0.20 b	2.27 ± 0.18 b	2.64 ± 0.25 b	5.54 ± 0.48 a	***
20:3n-6	2.60 ± 0.24 b	1.84 ± 0.24 b	2.30 ± 0.30 b	4.66 ± 0.38 a	***
20:4n-6	16.24 ± 1.09 b	10.08 ± 0.75 b	13.45 ± 0.92 b	46.10 ± 2.62 a	***
18:3n-3	2.76 ± 0.17 b	2.14 ± 0.23 b	2.81 ± 0.22 b	6.70 ± 0.65 a	***
20:3n-3	3.79 ± 0.31 b	3.41 ± 0.25 b	3.95 ± 0.20 b	6.08 ± 0.46 a	***
20:4n-3*	3.56 ± 0.36 b	2.73 ± 0.27 b	3.50 ± 0.25 b	8.68 ± 0.88 a	***
20:5n-3	23.23 ± 2.40 b	13.71 ± 0.57 b	19.04 ± 1.52 b	56.64 ± 3.26 a	***
22:3n-3	7.60 ± 0.66 b	9.85 ± 1.12 b	11.06 ± 0.75 b	24.52 ± 1.37 a	***
22:5n-3	10.00 ± 0.76 b	9.40 ± 0.97 b	13.50 ± 1.41 ab	20.32 ± 1.52 a	***
22:6n-3	170.71 ± 11.77 b	85.07 ± 5.32 c	120.97 ± 6.29 bc	415.71 ± 22.40 a	***
n-3	228.23 ± 16.57 b	126.72 ± 11.83 b	176.97 ± 11.27 b	546.32 ± 31.27 a	***
n-6	30.14 ± 2.33 b	22.20 ± 1.89 b	28.03 ± 1.83 b	79.13 ± 5.50 a	***
n-3/n-6	7.73 ± 0.17 a	5.92 ± 0.20 b	6.86 ± 0.29 ab	7.15 ± 0.22 a	**
Total PUFAs	**258.37 ± 18.81 b**	**148.92 ± 10.01 b**	**205.00 ± 13.35 b**	**625.45 ± 36.34 a**	***
iso18:0	3.11 ± 0.21 b	3.15 ± 0.23 b	3.35 ± 0.29 b	6.81 ± 0.33 a	***
iso15:0*	0.44 ± 0.04 b	0.44 ± 0.01 b	0.62 ± 0.06 b	1.46 ± 0.14 a	***

iso16:0*	0.94 ± 0.08 b	0.86 ± 0.07 b	1.03 ± 0.08 b	1.87 ± 0.09 a	***
iso17:0*	0.92 ± 0.07 b	0.95 ± 0.08 b	1.10 ± 0.12 b	2.66 ± 0.15 a	***
Total BCFAs	**5.43 ± 0.40 b**	**5.38 ± 0.39 b**	**6.10 ± 0.52 b**	**12.80 ± 0.60 a**	***
PUFA/SFA	**0.83 ± 0.02 a**	**0.49 ± 0.02 c**	**0.64 ± 0.02 b**	**0.89 ± 0.04 a**	***

SFAs saturated fatty acids, *BCFAs* branched-chain fatty acids, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids

[a,b,c]values in the same row with a different letter are significantly different (ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$) * = these compounds were tentatively identified by comparing their *m/z* spectra with those available in mass spectral libraries

Fatty acids signature is widely employed to explore trophic variations in marine fish, including tuna (Parrish et al., 2015), being fatty acids good indicators of dietary and ecosystem characteristics. Such an example, in the marine food web SFAs are highly represented in particulate matter and microzooplankton assemblages, while MUFAS are highly represented both in phyto-plankton biomasses (such as palmitoleic acid) or in animal fat (mainly, oleic acid, considered a biomarker of carnivore diet). Similarly, BCFAs have been identified in several fish species (including tuna), suggested to be formed primarily in zooplankton or coming from bacterial infestation (Ackman & Sipos, 1965; Hauff & Vetter, 2010). Finally, the marine environment is naturally enriched in long chain PUFAs of the n-3 series, mainly EPA, DPA, and DHA. These FAs are maintained through the food chain and preferentially stored into the phospholipid fraction of fish muscle (Dalsgaard et al., 2003). Furthermore, literature shows that the muscle of migratory fish, including tuna, contains higher amounts of DHA compared to non-migratory species (Medina et al., 1995; Murase & Saito, 1996).

Unsaturated fatty acids (UFAs) are more prone to degradation and, in muscle tissues, they are those generated in higher proportion as FFAs. According to this, in our study we found the sum of MUFAs and PUFAs accounting for ~ 57% of total FFAs in *mojama*. UFAs can act as forerunners of oxidative reactions, influencing the quality of the final product as precursors of volatile compounds responsible for the final aroma in dry-cured products (Toldrá, 1998; Zhang et al., 2016). Actually, most of the volatile compounds previously detected in fish products, such as aldehydes, alcohols, and ketones, have been associated to the primary and secondary oxidation of UFAs (Zhang et al., 2020; Moretti et al., 2017). Generally, the threefold increase observed for the amount of FFAs between the first stages of manufacturing (fresh tuna, salting, and salt-washing) and the final product (*mojama)* suggests that FFAs accumulated during the processing of dry-cured tuna, similarly to what observed in literature for processed products obtained by dry-curing of other muscle tissues (Jin et al., 2010; Toldrá, 1998). Actually, the concentration of FFAs in salt and dried products is mainly related to the lysis of the different lipids fractions at different rates, through the hydrolytic activity of the lipases, the microbial metabolic processes and the oxidation occur- ring during the production process, with phospholipids suggested as main source of FFAs in many dry-cured meat products (Toldrà, 1998; Jin et al., 2010). This phenomenon could be particularly relevant even in marine fish products since, due to the high content of long-chain PUFAs, their lipids could be more susceptible to oxidation. Previous studies (Moretti et al.,

2017; Zhang et al., 2020, 2021) suggested the role of lipolytic reactions in salted fish products in the lipid leakage, with free fatty acids formation and consequent development of volatile compounds responsible for typical flavors. Particularly, the content of FFAs has been suggested as an indicator of the extent of oil hydrolysis in aquatic products (Zhang et al., 2016).

The n-3/n-6 ratio observed in this study ranged from 5.92 ± 0.20 (salting) to 7.73 ± 0.17 (fresh tuna), with the only significant difference observed in the salting stage ($p < 0.01$). This difference could be related to the different rates of lipolysis and FFAs accumulation for the different classes of fatty acids (n-6 and n-3) during dry-cured tuna manufacturing, even if not associated to any particular factor. This trend was also observed in the PUFA/SFA ratio, observing a significant decrease in stage salting and washing. Even if not directly related to a nutritional interest, because of the minor proportion of FFAs over the total lipids, the high n-3/n-6 ratio found in this study may reflect the prevalence of n-3 fatty acids over the n-6 counterpart in the precursor lipid fractions, undergone to lysis, according to the typical composition of tuna flesh (Roy et al., 2010; Garaffo et al., 2011). Likewise, the PUFA/SFA ratio was 0.8, higher than the nutritional recommendations (> 0.4); however, it must be considered that this value was calculated for a fraction of the total lipids (FFAS) (Yerlikaya et al., 2022).

The biochemical changes characterized by lipolysis are strictly connected with the quality of the final product, since intramuscular phospholipids and FFAs are essential factors in the flavor and aroma development of food products (Xu et al., 2008). In detail, FFAs accumulate during production and, subsequently, they are transformed into volatile com- pounds (Toldrá, 1998). The intensity of these phenomena is related to both the composition of the raw material used and the production processes followed, in addition to the ingredients and the additives employed. In the case of Spanish *mojama* production, tuna loins are stored at − 18 °C in fishing vessels and arrive frozen at the transformation plants (Esteves & Anibal, 2019), where they are submitted to the addition of food grade salt, followed by salt-washing and drying steps. Ashby et al. (1973) reported that the frozen storage in the standard production of hams increased their porosity after thawing and resulted in a better penetration of the curing ingredients in the salting stage. It could be suggested that, similar for meat products, the frozen storage of tuna loins could be a relevant step in traditional *mojama* processing, but further studies are needed to asses this. Finally, it was reported that other processing-related factors, such as the addition of sodium chloride and the ambient ripening temperature, could intensify lipolysis in dry-cured meat and fish products (Andres et al., 2005; Moretti et al., 2017). Taking into account this assumption, it could be suggested that the traditional manufacturing process of *mojama*, consisting of salting and drying tuna loins kept at a controlled temperature, promoted a moderate lipolysis phenomenon and thus the formation of FFAs, which are known among the main precursors of volatile organic compounds with a significant impact on typical flavor development in fish and fish products.

Conclusions

This study evaluated the formation of biogenic amines and free fatty acids during the traditional production process of the Spanish fish product named *mojama,* elaborated with yellowfin tuna. This process includes fresh tuna, salting, salt-washing, and drying steps, and during each one the histamine

concentration was far below the regulated maxi- mum limits of 100–200 and 50 mg/kg established in the EU and FDA regulations, respectively, concluding that the overall production process of Spanish *mojama* meets food safety standards for biogenic amines. Moreover, the levels of spermidine and spermine, amines that perform vital functions in the life of the yellowfin tuna, were higher in fresh tuna, and progressively decreased in the following stages. Other biogenic amines such as PUT, PHE, or TRYP were not detected. Moreover, during the production process, a significant increase was observed in the fraction of FFAs. A prevalence of the group of unsaturated fatty acids was evidenced, particularly in the intermediate and final (salting and drying) stages of the fish product elaboration. The increment in unsaturated FFAs during the production process of this kind of food product is considered of primary importance since these compounds are among those largely responsible for the development of the flavor in seafood and fish products.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests. The authors declare no competing interests.

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