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Evolution of Volatile Compounds and Biogenic Amines throughout the Shelf-life of Marinated and Salted Anchovies (*Engraulis encrasicolus*)

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ABSTRACT

Producers of processed anchovies have developed hazard analysis and critical control points (HACCP) to guarantee the quality of their products. Nonetheless there is a lack of objective data to determine products shelf-life. The quality of a product is usually established based on its safety and organoleptic properties. These parameters were assessed by monitoring the profiles of volatile compounds and quantitating six biogenic amines in samples of two types of processed anchovies during their shelf-life. Regarding biogenic amines, quantities were below the regulatory limits throughout shelf-life, except when a temperature abuse was applied for marinated samples. Moreover, this work highlights an optimum volatile profile at five and six months of storage for salted and marinated anchovies, respectively. This is the result of higher content of six aldehydes and nine ketones compounds, mainly coming from the lipid oxidation.

KEYWORDS: Anchovies, Shelf-life assessment, Volatile compounds, Biogenic amines, SPME-GC/MS

INTRODUCTION

Anchovies are one of the most caught fish species in the world with an estimated 3.5 million tons landed in 2010[1] and quantities consumed estimated at 369,000 tons in 2008. Anchovies can be processed using a wide range of techniques, as salt ripening or marination in vinegar. Consumers are more and more aware of food quality and food safety. In a way to manage such issues, the European Union has encouraged the application of good manufacturing practices and the development of hazard analysis and critical control points (HACCP). This approach has been developed in industry with specific applications for salted and marinated anchovies.

After their catch, anchovies can be ripened in salt or marinated in vinegar for a specified period and preserved until consumption. The shelf-life of processed anchovies has only been empirically estimated and there is a real lack of objective information on how the properties of products change during storage. The assessment of optimal shelf-life will ensure to consumers a safe product with good quality.

Numerous studies have described the microbial flora present in salted and marinated anchovies. *Lactobacillus* and *Micrococcus* genera have been identified as the main bacteria present in processed marinated anchovies, [2] presence of *Enterobacteriaceae* was also demonstrated.[3,4] In salted anchovies, potential histaminogenic flora, as *Enterobacteriaceae* and *Staphylococcus*, has been reported.[3-5] In a study on brined anchovies,[6] halophilic and psychrophilic flora increased respectively at room temperature and in chilled conditions, for samples conserved in low NaCl concentrations brines. A consensus is observed from previous authors to conclude that hygiene management provides a better control of these bacteria. In addition, a study on the ingredients added during the marination process showed that they have no impact on microbiological quality.[7]

Histamine is a biogenic amine resulting from the enzymatic decarboxylation of histidine. This molecule is responsible for many cases of food poisoning, with various symptoms.[8] Due to the high histidine content in their flesh, numerous fish families (Scombridae, Clupeidae or Engraulidae) show a high histamine risk. This is the major food safety risk encountered in such products. European regulation (Commission Regulation (EC) No 1441/2007 & Commission Regulation (EC) No 1019/2013) has defined a limit of 400 $mg.kg^{-1}$ of histamine for a single products having undergone enzymatic maturation treatment in brine.[9,10]. Other biogenic amines have been reported, especially tyramine, putrescine, cadaverine, spermine and spermidine.[11-13] Studies tracing biogenic amines during the storage period indicate changes in tyramine, histamine and tryptamine.[11,13,14] Very few studies have been carried out on the storage of marinated samples and focus mainly on the marination step itself.[2,12] Furthermore, refrigeration limits the biogenic amine content.[6,13,14] Analysis of volatile compounds in different food matrices has been increasing over the last decade.[15-17] The volatilome of a matrix can be analyzed by trapping volatiles on a solid phase micro-extraction (SPME) fiber, in the headspace of a vial. [18] At last, volatiles can be quantitated thanks to GC/MS device. To date, most studies on the volatile compounds of processed anchovies have been carried out to assess the maturation step of salted anchovies. Thus, indicators of maturation have been defined for salted anchovies as aldehydes, alkadienals, alcohols and ketones.[19,20] Acetaldehyde, 2-methyl-butanal and 3-methyl-propanal have been described as the most odorant compounds in ripened anchovies.[21] Based on sensory analysis, the optimal maturation of salted anchovies has been estimated at close to 160 days.[22]

As anchovies processing is well documented, the goal of this study was focused on the less studied shelf-life assessment of processed anchovies. To this end, both salt-ripened and marinated anchovies stored in different conditions, with and without temperature change, were

studied. The first half of the study was focused on volatilome monitoring throughout storage whereas the other half dealt with safety issues by quantitating biogenic amines.

MATERIALS AND METHODS

Chemicals

NaCl used for the headspace analysis was obtained from Oxoid (Dardilly, France). An *n*-paraffin mix, C_5 to C_{15}, used for the calculation of the linear retention index (LRI) was acquired from Sigma-Aldrich (St-Quentin-Fallavier, France). All pure compounds used to improve identification reliability including: ethanol, dimethyl sulfide, 2-methyl-propanal, 2,3-butanedione, butanal, 3-methyl-butanal, 2-methyl-butanal, 1-penten-3-ol, 2-ethylfuran, 1-pentanol, 2,3-hexanedione, hexanal, heptanal, benzaldehyde, 1-octen-3-ol, (E,E)-2,4-heptadienal, octanal, benzeneacetaldehyde, nonanal, (E,Z)-2,6-nonadienal and decanal were purchased from Sigma-Aldrich (St-Quentin-Fallavier, France). All chemicals used for quantitating biogenic amines were obtained from Sigma-Aldrich (St-Quentin-Fallavier, France), except perchloric acid purchased from Carlo Erba (Val-de-Reuil, France), and toluene and acetonitrile from VWR (Fontenay-sous-Bois, France).

Anchovy processing and preservation

Samples of anchovies were provided by the Association of French Fish Processing Industries (*Confédération des Industries de Traitement des Produits de la Pêche Maritimes* (CITPPM - Paris)) and held at the innovation platform *PFI-Nouvelles Vagues* (Boulogne-sur-Mer, France). Samples of anchovy fillets (*Engraulis encrasicolus*) consisted of processed, packed, salted or marinated anchovies.

For salted anchovies, samples were prepared by immersion in brine. Fish were gutted and beheaded and then transferred to vats, with alternating layers of salt and anchovies. Saturated

brine was then added. Vats were covered and pressure was applied to the lids to remove trapped air. Anchovies were ripened at room temperature for several months (a minimum of 3 months). After ripening, samples were packed in glass jars.

Marinated fish samples were produced from fresh anchovies that were cleaned, beheaded, gutted and filleted. After 24 hours freezing to manage parasite risk, anchovies were then immersed in a vinegar solution. A marination period of a few days (with a minimum of 24 hours) under refrigeration was carried out. Anchovies were then covered with oil and vacuum packed.

All storage time applied in this study were set to exceed the shelf-life advocated by the guide to good hygiene practices. Two types of temperature of storage were applied to samples: with (wR) or without rupture (w/oR). Concerning marinated samples, storage without any change of temperature consisted of incubation at 4°C. Storage with breaks in the temperature conditions consisted of one-third of the time at 4°C and the remaining two-thirds at 8°C. For the salted anchovies, storage without any change of temperature consisted of incubation at 15°C, to insure their inherent shelf stability. Rupture applied in the temperature conditions consisted of two-thirds of the time at 15°C and the remaining one-third at 22°C.

Analysis of the headspace by SPME-GC/MS

Analysis of the headspace of vials containing anchovy samples was carried out as described by Duflos *et al.*[17]. Briefly, 50 g of flesh were added to 100 mL of NaCl solution in ultrapure water at 300 $g.kg^{-1}$ then ground with a stomacher at 300 rpm for 2 min. Then, 45 g of filtrate was centrifuged at 10,000 ×g, for 10 min at 4°C. The supernatant (11 mL) was then distributed in 20 mL SPME vials and tightly sealed. SPME vials were placed at 4°C on a refrigerated tray. The vials were handled using a combiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The vial was placed for 10 min at 50°C and shaken at 500 rpm. A 75 µm CAR/PDMS fiber

(Supelco, Lyon, France) was introduced into the headspace of the vial to collect volatile compounds for 40 min at 50°C under the same conditions. The fiber was then removed and placed 1 min in a Merlin Microseal injector (at 250°C)in splitless mode, of a gas chromatograph GC-2010 equipped with a QP2010 Plus mass spectrometer (Shimadzu, Kyoto, Japan). The GC was equipped with a SLB-5 MS capillary column (60 m × 0.25 mm × 0.25 µm) from Supelco (St-Quentin-Fallavier, France) and helium was used as carrier gas at a flow of 1.78 mL/min. The temperature conditions applied in the oven were as follows: 5 minutes at 35°C then increased to 100°C at a rate of 10°C/min, then increased to 280°C at a rate of 20°C/min and maintained for 5 minutes. Spectrometric analysis was performed with the following conditions: interface temperature at 280°C, ionization voltage at 70 eV, mass range from 33 to 200 m/z and a scan frequency of 500 Hz. After each injection, the fiber was reconditioned at 300°C with helium flow for 10 min. Volatile compounds were automatically integrated by GC/MS Postrun analysis (Shimadzu, Kyoto, Japan), with a given retention time, a target ion and reference ions for each volatile compound (**Table 1**). A normalization of each area (*i.e.* division of the area by the sum of areas of all compounds) was realized. Compounds were identified by comparing them with mass spectra in the NIST 08 library database and with LRI found in the literature,[23] calculated using the equation in H. van den Dool and D. J. Kratz.[24] The headspace analysis was performed in duplicate for all samples.

Separation and quantitation of biogenic amines

Quantitative assay of biogenic amines was carried out using the method recommended by Commission Regulation (EC) No 1441/2007 with high performance liquid chromatography (HPLC).[25,26] Briefly, to extract biogenic amines, 5 g of anchovies were added to 100 µL of 1,3-diaminopropane used as internal standard and 10 mL of 0.2 M perchloric acid. The mix was blended with an Ultraturrax homogenizer and centrifuged at 7000 ×g for 5 min at 4°C. Then,

100 µL of supernatant was transferred in 300 µL of sodium carbonate and 400 µL of dansyl chloride, homogenized and kept in the dark at 60°C for 5 min. After derivatization, 100 µL of L-proline was added to remove excess dansyl chloride and kept in the dark at room temperature for 15 min. Then, 500 µL of toluene was added; after settling, the aqueous phase was frozen and the organic phase containing dansyl derivates was specifically recovered and evaporated under nitrogen flow for 5 min. The dry residue was dissolved in 200 µL of acetonitrile, then filtered at 0.2 µm and injected in a high-performance liquid chromatograph. Biogenic amines were separated on a Kromasil C18 reverse phase column (250 mm x 4.6 mm x 5µm) from Alltech (Deerfield, IL, USA) with a water/acetonitrile gradient and a flow of 1 mL/min. After 30 min of separation, the chromatogram showed the peaks of the six biogenic amines: putrescine, cadaverine, histamine, tyramine, spermidine and spermine and the internal standard (1,3-diaminopropane). Quantitation of biogenic amines was performed calculating each response factor against 1,3-diaminopropane and using a calibration curve.

Experimental design and statistical analysis

For the analysis of volatile compounds, marinated samples were studied for 9 months and salted samples for 18 months, to reach or exceed the shelf life recommended by good manufacturing practices. Samples were irregularly supplied by producers but a minimum of two samples was analyzed in duplicate each month for the duration of the tested shelf-life. A principal component analysis (PCA) was carried out on the data using open-source data-mining software Tanagra 1.4.[27] For each PCA, the two first PC were retained for interpretations. Correlations with principal components resulting from analysis determined which volatile compounds best characterized each group, as visualized by PCA.

Experiments on biogenic amines were designed as follows: for each pair of parameters (methods of preparation and storage conditions), five samples were taken for initial

measurements (M0) and three samples for remaining month. Samples were analyzed every two months for 8 months (marinated samples) and 14 months (salted anchovies). For each analysis, measures were performed in duplicate. Comparisons of average results of assays, for biogenic amines, were carried out using non-parametric Kruskal-Wallis tests and *post hoc* pairwise Wilcoxon rank sum tests because the results did not follow a normal distribution and there was not homoscedasticity of variances. The tests were performed using the package "stats" from the R software version 3.0.0. Kruskal-Wallis tests leading to significant results (p-value < 0.05) indicated that significant differences were observed for the concentration of the biogenic amines with respect to storage time. Pairwise Wilcoxon rank sum tests with p-values < 0.05 indicated that the two compared means were significantly different.

RESULTS

Selection of the volatile compounds for PCA analysis

From the whole dataset obtained by chromatogram integration, 36 compounds were selected as useful for our analysis (**Table 1**). These mostly included aldehydes, ketones and alcohols and three compounds that do not belong to these chemical classes: 2-ethylfurane (furan), dimethyl sulfide (organosulphur) and trimethylamine (TMA) (amine).

Among the compounds selected for statistical analysis, none allowed to differentiate storage conditions with or without rupture (data not shown). This study thus focused on the change in the volatilome with regard to each method of anchovies preparation.

Volatile compounds in marinated samples

Integration failed to retrieve all the volatile compounds for each sample. Compounds or sample duplicates with missing values were removed from the dataset. Thus, on the 36 compounds, only 13 were analyzed as shown in **Table 1**. Among the samples, only one sample was

194 completely removed (*i.e.* both duplicates): a sample preserved for 9 months without any change
195 in storage temperature. For the remaining samples, only four samples had data from only one
196 duplicate.

197 **Figure 1** illustrates the results of the PCA according to storage time. The global analysis
198 (**Figure 1 a**) showed that samples were relatively homogeneous, especially in the first months
199 of storage (from M0 to M4). Groups of sample storage were differentiated primarily along the
200 x-axis. The composition of volatile compounds appeared to change during the first five months
201 of preservation and the product appeared to stabilize in the following months. **Table 2** shows
202 that the samples of the fifth month stand out from the other months with higher proportions of
203 (E,E)-2,4-heptadienal, (E,Z)-2,6-nonadienal, 2,3-octanedione, (E)-2-heptenal, hexanal, heptanal,
204 benzeneacetaldehyde and benzaldehyde. The fifth month may be designated as the month when
205 the volatile compounds reach their optimum with regard to the storage of marinated anchovies.

206 Based on this five-month optimum, the dataset was then split into a pre-optimal phase and a
207 post-optimal phase. The pre-optimal phase (**Figure 1 b**) shows that months 0 to 4 are
208 characterized by smaller proportions in the above-mentioned compounds compared to the fifth
209 month, except for hexanal and heptanal. Moreover, months 4 and 5 have higher proportions of
210 2-ethylfuran, (E)-2-hexenal and (E)-2-pentenal than the other months. In the post-optimal phase
211 (**Figure 1 c**), groups were differentiated only along the x-axis. Thus from month 6 to month 9,
212 proportions of 2,3-octanedione, (E,E)-2,4-heptadienal, 2-ethylfuran, (E)-2-hexenal, (E)-2-
213 pentenal, (E)-2-heptenal, hexanal, heptanal and benzeneacetaldehyde decreased.

214 Splitting the dataset into two phases according to this putative month 5 optimum showed an
215 overall decrease in the proportions of 2-ethylfuran, (E)-2-hexenal and (E)-2-pentenal throughout
216 the study. However, for some compounds, proportions increased up until month 5 before
217 decreasing, e.g. 2,3-octanedione, 2,4-heptadienal, (E)-2-heptenal and benzeneacetaldehyde.

Volatile compounds in salted samples

For these samples also, integration failed to retrieve all the volatile compounds for each duplicate. Samples with missing values were discarded. Ultimately, data from 24 compounds out to 36 were analyzed as shown in **Table 1**. Five duplicates were discarded due to missing data.

Figure 2 shows the results according to storage time. The storage month groups were differentiated mainly along the x-axis (**Figure 2 a**). Groups were relatively homogeneous especially during the first months of storage (from M0 to M4). This can be observed, in particular, for samples analyzed at the initial stage. A change in the profile of volatile compounds was recorded at six months. Month 6 thus appeared to correspond to the volatile compound optimum for salted anchovies. **Table 2** shows that samples from month 2 stand out with higher proportions of 2-pentenal. Samples from month 6 differ from the other groups, probably due to higher relative quantities in the following compounds: (E,E)-2,4-heptadienal, (Z)-4-heptenal, (E)-2-heptenal, benzeneacetaldehyde, 2-nonanone, (E,E)-3,5-octadien-2-one, heptanal, hexanal, 2,3-octanedione and benzaldehyde. Finally, samples corresponding to the last few months (10, 16 and 18) do not appear different from each other.

According to this putative optimum, the dataset was divided into two phases, a pre-optimal phase and a post-optimal phase. The PCA of the pre-optimal phase (**Figure 2 b**) shows that months 0, 2 and 6 were characterized by higher proportions of (E)-2-hexenal, 2-methylbutanal and 3-methylbutanal compared to month 4. In the same phase, the month 6 differs from the other months, showing higher levels for the compounds described for the overall dataset and lower proportions of 1-penten-3-ol. The PCA of the post-optimal phase (**Figure 2 c**) shows that month 8 stands out due to higher levels of 2,3-octanedione, (E,E)-2,4-heptadienal, (E)-2-pentenal, (E,E)-3,5-octadien-2-one, (Z)-4-heptenal, (E)-2-heptenal, benzeneacetaldehyde,

heptanal and hexanal. Moreover, months 10 to 16 show higher relative quantities in TMA. Lastly, there seems to be no differences between month 16 and month 18: they both show higher proportions of 2-methylbutanal, 3-methylbutanal and 2-nonanone compared to previous months.

During the two phases, relative quantities of 2,3-octanedione, (E,E)-2,4-heptadienal, (Z)-4-heptenal, (E)-2-heptenal, (E,E)-3,5-octadien-2-one, hexanal, heptanal and benzeneacetaldehyde appeared to increase until reaching the optimum level before decreasing. During the same period, a continuous increase of 2-nonanone was recorded.

Changes in biogenic amines

The overall results are summarized in **Table 3**. In the large majority of cases, no samples ever exceeded the limit defined by the regulation (Commission Regulation (EC) No 1441/2007 & Commission Regulation (EC) No 1019/2013) during the study. Only marinated anchovies that underwent a break in the cold chain showed a significant change (p-value < 0.001) in the quantity of histamine, exceeding of the prescribed limit from month 6. Regarding putrescine, cadaverine and tyramine, a significant change (p-value ≤ 0.003) was also demonstrated with a maximum concentration of nearly 50 mg/kg, between month 4 and 6. Finally a significant change (p-value < 0.001) in the concentrations of spermidine and spermine was recorded, although levels remained low, near 20 mg/kg. The experiment carried out on samples that did not undergo a break in the cold chain shows similar patterns, although all the amine concentrations were lower. Thus the concentrations of histamine, putrescine, cadaverine, tyramine, spermidine and spermine did not exceed the threshold values of 40 mg/kg.

Regarding the salted anchovies, although significant changes were recorded throughout the storage of samples, the threshold of 20 mg/kg was never reached for any amine at any storage time or either storage condition.

DISCUSSION

Principal component analysis helped to visualize a significant proportion of the variance contained in the datasets analyzed. For each analysis, principal components explained at least 64% of the variability in the whole dataset, which is an acceptable level (**Figure 1** & **Figure 2**).

In these analyses, a difference in the volatile profile between marinated and salted samples was observed. Generally, it was found that the marinated samples did not show as many compounds as those identified for salted anchovy samples. As a result, multivariate statistical analysis could not be carried out on the same number of compounds for both types of processing methods. A significant amount of acetic acid in the headspace of marinated samples may explain the lower number of compounds recovered, possibly due to saturation of the SPME fiber.

Regarding the nature of compounds allowing the discrimination among samples, a large majority originated from the degradation of fatty acids of the ω-3 and 6 series (**Table 4**). This origin is consistent with observations of increased levels of free fatty acids throughout the maturation process of anchovies.[3,28] The presence of products of the catabolism of amino acids was also recorded as well as some products such as TMA or 2-ethylfuran, which are frequently observed in fish matrices. All compounds selected for the analysis of marinated samples were used in the statistical analysis of salted anchovy dataset. Among the compounds included in the analysis of salted samples, five come from the degradation of fatty acids, three from the catabolism of amino acids, two from sugar degradation and one from the degradation of trimethylamine N-oxide (TMAO) (**Table 4**).

The PCA traced the changes in the composition of the headspace over time. From those analyses, volatilome optima appear to occur at six months for salted anchovies and five months for marinated anchovies. Only a small change in the composition of volatile compounds was recorded after this period of storage.

The results of salted samples corroborate those of a study carried out on the selection of sensory attributes for the evaluation of the maturation of processed anchovies: the smell of Iberian ham is a reliable marker of maturation of salted anchovies.[22] SPME-GC/MS studies have specifically shown the presence of heptanal, with a maximum at the sixth month of storage. This compound is described in the literature as having a characteristic odor of ham, fat, even rancidity.[29,30] Moreover, higher proportions of 2,3-octanedione, 2-nonanone, (Z)-4-heptenal, (E)-2-heptenal, benzaldehyde, hexanal, benzeneacetaldehyde, (E,E)-2,4-heptadienal and (E,E)-3,5-octadien-2-one were recorded for these samples. After six months, the proportions of all compounds decreased except for benzaldehyde. Among the above-mentioned compounds, only 2,3-octanedione and 2-nonanone have never been described in the total volatile profile, in previous studies on ripened anchovy maturation.[19-21] Based on the aromatic qualifiers defined by Leduc *et al.*[16], odors can be divided into the following classes: earthy/woody smell, vegetable odor and floral smell.[30-32] Moreover, anchovy samples from the tenth to eighteenth month are characterized by higher proportions of TMA compared to the eighth month. This suggests that the increase in total volatile basic nitrogen (TVB-N), the chemical class to which TMA belongs[33] and recorded during the anchovy maturation process,[3] continues during storage.

Regarding marinated anchovies, the fifth month appears to mark the volatilome optimum of the product. These samples were characterized by a high proportion of benzeneacetaldehyde, (E,E)-2,4-heptadienal, (E)-2-heptenal, benzaldehyde, (E,Z)-2,6-nonadienal and 2,3-octanedione compared with other months. Only 2,3-octanedione has never been described in other studies on salted anchovies[19-21] (there are no published studies on the volatilome of marinated anchovies). The proportion of the above-mentioned compounds decreased except benzaldehyde. These compounds are characterized by respective odors of moss/solvent,[32] fat/soap,[34,35] cardboard/fat,[30,31] almond/woody[29,30] and cucumber for the latter two.[31,34]

Five compounds were present in maximum amounts at the volatilome optimum of processed anchovies, whatever the method of preparation: (E,E)-2,4-heptadienal, benzeneacetaldehyde, (E)-2-heptenal, benzaldehyde and 2,3-octanedione. To our knowledge, this is the first time that the latter compound has been identified, only by MS and LRI, as component of volatiles compounds in anchovies. 2-nonanone, hexanal and (E,E)-3,5-octadien-2-one appeared to be more specific to salt-ripened anchovies.

Analysis of biogenic amines showed high variability in quantities in similar samples principally in marinated anchovies stored with rupture in the temperature conditions. This probably reflects the heterogeneity of the fish that make up the samples. The initial microbiological load of the product, hygiene measures and the presence of bruises are all factors that may influence the degradation of anchovies. Despite the high variability of two samples with rupture in the storage conditions, the lower quantities observed exceeded 50 $mg.kg^{-1}$, concentration where the first allergic symptoms could be observed.

The type of anchovy processing has an impact on the quantity of biogenic amines, because marinated samples had higher concentrations of histamine, cadaverine, putrescine and tyramine than salted anchovies. This fact can be explained by the bacteriostatic action of salt ripening, while the development of lactic flora, potential producers of biogenic amines, is still possible in acidic conditions.[2]

The major factor that determines the production of biogenic amines, as highlighted in our study and previous papers,[6,13,14] is storage temperature. If the cold chain is broken during storage of marinated anchovies, the regulatory threshold of 400 $mg.kg^{-1}$ is exceeded. However, it should be noted that the producing process, mainly in salt ripening treatment, limits the bacterial development at the origin of histamine production. [52]

The present work helps to visualize the changes in the profile of volatile compounds and safety for the both types of anchovy processing techniques. It was shown that irregularities in

339 temperatures of storage lead to the development of biogenic amines in marinated samples,
340 whereas no risk was observed in salted samples. In contrast, the modification of temperature
341 conditions did not impact the volatile profile of marinated or salted samples for which the
342 volatilome optimum is respectively reached after five and six months. Considering these results,
343 none of them lead to conclude to a food safety risk caused by biogenic amines when anchovies
344 are stored in good temperature conditions. However, the study of volatilomes suggests that
345 marinated and salted anchovies should be consumed within five to six months after maturation,
346 respectively, so as to guarantee a product with optimal flavor for consumers.

ABBREVIATIONS USED

ALA: α-Linolenic acid, CAR/PDMS: Carboxen/polydimethylsiloxane, DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, GC: Gaz chromatography, HACCP: Hazard analysis critical control point, HPLC: High performance liquid chromatography, LA: Linoleic acid, LRI: Linear retention index, MS: Mass spectrometry, MUFA: Mono-unsaturated fatty acid, PCA: Principal component analysis, PUFA : Poly-unsaturated fatty acid, SPME: Solid phase microextraction, TMA: Trimethylamine, TMAO: Trimethylamine N-oxide, TVB-N: Total volatil basic nitrogen, wR: with rupture & w/oR: without rupture.

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FIGURE CAPTIONS

Figure 1: PCA obtained on the marinated anchovy samples for the whole dataset (a), from the pre-optimal phase (b) and from the post-optimal phase (c). Symbols indicate the tested month (M) of storage: (M0●, M2●, M3●, M4●, M5●, M6●, M7●, M8● and M9■).

Figure 2: PCA obtained on the salted samples for the whole dataset (a), from the pre-optimal phase (b) and from the post-optimal phase (c). Symbols indicate the tested month (M) of storage (M0●, M2●, M4●, M6●, M8●, M10●, M16● and M18■).

TABLES

Table 1: Settings used to perform automatic integration of the chromatograms.

compound [a]	LRI [d]	reliability [e]	ions used for integration (m/z) [f]
ethanol [c]	477	MS, LRI, Std	**45** (46 43)
dimethyl sulfide	516	MS, LRI, Std	**62** (47 45 46 61)
trimethylamine [c]	522	tentative	**58** (59 42)
2-methyl- propanal [c]	549	MS, LRI, Std	**43** (41 72)
2,3-butanedione [c]	586	MS, LRI, Std	**43** (86)
butanal	591	MS, LRI, Std	**44** (43 72 57 41)
2-butanone [b,c]	595	Tentative	**43** (72 57)
3-methyl- butanal [c]	652	MS, LRI, Std	**41** (44 58 43 39)
2-methyl- butanal [c]	662	MS, LRI, Std	**41** (57 58 39)
1-penten-3-ol [c]	684	MS, LRI, Std	**57** (41 43 39 58)
2,3-pentanedione	697	Tentative	**43** (57 100)
2-ethyl-furan [b,c]	700	MS, LRI, Std	**81** (96 53)
3-methyl-1-butanol	753	Tentative	**55** (42 43 70)
(E)-2-pentenal [b,c]	755	Tentative	**55** (84 83 41)
1-pentanol	768	MS, LRI, Std	**42** (55 41 70)
2,3-hexanedione	785	MS, LRI, Std	**43** (41 71 57)
hexanal [b,c]	799	MS, LRI, Std	**44** (56 41 43 72 82)
(E)-2-hexenal [b,c]	855	Tentative	**41** (42 39 83 69)
(Z)-4-heptenal [c]	898	Tentative	**41** (68 55 84 43)
heptanal [b,c]	900	MS, LRI, Std	**70** (41 55 57 81 44)
6-methyl-2-heptanone	956	Tentative	**43** (58 71 95 110 41)
(E)-2-heptenal [b,c]	960	MS, LRI, Std	**41** (68 55 84 43)
benzaldehyde [b,c]	970	MS, LRI, Std	**77** (106 105 51)
1-octen-3-ol	981	MS, LRI, Std	**57** (43 72 55 85)
2,3-octanedione [b,c]	983	Tentative	**43** (71 99)
(E,E)-2,4-heptadienal [1] [b,c]	997	MS, LRI, Std	**81** (110 53 41 67 79)
octanal	1002	MS, LRI, Std	**43** (44 56 84 69 100)
(E,E)-2,4-heptadienal [2] [b,c]	1014	tentative	**81** (110 53 41 67 79)
benzeneacetaldehyde [b,c]	1053	MS, LRI, Std	**91** (120 92 65)
(E)-2-octenal	1064	Tentative	**70** (55 41 83 57 69)
(E,E)-3,5-octadien-2-one [1] [c]	1073	tentative	**95** (43 81 109 124)
2-nonanone [c]	1091	Tentative	**43** (58 71 57 41)
(E,E)-3,5-octadien-2-one [2] [c]	1096	Tentative	**95** (43 81 109 124)
nonanal	1106	MS, LRI, Std	**57** (41 56 70 82 98)
(E,Z)-2,6-nonadienal [b,c]	1159	MS, LRI, Std	**41** (69 70 53)
decanal	1224	MS, LRI, Std	**43** (57 70 82 95 112)

[a] Numbers in brackets correspond to identifiers of peaks when a double peak is recorded on chromatograms for one identified compound. [b] Compounds used for multivariate analysis of marinated samples. [c] Compounds used for multivariate analysis of salted samples. [d] Linear retention index (LRI). [e] MS, LRI, Std: identification confirmed by mass spectrometry, LRI and pure standard; Tentative: tentative identification by mass spectrometry and LRI; tentative: tentatively identified compounds by mass spectrometry only. [f] **43**: target ion for the method. (41 72): ions of reference to improve the integration process.

Table 2: Correlation coefficients between chemical compounds and the two first axes determined by PCA. Three PCA were carried out per maturation process.

	Marinated anchovies		
	Whole dataset	***Pre-optimum phase***	***Post-optimum phase***
PC 1	benzaldehyde **(0.93)** [a] 2,3-octanedione **(0.88)** (E,E)-2,4-heptadienal [2] [b] **(0.86)** (E)-2-heptenal **(0.85)** benzeneacetaldehyde (**0.83)** heptanal **(0.82)** (E,Z)-2,6-nonadienal **(0.75)** hexanal **(0.71)**	benzaldehyde **(0.95)** (E)-2-heptenal **(0.93)** 2,3-octanedione **(0.9)** (E,Z)-2,6-nonadienal **(0.86)** (E,E)-2,4-heptadienal [2] **(0.81)** benzeneacetaldehyde **(0.78)** (E,E)-2,4-heptadienal [1] **(0.75)**	(E,E)-2,4-heptadienal [2] **(0.96)** heptanal **(0.95)** hexanal **(0.83)** (E)-2-heptenal **(0.83)** (E)-2-hexenal **(0.83)** (E)-2-pentenal **(0.8)** 2,3-octanedione **(0.76)** 2-ethyl-furan **(0.74)** benzaldehyde **(0.71)** (E,E)-2,4-heptadienal [1] **(0.74)**
(Inertia)	*(49.3%)* [c]	*(48.8%)*	*(56.4%)*
PC 2	(E)-2-pentenal **(0.92)** 2-ethyl-furan **(0.92)** (E)-2-hexenal **(0.84)**	(E)-2-hexenal **(-0.95)** (E)-2-pentenal **(-0.91)** 2-ethyl-furan **(-0.9)**	(E,Z)-2,6-nonadienal **(-0.9)**
(Inertia)	*(27.9%)*	*(29.9%)*	*(25.6%)*
	Salted anchovies		
	Whole dataset	***Pre-optimum phase***	***Post-optimum phase***
PC 1	(E,E)-3,5-octadien-2-one [1] **(0.97)** (Z)-4-heptenal (**0.96)** (E,E)-3,5-octadien-2-one [2] **(0.95)** heptanal **(0.95)** (E)-2-heptenal **(0.94)** hexanal **(0.94)** 2-nonanone **(0.92)** (E,E)-2,4-heptadienal [2] **(0.92)** 2,3-octanedione **(0.87)** benzaldehyde **(0.85)** (E,E)-2,4-heptadienal [1] **(0.79)** benzeneacetaldehyde **(0.77)**	(E,E)-3,5-octadien-2-one [1] **(0.98)** heptanal **(0.97)** 2-nonanone **(0.97)** (Z)-4-heptenal **(0.97)** (E,E)-3,5-octadien-2-one [2] (**0.97)** (E)-2-heptenal **(0.95)** hexanal **(0.95)** benzaldehyde **(0.92)** (E,E)-2,4-heptadienal [2] **(0.9)** benzeneacetaldehyde (**0.9)** 2,3-octanedione **(0.87)** (E,E)-2,4-heptadienal [1] **(0.72)** 1-penten-3-ol **(-0.7)**	(E,E)-3,5-octadien-2-one [2] **(0.95)** (E,E)-3,5-octadien-2-one [1] **(0.93)** (E,E)-2,4-heptadienal [1] **(0.91)** (E,E)-2,4-heptadienal [2] **(0.91)** (Z)-4-heptenal **(0.89)** (E)-2-heptenal **(0.85)** (E)-2-pentenal **(0.82)** benzeneacetaldehyde **(0.78)** hexanal **(0.75)** heptanal **(0.73)** 2,3-octanedione **(0.71)** trimethylamine **(-0.84)**
(Inertia)	*(52.9%)*	*(57.7%)*	*(45.4%)*
PC 2	(E)-2-pentenal **(-0.83)**	3-methyl-butanal **(-0.78)** 2-methyl-butanal **(-0.74)** (E)-2-hexenal **(-0.72)**	3-methyl-butanal **(0.85)** 2-nonanone **(0.76)** 2-methyl-butanal **(0.73)**
(Inertia)	*(12.0%)*	*(15.2%)*	*(18.2%)*

[a] Coefficient in parenthesis correspond to the correlation of the volatile compound with the PC concerned (row) for a given PCA (column). [b] Numbers in brackets correspond to identifiers of peaks when a double peak is recorded on chromatograms for one identified compound. [c] Percentage in parenthesis correspond to the inertia harbored by the PC concerned for a given PCA (e.g 49.3% means that PC1 harbored 49.3% of the dataset inertia in the PCA achieved on the marinated anchovies whole dataset).

Table 3: Changes in the concentrations of biogenic amines throughout a shelf-life of 14 months[a]. Values are listed according to curing method and storage conditions with (wR) or without (w/oR) rupture in the temperature of storage.

	histamine (mg.kg^{-1}) [b]				**putrescine (mg.kg^{-1})**			
	marinated		**salted**		**marinated**		**salted**	
	wR	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**
M0	4 ± 1 a	4 ± 1 a	1 ± 1 a	1 ± 1	4 ± 1 a	4 ± 1 a	3 ± 1 a	3 ± 1 a
M2	11 ± 2 b	6 ± 1 a	n.d. [d]	2 ± 2	4 ± 0 a	3 ± 1 a	2 ± 2 a	3 ± 2 ab
M4	28 ± 15 b	21 ± 11 b	1 ± 2 a	1 ± 2	25 ± 14 b	17 ± 8 b	3 ± 1 a	2 ± 0 ab
M6	2826 ± 2356 c	4 ± 2 a	n.d	n.d	48 ± 13 b	4 ± 0 a	7 ± 1 b	7 ± 1 b
M8	369 ± 269 c	7 ± 4 ab	n.d	n.d	21 ± 6 b	4 ± 1 a	3 ± 1 a	4 ± 0 b
M10			n.d	n.d			6 ± 4 ab	5 ± 1 ab
M12			1 ± 1 a	n.d			8 ± 1 b	6 ± 1 b
M14			1 ± 1 a	n.d			4 ± 0 a	4 ± 1 ab
p [c]	< 0.001	0.003	0.007	0.291	< 0.001	0.002	< 0.001	< 0.001
	tyramine (mg.kg^{-1})				**cadaverine (mg.kg^{-1})**			
	marinated		**salted**		**marinated**		**salted**	
	wR	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**
M0	14 ± 3 a	14 ± 3 a	7 ± 2 a	7 ± 2 a	15 ± 2 a	15 ± 2 a	12 ± 2 a	12 ± 2 a
M2	4 ± 0 b	5 ± 2 b	7 ± 1 a	7 ± 1 a	n.d.	12 ± 1 a	2 ± 1 b	2 ± 2 ab
M4	38 ± 25 a	20 ± 11 b	4 ± 1 a	3 ± 1 a	48 ± 23 b	36 ± 13 b	3 ± 0 b	4 ± 1 ab
M6	6 ± 3 b	2 ± 1 a	10 ± 1 a	9 ± 1 a	36 ± 8 b	38 ± 1 b	7 ± 1 ab	8 ± 1 ab
M8	10 ± 9 ab	3 ± 2 b	5 ± 1 a	4 ± 0 a	14 ± 4 a	14 ± 1 a	4 ± 1 b	3 ± 0 b
M10			8 ± 2 a	5 ± 1 a			7 ± 2 ab	5 ± 1 ab
M12			10 ± 2 a	5 ± 0 a			6 ± 1 ab	4 ± 1 b
M14			5 ± 0 a	4 ± 0 a			4 ± 0 ab	3 ± 0 b
p	0.003	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	spermidine (mg.kg^{-1})				**spermine (mg.kg^{-1})**			
	marinated		**salted**		**marinated**		**salted**	
	wR	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**	**wR**	**w/oR**
M0	10 ± 3 a	10 ± 3 a	18 ± 3 a	18 ± 3 a	2 ± 1 a	2 ± 1 a	4 ± 1 a	4 ± 1 a
M2	22 ± 2 b	20 ± 5 b	15 ± 4 ab	14 ± 3 ab	13 ± 2 b	12 ± 4 b	11 ± 4 ab	11 ± 3 b
M4	8 ± 3 a	13 ± 4 ab	9 ± 4 ab	13 ± 1 ab	6 ± 3 ab	8 ± 2 b	3 ± 0 ab	2 ± 1 ab
M6	7 ± 3 a	10 ± 1 a	8 ± 1 b	8 ± 1 b	n.d.	1 ± 1 a	n.d.	n.d.
M8	15 ± 3 ab	11 ± 1 a	8 ± 1 b	7 ± 1 b	1 ± 1 a	5 ± 2 b	2 ± 2 b	4 ± 1 ab
M10			11 ± 2 ab	9 ± 1 ab			n.d.	n.d.
M12			9 ± 2 b	8 ± 0 b			n.d.	2 ± 1 ab
M14			8 ± 0 b	7 ± 2 b			3 ± 0 b	4 ± 0 ab
p	< 0.001	0.013	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a] Means with different letters are significantly different ($p < 0.05$) throughout the tested period. [b] Means of the concentrations ± 2 standard-error of the mean (95% confidence interval). [c] p value calculated with the Kruskal-Wallis test. [d] n.d.: values below the limit of quantitation.

Table 4: Compounds used in the multivariate statistical analysis of anchovies and their assumed origins.

Compounds	Assumed origin
ethanol	Fermentation of sugar
trimethylamine	Trimethylamine-N oxide (TMAO) reduction, degradation of choline[36]
2-methyl-propanal	Amino-acid catabolism (L-valine)[37]
2,3-butanedione	Fermentation of sugar[38,39]
2-butanone	Degradation of 2,3-butanedione[40]
3-methyl-butanal	Amino-acid catabolism (L-leucine)[37]
2-methyl-butanal	Amino-acid catabolism[41]
1-penten-3-ol	Eicosapentaenoic acid (EPA) (ω-3) oxidation[42]
(E)-2-pentenal	α-Linolenic acid (ALA) (ω-3) oxidation[43]
hexanal	Linoleic acid (LA) (ω-6) oxidation and 2,4-decadienal degradation[43-45]
2-ethyl-furan	ω-3 fatty acid oxidation (ALA, EPA, Docosahexaenoic acid (DHA))[46]
(E)-2-hexenal	EPA (ω-3) oxidation[42]
(Z)-4-heptenal	EPA (ω-3) oxidation[42]
(E)-2-heptenal	Linoleic acid (LA) (ω-6) oxidation[47]
heptanal	Auto-oxidation of MUFA and PUFA (ω-6)[36,48]
benzaldehyde	Amino-acid catabolism[37,40]
2,3-octanedione	Lipid oxidation[49]
(E,E)-2,4-heptadienal	EPA and ALA (ω-3) oxidation[42,50]
benzeneacetaldehyde	Amino-acid catabolism[40]
2-nonanone	Lipid auto-oxidation
(E,E)-3,5-octadien-2-one	EPA (ω-3) auto-oxidation[42]
(E,Z)-2,6-nonadienal	ω-3fatty acid oxidation (ALA, EPA, DHA)[51]

FIGURES

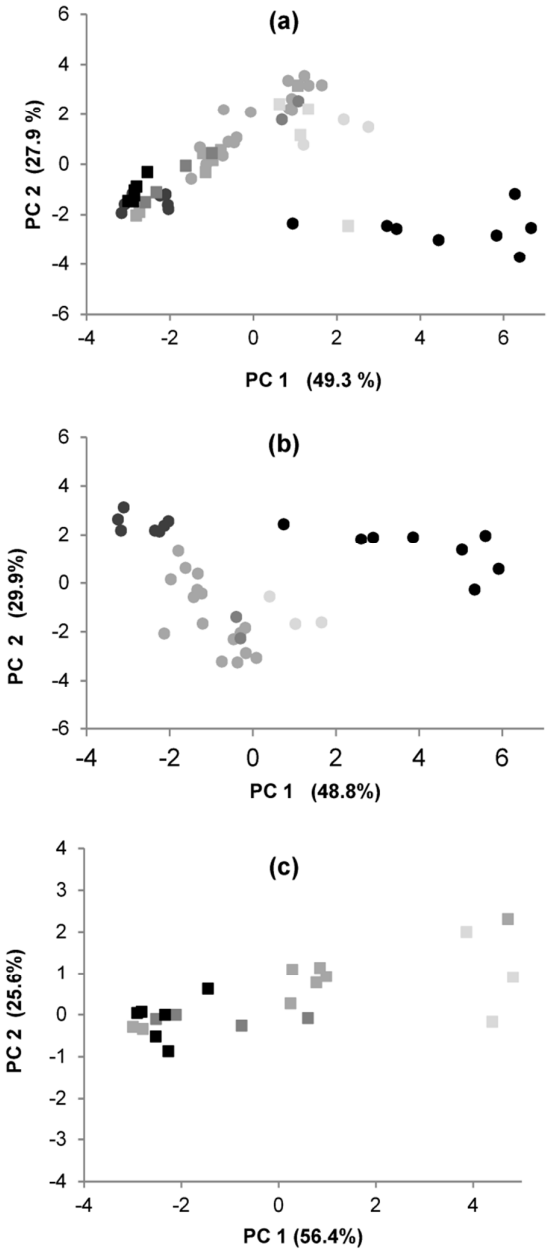


Figure 1

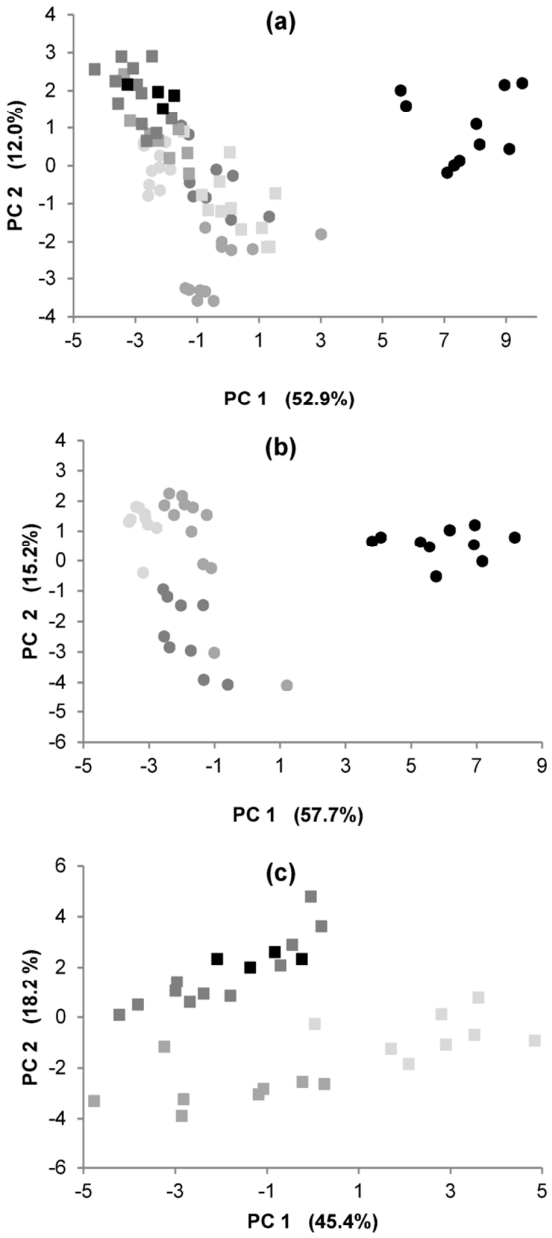
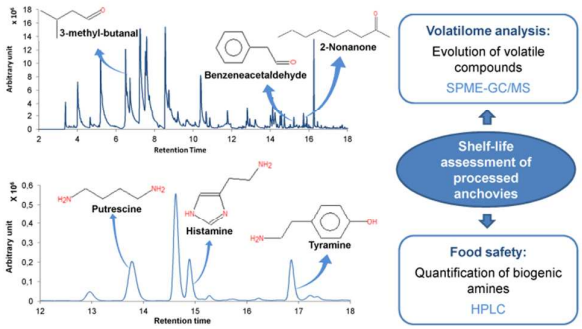


Figure 2

TOC GRAPHIC


3-methyl-butanal
Benzeneacetaldehyde
2-Nonanone
Arbitrary unit
Retention Time
Putrescine
Histamine
Tyramine
Retention time
Volatilome analysis:
Evolution of volatile compounds
SPME-GC/MS
Shelf-life assessment of processed anchovies
Food safety:
Quantification of biogenic amines
HPLC