PAPER

THE IMPACT OF LEMON JUICE ON THE MARINATION OF ANCHOVY (*ENGRAULIS ENCRASICOLUS*): CHEMICAL, MICROBIOLOGICAL AND SENSORY CHANGES

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ABSTRACT

This paper investigates the qualitative changes (physical, biochemical, microbiological and sensory) in anchovy fillets during the maturation, in a marinade made from fresh lemon juice and olive oil with the addition of acetic acid. Marination was carried out at 4°C and it required 7 days until sensory acceptability of the product was achieved. At marination end point, a pH value of 4.2, a_w of 0.85 and NaCl content of 4% were measured for the products. Volatile and non-volatile amines were extracted from the marinated anchovy fillets, and the detected concentration implies that the accumulation of these components during processing was weak (<14 mg TVB-N/100 g, <0.5 mg TMA/100 g, <30 mg biogenic amines/kg). The lemon juice showed good preservative effect, it reduced the bacterial count in the products, while the addition of 0.5% acetic acid improved the sensory attributes of the product.

Keywords: marination, anchovies, quality control, TVB-N, TMA, biogenic amines, lemon juice

1. INTRODUCTION

Marinating is one of the oldest chemical preservation methods used for fish. During the process, the preservative effect is achieved by different concentrations of organic acids and salt. This effect is based on reducing the product's pH value and water activity, and increasing the salt content, resulting in a retardation of bacterial activity and enzymes. Marination promotes the structural characteristics of fish flesh, giving the product a characteristic flavour and aroma, and tender texture, but a limited shelf life of 1 to 6 months in refrigerated storage. Cold marinades achieve fish ripening in acetic acid and salt solution without any heat treatment. After maturation has been completed, the product is packaged in oil, sauces or a new salt/acid solution with the addition of spices (MCLAY, 1972). The quality of the final product is directly influenced by the initial quality of the raw material. In promoting the Mediterranean diet as a healthy way of living, minimally processed seafood products have become extremely popular. Thus, there is a growing interest in marinated fish products, and especially traditional carpaccio-like products (cold-marinated fish in fruit juice citric acid and salt). So far, the time required for the maturation of fish fillets in citric juice has been estimated empirically. Depending on the thickness of the raw material used for marination, it is generally considered that thin slices of bigger fishes (such as tuna) are ready for consumption after two hours, while the fillets of smaller fish (anchovy or sardine) need at least two days of maturation in a lemon juice-oil mixture. There is a real lack of information on how the properties of fillets change during maturation in citric juice, which would be useful for the industrial production of carpaccio-like products. In food preparation processes, citric acid has shown a positive effect on the texture and colour of raw materials (TOPUZ *et al*., 2016). Fresh lemon juice appears to be a good natural choice for marinating processes. It contains about 3% sugars, ascorbic acid, minerals such as potassium and phenolics, all of which have health-promoting properties and antioxidant effects (GONZÁLEZ-MOLINA *et al*., 2009).

The only European representative of the Engraulidae family, the anchovy (*Engraulis encrasicolus*, L.), belongs to the group of small pelagic fish caught along the Mediterranean shores and widely distributed in the Adriatic Sea. They are characterized by a gentler texture and lower fat content than sardines (ŠIMAT and BOGDANOVIĆ, 2012), which makes them a good raw material for minimally processed fish products. On the other hand, anchovies present a risk of infestation by *Anisakis* spp. (MLADINEO *et al*., 2012; ŠIMAT *et al*., 2015); thus, they must be frozen prior to processing. The marinating of thawed fish presents a challenge with respect to fillet texture, juiciness and colour, since the freezing process and storage in a freezer result in denaturation of muscle proteins. Anchovy carpaccio is a minimally processed, natural fish product traditionally prepared from fish fillets, marinated in lemon or lime juice and salt, with the addition of oil and spices. During maturation, fillets become glossy white, juicy and tender with a gentle acidic taste. The blood stains on the belly part of the fillet appear on the fillet as a result of the freezing process and ruin the appearance and overall acceptability of the product; therefore, the quality of the raw material is of key importance for the production of high quality products. Since lightly preserved fish products, in general, have a low salt content (<6 % NaCl w/w) and their pH value usually exceeds 5.0, carpaccio-like products are on the very edge of that definition; they have a low salt content and a pH value below 5. After processing, these products are stored at refrigerated temperatures (4-6°C) and consumed without prior heating.

Consequently, this research was conducted in order to investigate the optimal duration of maturation as well as qualitative changes (physical, chemical, microbiological and sensory) in anchovy fillets during the maturation process in a marinade made from fresh lemon juice and olive oil. Additionally, the effect of the addition of acetic acid (final

concentration of 0.5% and 1% in the marinating bath) to the lemon juice and olive oil marinade was investigated. The aim was to determine the time required for completion of the maturation process, and the physical, chemical and microbiological changes underlying the maturation process. The objectives of the maturation process were to obtain sensory acceptability of the product, glassy-white colour and soft fillet texture, characteristic flavour, satisfactory salt content and decrease in the pH and water activity values, and to investigate the preservative effect of lemon juice.

2. MATERIAL AND METHODS

2.1. Raw material preparation and marinating

Anchovies used for marinating were caught in southern part of the Adriatic Sea (FAO fishing area 37.2.1.), placed on ice onboard, transported to a local factory where they were frozen in a brine-air blast freezer and stored at -20°C for four months.
The traditional method of preparing carpaccio marinade was somewhat modified to attain a better quality product. The fish were thawed, beheaded, gutted and filleted, and the fillets transferred to a NaCl (200 g/L) solution (ratio 1:1) for 22 minutes to remove excess blood, intestines or scales. The fillets were then washed in icy water and drained. Marination was carried out in round polypropylene containers, in three lots. The first lot, 140 g of brined filets, were packed with 70 mL fresh lemon juice and 70 mL olive oil per container. Lots two and three were processed in the same way, with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath, respectively. The containers were closed, stored at 4±2°C and shaken several times before the first sampling for analyses. Preliminary investigation results were used for preparation of the sampling plan; thus, five randomly chosen containers were sampled on days 2 and 7 in order to investigate physical, chemical, microbiological and sensory qualitative changes.
Before the analyses, the liquid was drained from all the containers and the fillets were homogenized using a laboratory homogenizer (KINEMATICA Microtron MB 550, Switzerland). The homogenized mass of three containers was used for physical and chemical analyses. The remaining two containers were used for microbiological analysis and sensory assessment.

2.2. Chemical composition analyses

The chemical composition of anchovy fillets was determined as moisture content by gravimetric method, crude protein (AOAC, 2000), crude lipid (BLIGH and DYER, 1959) and crude ash (AOAC, 2000). All analyses were repeated six times and the results were presented on a wet weight basis (percentage).

2.3. Physical and chemical analysis

For the pH measurements, 10 g of fillets were homogenized with 10 mL distilled water. The pH value of the fish homogenate was measured using a digital Iskra pH-meter MA 5705 with a combined glass electrode (Iskra Model 0101, Slovenia). Water activity (a_w) was determined at room temperature using an a_w meter (Rotronic AG, HygroPalm AW1-set 40, RotronicInstrument Corp., Basserdorf, Germany). Total volatile basic nitrogen (TVB-N, mg/100 g) and trimethylamine (TMA, mg/100 g) were analysed according to ŠIMAT *et al.* (2009) using a Kjeldahl distillation unit (model B-324, Büchi, Switzerland) and automatic titration (Methrom 702 SET / MET titrino). The 2-thiobarbituric acid (TBA, mg

malonaldehyde/kg) assay, as an index of lipid oxidation, was performed according to the procedure of VYNCKE (1970) and LEMON (1975).
The total of eight biogenic amines, namely β-phenyletylamine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine and tyramine, were determined in samples using high-performance liquid chromatography (HPLC), according to the method described by EEROLA *et al*. (1993) and modified for smaller particle size column (ŠIMAT and DALGAARD, 2011). The standards of biogenic amines that were used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HPLC analyses were carried out using a classical Agilent 1200 Series LC system (Agilent Technologies Inc., Waldbronn, Germany) and the separation was performed on a C18 Agilent column (Zorbax Eclipse XDB 50 × 4.6 mm ID, 1.8 μm particle size). All analyses were done in triplicate and the results expressed as mg/kg of the sample.

2.4. Microbiological analysis

Samples and decimal dilutions were prepared according to the method described by ÖZOGUL *et al*. (2006). For all microbial counts, 25 g of anchovy fillet was taken and homogenized with 225 mL of buffered peptone water (BPW, Biolife Italiana). A series of decimal dilutions were obtained and inoculated on appropriate media. Total viable count was enumerated on Plate Count Agar (PCA, Biolife Italiana) at 30°C for 24-48 h using the pour plate method. Psychrotrophic microorganisms were also enumerated using PCA as the medium. Plates were incubated at 7°C for 10 days. The lactic acid bacteria count was determined using the pour plate method on De Man, Rogosa and Sharpe Agar (Biolife, Italiana) after incubation at 30°C for 72 hours. *Enterobacteriaceae* were enumerated from Violet Red Bile Glucose Agar (VRBGA, Biolife Italiana) after incubation at 37°C for 48 hours. The number of colony forming units (CFU) per gram of sample was calculated from the number of colonies obtained on selected plates retained from three successive dilutions and the results were presented as log CFU/g. Each dilution was poured in triplicate.

2.5. Sensory analysis

The sensory attributes of the marinated fish fillets were evaluated by a panel of 8 trained panellists according to the method described by SALLAM *et al*. (2007). The panellists, composed of MSc students, University staff and fish industry technologists, were educated on specific positive and negative sensory aspects of cold marinated fish. Every day, during seven evaluation days, fillet samples of two different products were served to each panellist in covered porcelain dishes coded with 3-digit randomly chosen numbers. An eight-point hedonic scoring scale was used for evaluation of the appearance (8 = extremely acceptable to 1 = extremely unacceptable), colour (8 = faint pink to 1 = deep brown), texture (8 = firm and consistent to 1 = extremely soft or extremely hard), odour (8 = characteristic saury odour to 1 = extreme off-odour), rancidity (8 = no rancidity to 1 = extremely rancid), juiciness and tenderness (8 = extremely juicy/tender to 1 = extremely dry/tough), sour and salty taste (8 = not sour/salty to 1 = extremely sour/salty), as well as flavour and aftertaste (8 = characteristic saury flavour to 1 = extreme off-flavour). A nine-point hedonic scale (9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely) was used for evaluation of overall acceptability. The maturation process was performed at refrigerated temperature (4±2°C) and it was considered complete when sensory acceptability of the product was satisfactory (glassy-white colour, soft fillet texture, favourable effect in terms of odour and flavour). This was

achieved after seven days of maturation (preliminary study, data not shown). Thus, the sensory analysis 2-day data (generally accepted end of maturation) and 7-day data (end of maturation based on preliminary study) are presented in this paper.

2.6. Statistical analyses

In order to establish significant differences between data obtained for different marinating baths (with and without acetic acid), one-way variance analysis (ANOVA) was performed using the Statgraphics Centurion XVI 16.1 (StatPoint technologies, Inc.) statistical package, and Fisher's least significant difference (LSD) procedure at p value of <0.05.

3. RESULTS AND DISCUSSION

Taking into account the economic value of anchovy and increasing production of lightly preserved and uncooked fish products, knowledge on quality changes during processing will ensure standardization of the production processes as well as general safety requirements for consumers. In marinated products, a synergic effect of salt and acid content ensures specific product characteristics, such as taste, appearance or texture, but also environmental conditions that delay the growth of microorganisms causing spoilage. The addition of 0.3% acetic acid is considered to have a bactericidal effect, especially against Gram-negative bacteria (RAY and BHUNIA, 2008). Carpaccio products are prepared from raw fish fillets, thinly sliced and marinated in lemon juice and olive oil. Anchovy is a small pelagic fish whose fillets are thin and suitable for this type of product; however, the European anchovy in the Adriatic Sea represents a moderately highly-infected paratenic host of *Anisakis pegreffii* (MLADINEO *et al*., 2012; ŠIMAT *et al*., 2015) and, therefore, freezing of the fish before the cold marinade processing is obligatory. During freezing and storage, fish changes such as protein denaturation and lipid oxidation may influence the marinating process, which means that the overall acceptability of such products is usually lower, the colour and texture being less appealing to consumers.
Table 1 shows the moisture, protein, lipid and ash content of thawed, brined and marinated anchovy fillets after two and seven days of maturation at 4±2°C. The moisture content in thawed fillet was lower than in raw fish (0.78%, data not shown) due to the combined brine/air blast system used for freezing the fish. It is an individually quick freezing technology that uses a brine solution chilled at -21°C for freezing and air blast freezer for glaze fixation and final freezing of the product. Small pelagic fish such as anchovy reach a temperature of -18°C in 8-9 minutes but this results in lower moisture and higher NaCl content (0.23% in raw material) than in fresh samples. The lipid content of anchovies used in this study was lower than in previous reports for this species (DUYAR and EKE, 2009; KOCATEPE and TURAN, 2012; ÖZOGUL *et al*., 2017). Atlantic or Black Sea representatives of this species, in general, have different proximate composition and higher lipid content than anchovies caught in the Adriatic Sea (ŠIMAT and BOGDANOVIĆ, 2012). The brining of the fillets also decreased moisture content and this remained relatively constant during the marinating process. The statistical analysis attempted to determine the interaction of the investigated parameters, i.e. whether the chemical composition was dependent on the particular stage of production as well as the acetic acid addition at the beginning (day 2) and at the end of the maturation process (day 7). The brining of the fish before the marinating process had a statistically significant effect ($p<0.05$) on the chemical composition of the fillets (Table 1). Both brining and marinating significantly affected the lipid content (Table 1) when compared to thawed fillets. According to the statistical analysis, the difference between three groups of marinated

products was found to be significant ($p < 0.05$). The protein and ash content was higher in marinated samples, but did not change significantly during the marinating process. For fillet brining, strong brine (200 g/L) was used to avoid swelling of the flesh (weak brine) or surface crystallisation of filets (if saturated brines are used).
The marinating process also significantly influenced the changes of all the investigated chemical parameters (Table 2).
Table 2 shows changes in the physical and chemical parameters of anchovy fillets during marination in lemon juice, and lemon juice with added acetic acid (0.5 and 1%). The results allow concluding that the brining step ensured satisfactory content of NaCl in the fillets (4.12%) while lemon juice ensured pH values of 4.2 in all marinated products. When the brining step (dipping the fillets in NaCl solution for 22 minutes) was applied to raw fresh fillets, the salt content was higher than 6% (data not shown), which makes the fillets too salty in sensory terms. The brining step made the thawed fillets whiter, hardened the consistency of the fillets by drawing some water out of them and achieved appropriate salinity of the product (Tables 1, 2 and 4). The addition of acetic acid did not affect the NaCl content at the end of maturation (7 days). In the literature, significantly higher salt contents were found in marinated fish products. KILINC and CAKLI (2005) marinated sardine (*Sardina pilchardus*) in a solution containing 7% acetic acid and 14% salt. Marination was preceded by brining of the fish in 10% aqueous salt solution. The authors reported a statistically significant change in the salt content during marination and storage of marinated sardines. On the first day of maturation, the salt content was 4.93% and after 22 days 7.12%. The marinating process used for *Engraulis anchoitae* (brined in 10% NaCl, marinated in a solution containing 3% acetic acid and 10% salt) resulted in 4.97% NaCl in the fish meat on the first day of storage (YEANNES and CASALES, 2008).
Citric acid has shown to be an excellent choice of natural additive for fish maturation and preservation as it controls the acidity of the product, softens the consistency of muscle, gives it a lighter colour and sour taste, and enhances the impact of conservation. The pH value of the prepared lemon juice marinade was 2.35±0.15. The addition of acetic acid in the proposed concentrations did not have any effect on the pH of the marinade or the fillets during maturation. The combined effect of aw (< 0.9) and pH (4.2) in marinated anchovies places these products in the inhibition zone of growth of pathogenic bacteria and many of the deteriorant microorganisms (CABRER *et al*. 2002). Low pH values of fish marinated in acetic acid and salt solutions are recorded in the scientific literature. The pH values of 3.9 were recorded in anchovies marinated with acetic acid (concentration of 50-100 g/L) in a study by POLIGNE and COLLIGAN (2000). On the 20th day of storage, the pH value reached its maximum (4.21) and thereafter remained constant for 60 days. GÖKOĞLU *et al*. (2009) recorded decreasing pH in fresh anchovy, from 6.3 to 4.57, during marination in 30 g acetic acid/L and 150 g NaCl/L. KILNIC and CAKLI (2004) marinated sardine (pH = 6.72) in a solution with a pH value of 3.86, while in the marinated fillet they achieved a pH value of 4.23 at the beginning and 4.11 at the end of maturation (22 days). During 7 days of maturation in a bath prepared from 30 g acetic acid/L, FUSELLI *et al*. (1994) obtained a pH value of 4.2 for *E. anchoita* fillets.
Freshness, deterioration of freshness, and spoilage of seafood products can be monitored over time through the dynamics and speed of changes in biochemical parameters (ÓLAFSDÓTTIR *et al*., 1997) by numerous biochemical tests that have been developed. TBA has been used as an indicator of lipid oxidation of fish through the determination of secondary lipid oxidation products generated by hydroperoxide decomposition, while TVB-N and TMA values are widely used as criteria of freshness and degree of spoilage of fish muscle (ÓLAFSDÓTTIR *et al*., 1997).

Table 1. Changes of proximate composition of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=6).

	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
% Moisture	74.88±0.26 [a]	70.05±0.34 [b]	70.07±0.34 [b]	69.68±0.24 [c]	70.75±0.18 [b]	69.19±0.36 [c]	70.75±0.11 [b]	69.42±0.14 [c]
% Protein	20.11±0.11 [a]	20.89±0.12 [a]	23.32±0.06 [b]	23.14±0.10 [b]	23.47±0.12 [b]	23.41±0.21 [b]	21.47±0.12 [c]	21.49±0.18 [c]
% Lipid	2.79±0.14 [a]	2.32±0.12 [b]	1.68±0.10 [c]	1.69±0.06 [c]	1.54±0.05 [d]	1.52±0.06 [d]	1.74±0.05 [e]	1.71±0.06 [e]
% Ash	2.31±0.01 [a]	4.62±0.06 [b]	4.07±0.03 [c]	4.24±0.02 [d]	3.94±0.03 [c]	4.18±0.02 [d]	3.35 ±0.03 [e]	4.47±0.02 [b]

[a-e]mean value±standard deviation in the same raw followed by different superscript are significantly different (p<0.05).

Table 2. Changes in the physical and chemical parameters of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=6).

Chemical analyses	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
pH	6.22±0.02 [a]	5.67±0.03 [b]	4.23±0.04 [c]	4.22±0.11 [c]	4.24±0.04 [c]	4.21±0.06 [c]	4.21±0.04 [c]	4.23±0.08 [c]
a_w	0.88±0.01 [a]	0.77±0.01 [b]	0.85±0.04 [c]	0.85±0.07 [c]	0.85±0.08 [c]	0.86±0.06 [c]	0.84±0.04 [c]	0.86±0.02 [c]
NaCl (%)	1.34±0.12 [a]	4.12±0.21 [b]	4.02±0.2 [c]	4.10±0.14 [b]	3.87±0.12 [d]	4.02±0.14 [c]	3.61±0.12 [e]	4.27±0.26 [f]
TBA (mg malonaldehyde/kg)	2.04±0.14 [a]	2.31±0.18 [b]	3.67±0.21 [c]	3.37±0.17 [d]	4.06±0.21 [e]	3.65±0.12 [c]	2.94±0.14 [e]	2.10±0.19 [a]
TVB-N (mg/100 g)	12.41±0.21 [a]	14.04±0.36 [b]	10.35±0.32 [c]	10.47±0.24 [c]	8.05±0.26 [d]	9.59±0.32 [e]	8.24±0.16 [d]	8.75±0.12 [d]
TMA(mg/100 g)	0.36±0.04 [a]	0.32±0.04 [a]	0.29±0.02 [b]	0.27±0.04 [b]	0.25±0.08 [c]	0.22±0.05 [d]	0.24±0.08 [c]	0.21±0.05 [d]

[a-f] mean value±standard deviation in the same raw followed by different superscript are significantly different (p<0.05).

Changes in biochemical parameters of anchovy fillet during marination in lemon juice, and lemon juice with the addition of 0.5 and 1% acetic acid are shown in Table 2. Regarding the TBA test, quality fish products are limited to 5 mg malonaldehyde (MDA)/kg, while the fish is considered safe for consummation up to a maximum level of 8 mg MDA/kg (SALLAM *et al*., 2007).

The TBA values of thawed and brined fillets were low, <2.5 mg MDA/kg indicating good quality of the raw material. Both brining and marination processes significantly (p<0.05) increased TBA (>3 mg MDA/kg) values, while over 7 days of maturation these values were slightly lower. The marination bath was prepared using fresh lemon juice and olive oil, which might influence the TBA values in marinated fillets. The data suggest that the TBA values of marinated fish are within the good quality limits after 7 days of maturation.

The TVB-N value is related to species, catching season and region, age and sex of the fish (KILINC and CAKLI, 2004). According to HUSS (1988), the TVB-N value in fresh fish is typically between 5 and 20 mg/100 g of flesh, whereas 30 to 35 mg/100 g is considered a limit of acceptability. In our study, the TVB-N value of thawed anchovies was 12.41 mg/100 g, while data reported in the literature ranged from 7.32 to 12.77 mg/100 g (PONS-SÁNCHEZ-CASCADO *et al*., 2005b; OLGUNOĞLU *et al*., 2009; ÖZOGUL *et al*., 2010; TOPUZ *et al*., 2016). After the brining step, the TVB-N value increased to 14.04 mg/100 g; however, two days in the lemon juice/olive oil mixture resulted in a significant reduction (p<0.05) of TVB-N values. Reduction of TVB-N through the marinating process has been reported in the literature and can be attributed to the action of acid and salt whereby the TVB-N components are extracted from marinated filets. KILINC and CAKLI (2004) reported a decrease in the amount of TVB, from 10.24 to 6.53 mg/100 g, during marination of sardine (*S. pilchardus*) in 7% acetic acid and 14% salt bath. AKSU *et al*. (1997) report the same for anchovy; and the value of TVB decreased from 8.7 in the raw material to 7.41 mg/100 g in the final product (6% oacetic acid, 14% NaCl). PONS-SÁNCHEZ-CASCADO *et al*. (2005a) detected a decrease of TVB-N, from 7.32 to 6.04 mg/100 g, during marination of anchovy in a wine vinegar marinade. In this study, lemon juice appeared to have a strong extractive effect on TVB-N components, which can be attributed to the presence of citric acid. During a shrimp marination process, a marination bath containing citric acid (2%) and salt (4%) resulted in a lower TVB-N value, from the initial 27.5 to as low as 7 mg/100 g shrimp flesh (CADUN *et al*., 2008). A similar lowering effect of lemon juice was observed for TMA, a non-protein fraction found in marine fish produced from the reduction of trimethylamine oxide by bacterial activity. The TMA values observed in thawed and brined fillet (0.36 and 0.32 mg/100 g) were significantly reduced during maturation (Table 2). Since TMA values in fresh fish were around 1 mg/100 g, and the limit of acceptability was found to be 5 mg/100 g (HUSS, 1988), the quality of the investigated fillets, in terms of TMA, was good and remained good during the entire maturation period. The range of TMA for small pelagic fish ranges from 0.49 to 3.21 mg/100 g (PONS-SÁNCHEZ-CASCADO *et al*., 2005b; KILINC and CAKLI, 2005; GÖKOĞLU *et al*., 2009). The content of TVB-N and TMA were not analysed in the marinade bath during maturation, but based on previous research conducted by PONS-SÁNCHEZ-CASCADO *et al*. (2005a). It can be assumed that lemon juice marinade has a strong extraction effect on the TVB-N and TMA components of anchovy fillets and, considering the low initial values, high levels should not be expected in such products. During the storage of marinated sardines in marinating baths with the addition of 2 and 4% acetic acid and 10% NaCl at 4°C, GÖKOĞLU *et al*. (2004) reported a statistically significant increase in the amount of TMA in the flesh of sardines; however, in both types of baths, this amount did not exceed 1.7 mg/100 g after 150 days of storage. Similarly, TMA did not exceed 2 mg/100 g in marinated anchovies (30 g/L acetic acid and 150 g/L salt) after several months of storage of the marinated anchovies in oil and tomato sauce at

a temperature of 4°C (GÖKOĞLU *et al*., 2009). KILINC and CAKLI (2004) recorded values lower than 2 mg TMA/100 g in marinated sardine flesh (7% acetic acid and 14% salt) during the marinating process (22 days). PONS-SÁNCHEZ-CASCADO *et al*. (2005a) recorded a slight increase in the amount of TMA after 3 months of storage of cold marinated anchovy in wine vinegar, but these quantities did not exceed 1 mg TMA/100 g of flesh.

The presence of biogenic amines (BAs) in seafood is involved in various toxicological reactions and can constitute a risk to consumer health. The accumulation of high levels of BAs is associated with a relevant growth (>7 log CFU/g) of decarboxylating microorganisms (GARDINI *et al*., 2016) and indicates a need for better hygiene and process control during production. The decarboxylation process can be catalyzed by naturally occurring endogenous amino acid decarboxylases present in animal or plant cells and by exogenous enzymes produced by various microorganisms under favourable conditions (HALÁSZ *et al*., 1994). There is no legal regulation on the BA content of foods (besides histamine); however, a profile of BA in foods provides a better overview of the quality of certain products. The accumulation of BAs during brining and marination of anchovy fillets is shown in Fig. 1. Out of eight BAs (β-phenyletylamine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine and tyramine) determined in this work, only cadaverine, spermine and tyramine were detected in thawed and marinated anchovy fillets. A total of 20.62 mg BA/kg was observed in thawed fillets, which corresponds to the quantities of BAs found in fresh/thawed anchovy reported in the literature (VECIANA-NOGUÉS *et al*., 1996; Pons-SÁNCHEZ-CASCADO *et al*., 2005a, ÖZOGUL *et al*., 2017). After the brining process, the cadaverine and spermine values remained unchanged while tyramine concentration increased from 4.32 to 14.86 mg/kg. This resulted in significantly higher (p<0.05) BA content (30.58 mg/kg). The lowest content of total BA during the marinating process was observed for the lemon juice/olive oil mixture, with 0.5% acetic acid, and was 15.22 mg/kg. Spermine was detected in small amounts (4-6 mg/kg) and its concentration remained unchanged during maturation. Together with spermidine (undetected in marinated filets), spermine is not related to bacterial spoilage but rather a physiological BA needed for cell growth (VECIANA-NOGUÉS *et al*., 1996). The increased levels of tyramine in anchovies during the marination process suggest that some enzymatic processes occur during lemon juice/olive oil marination that supports BA formation. In marinades, amino acid decarboxylation by bacterial enzymes is limited by the pH value, NaCl content and a_w value, which directly affect the growth of bacteria (PONS-SNÁCHEZ-CASCADO *et al*., 2005b). Furthermore, acidic conditions increase the activity of cathepsin, which results in an increase of free amino acids, the precursors for BA formation (MEYER, 1965; PONS-SÁNCHEZ-CASCADO *et al*., 2005b). Tyramine is often reported as dominant BA in anchovy products, both marinated and salt-ripened (VECIANA-NOGUÉS *et al*., 1996; PONS-SÁNCHEZ-CASCADO *et al*., 2005a) but also in fresh anchovies during storage (ÖZOGUL *et al*., 2017). Tyramine production is generally related to lactic acid bacteria and orally administered tyramine (concentrations over 125 mg/kg) can be potentially toxic (LADERO *et al*., 2010). No other BAs were detected in marinated fillets; however, the BA content associated with a lemon juice/olive oil marinating mixture was not analysed. During production of vinegar-marinated anchovies, PONS-SÁNCHEZ-CASCADO *et al*. (2005a) recorded a total of 9.80 mg/kg of BAs per kilogram of raw material (not including agmatine), and 35.44 mg/kg after 3 months of storage. The authors did not find any β-phenylethylamine or tryptamine in the raw material or marinated product. They concluded that vinegar acts as an extractive solvent of biogenic amines from fish, and that the levels of some BAs (cadaverine and putrescine) decreased in fish flesh during the marinating process in tandem with their increased levels in vinegar. According to VECIANA-NOGUÉS *et al*.

(1996), β-phenylethylamine and tryptamine are present only in fish with a high content of other BAs. In general, *Enterobacteriaceae* and lactic acid bacteria are associated with the accumulation of the main biogenic amines: histamine, cadaverine, putrescine and tyramine in seafood products (ÖZOGUL *et al*., 2017). The low TMA values and BA accumulation data obtained through microbiological analyses are given in Table 3. The lemon juice/olive oil mixture had an inhibitory effect on total viable bacteria count, psychrophilic bacteria count and lactic acid bacteria count. The addition of acetic acid inhibited the growth of total viable count and psychrophilic bacteria at first two days of maturation, but by the end of maturation the investigated bacteria adapted to the new conditions and their count increased. No *Enterobacteriaceae*, which are primarily responsible for cadaverine production (MARINO *et al*., 2000; ÖZOGUL *et al*., 2017), were detected in the carpaccio products, which corresponds to low BA production in marinated anchovy fillets. Total or partial inhibition of microorganisms by the application of a marinating process has been recorded previously by FUSELLI *et al*. (1994) in marinated anchovy (*E. anchoita*), with 3% acetic acid and 10% salt; AKSU *et al*. (1997) in marinated anchovy *E. encrasicolus* (6% acetic acid and 16% salt); KILNIC and CAKLI (2004) in sardine marinade (7% acetic acid and 14% salt); OLGUNOĞLU *et al*. (2009) in anchovy *E. encrasicolus* (4.5% alcoholic vinegar, 10% salt and 0.2% citric acid).

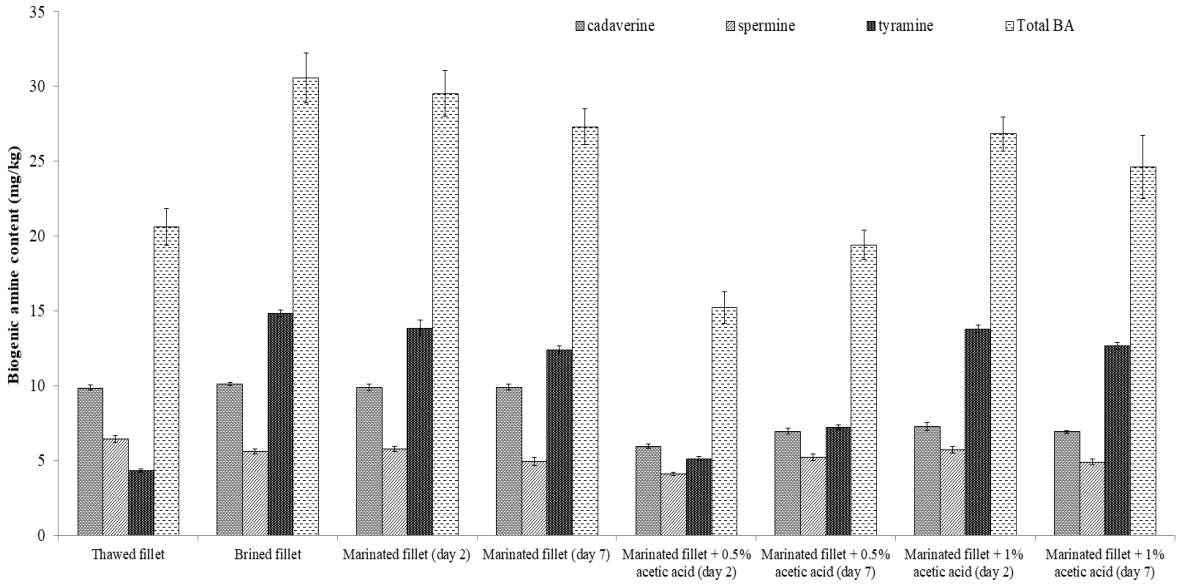

Figure 1. Biogenic amine formation in anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=3).

In general, the marinating process does not completely inactivate bacteria. They are able to continue to grow, faster or slower depending on their ability to adapt to the new media surrounding them (FUSELLI *et al*., 1994). Resistance of microorganisms to the acidic environment is variable and depends on their ability to adapt to new conditions. According to Ray and BHUNIA (2008), brief exposure of microorganisms under suboptimal conditions triggers their cellular mechanisms and allows them to be more resistant and better adapted to harsh environments. The authors state that acid tolerant bacteria may, by exposure to slightly acidic conditions (pH 5.0-5.8), exhibit resistance to survival at pH 2.4-4.0.

The results of sensory evaluation of marinated anchovies are given in Table 4.

Table 3. Changes in the microbiological count of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of acetic acid to reach a final concentration of 0.5% and 1% in the marinating bath (n=3).

Microbiological analyses (log CFU/g)	Thawed fillet	Brined fillet	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
Total viable count	1.13±0.62	ND	0.91±0.24	0.26±0.14	0.34±0.11	0.42±0.18	$<10^{-1}$	0.28±0.09
Psychrophilic bacteria	1.16±0.14	ND	0.56±0.11	$<10^{-1}$	$<10^{-1}$	0.94±0.09	$<10^{-1}$	0.56±0.12
Lactic acid bacteria	0.56±0.02	ND	0.65±0.28	$<10^{-1}$	0.62±0.12	$<10^{-1}$	0.73±0.12	$<10^{-1}$
Enterobacteriaceae	$<10^{-1}$	ND	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$	$<10^{-1}$

mean value±standard deviation, ND – not determined.

Table 4. Results of the sensory analysis of anchovy (*Engraulis encrasicolus*) fillet during marination in a lemon juice/olive oil mixture, and a lemon juice/olive oil mixture with the addition of 0.5 and 1% acetic acid (% on a wet weight basis) (n=6).

Sensory attribute	Marinated fillet (day 2)	Marinated fillet (day 7)	Marinated fillet + 0.5% acetic acid (day 2)	Marinated fillet + 0.5% acetic acid (day 7)	Marinated fillet + 1% acetic acid (day 2)	Marinated fillet + 1% acetic acid (day 7)
Appearance	7.87±0.92 ^{a}	7.90±0.46 ^{a}	7.90±0.62 ^{a}	7.98±0.08 ^{b}	7.76±0.91 ^{c}	7.90±0.85 ^{a}
Colour	6.90±1.08 ^{a}	7.47±0.88 ^{b}	6.73±0.81 ^{c}	7.93±0.17 ^{d}	6.87±0.74 ^{a}	7.83±0.65 ^{d}
Texture	7.92±0.94 ^{a}	7.93±0.68 ^{a}	7.53±0.99 ^{b}	8.00±0.08 ^{c}	8.00±0.02 ^{c}	7.47±0.62 ^{b}
Odour	7.41±0.94 ^{a}	7.94±0.68 ^{b}	7.62±0.74 ^{c}	7.81±0.56 ^{d}	7.96±0.99 ^{b}	7.38±0.87 ^{a}
Rancidity	6.97±1.12 ^{a}	7.42±0.74 ^{b}	7.24±0.42 ^{b}	7.45±0.99 ^{b}	7.79±1.21 ^{c}	7.45±0.91 ^{b}
Juiciness	7.92±0.81 ^{a}	7.93±1.18 ^{a}	7.53±0.60 ^{b}	7.98±0.22 ^{c}	8.00±0.69 ^{c}	7.47±0.88 ^{b}
Sour and salty taste	6.85±0.99 ^{a}	7.38±1.14 ^{b}	6.91±0.62 ^{a}	7.63±0.48 ^{c}	7.85±0.87 ^{d}	6.35±0.63 ^{e}
Flavour and aftertaste	5.61±0.88 ^{a}	6.27±0.97 ^{b}	5.45±0.71 ^{c}	7.81±0.87 ^{d}	7.72±0.49 ^{d}	6.48±0.47 ^{a}
Overall acceptability (scale from 1-9)	7.31±1.12 ^{a}	8.54±1.12 ^{b}	7.24±0.94 ^{a}	8.94±0.66 ^{c}	7.90±0.78 ^{d}	7.35±0.49 ^{a}

$^{a-e}$mean value±standard deviation in the same raw followed by different superscript are significantly different ($p<0.05$).

Based on the results of sensory analysis, the time required for maturation of anchovy fillets in the lemon/olive oil mixture was 7 days at 4°C. Depending on the acid and NaCl content, maturation temperature, bath:fish ratio etc., time needed for complete maturation of anchovy fillets ranges from 22 days at 4°C (PONS-SÁNCHEZ-CASCADO *et al*. (2005a) to 9 days at 20°C (CABRER *et al*., 2002). In general, better quality of fish is achieved when maturation takes place at lower temperatures over a longer period. Results for texture, juiciness and odour obtained on the second day of maturation in the lemon/olive oil mixture were high (7.9) and did not change during 7 days. However, products containing acetic acid showed improvement of these attributes over maturation. On day 2, all samples had a fishy (raw) flavour and a strong aftertaste and thus obtained lower grades from the panellists. Also, the colour was not glossy white, but yellowish and so colour grades were lower as well. After 7 days, the sensory acceptability of the product was satisfactory and grades were high for all investigated attributes. Glassy-white opaque colour, soft fillet texture, favourable effect in terms of odour and flavour were obtained. At this point, all assessed attributes (with the exception of odour) and overall acceptability of the product were the highest for the product in the lemon/olive oil mixture, with 0.5% acetic acid. The addition of 1% acetic acid resulted in an acidic aftertaste that covered the flavour and good aroma, thus changing the odour of the product and resulting in lower grades.
To the best to our knowledge, this paper presents the first results on the quality changes (physical, chemical, microbiological and sensory) of anchovy fillets during maturation in a lemon juice and olive oil marinade. The described process, consisting in the brining of fillets in a 200 g/L NaCl solution and marination of fillets in a lemon juice and olive oil mixture (50:50) over 7 days, achieved glassy-white colour and soft fillet texture, characteristic flavour and satisfactory salt content. The process resulted in a decrease of pH and water activity and good chemical characteristics of the final product. Low amine accumulation led us to assume that lemon juice has an extracting effect on volatile and non-volatile amines from anchovy fillets. Therefore, their role as an index of freshness/quality for carpaccio-like products is questionable. Further studies should include an analysis of the marination bath. Moreover, marination in lemon juice showed good preservative effects (total viable bacteria, psychrophilic bacteria and lactic acid bacteria count were reduced). The addition of 0.5% acetic acid improved the overall acceptability of the product. The results of this study could be of interest to the fish industry and contribute and enrich the offer of minimally processed seafood on the market.

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