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

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15 **ABSTRACT**

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

26

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

29 INTRODUCTION

30 Anchovies are one of the most caught fish species in the world with an estimated 3.5 million
31 tons landed in 2010¹ and quantities consumed estimated at 369,000 tons in 2008. Anchovies can
32 be processed using a wide range of techniques, as salt ripening or marination in vinegar.
33 Consumers are more and more aware of food quality and food safety. In a way to manage such
34 issues, the European Union has encouraged the application of good manufacturing practices and
35 the development of hazard analysis and critical control points (HACCP). This approach has
36 been developed in industry with specific applications for salted and marinated anchovies.

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

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

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

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

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

78 **MATERIALS AND METHODS**

79 **Chemicals**

80 NaCl used for the headspace analysis was obtained from Oxoid (Dardilly, France). An *n*-
81 paraffin mix, C₅ to C₁₅, used for the calculation of the linear retention index (LRI) was acquired
82 from Sigma-Aldrich (St-Quentin-Fallavier, France). All pure compounds used to improve
83 identification reliability including: ethanol, dimethyl sulfide, 2-methyl-propanal, 2,3-
84 butanedione, butanal, 3-methyl-butanal, 2-methyl-butanal, 1-penten-3-ol, 2-ethylfuran, 1-
85 pentanol, 2,3-hexanedione, hexanal, heptanal, benzaldehyde, 1-octen-3-ol, (E,E)-2,4-
86 heptadienal, octanal, benzeneacetaldehyde, nonanal, (E,Z)-2,6-nonadienal and decanal were
87 purchased from Sigma-Aldrich (St-Quentin-Fallavier, France). All chemicals used for
88 quantitating biogenic amines were obtained from Sigma-Aldrich (St-Quentin-Fallavier, France),
89 except perchloric acid purchased from Carlo Erba (Val-de-Reuil, France), and toluene and
90 acetonitrile from VWR (Fontenay-sous-Bois, France).

91 **Anchovy processing and preservation**

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

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

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

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

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

115 **Analysis of the headspace by SPME-GC/MS**

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

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

141 **Separation and quantitation of biogenic amines**

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

147 100 μL of supernatant was transferred in 300 μL of sodium carbonate and 400 μL of dansyl
148 chloride, homogenized and kept in the dark at 60°C for 5 min. After derivatization, 100 μL of L-
149 proline was added to remove excess dansyl chloride and kept in the dark at room temperature
150 for 15 min. Then, 500 μL of toluene was added; after settling, the aqueous phase was frozen and
151 the organic phase containing dansyl derivates was specifically recovered and evaporated under
152 nitrogen flow for 5 min. The dry residue was dissolved in 200 μL of acetonitrile, then filtered at
153 0.2 μm and injected in a high-performance liquid chromatograph. Biogenic amines were
154 separated on a Kromasil C18 reverse phase column (250 mm x 4.6 mm x 5 μm) from Alltech
155 (Deerfield, IL, USA) with a water/acetonitrile gradient and a flow of 1 mL/min. After 30 min of
156 separation, the chromatogram showed the peaks of the six biogenic amines: putrescine,
157 cadaverine, histamine, tyramine, spermidine and spermine and the internal standard (1,3-
158 diaminopropane). Quantitation of biogenic amines was performed calculating each response
159 factor against 1,3-diaminopropane and using a calibration curve.

160 **Experimental design and statistical analysis**

161 For the analysis of volatile compounds, marinated samples were studied for 9 months and salted
162 samples for 18 months, to reach or exceed the shelf life recommended by good manufacturing
163 practices. Samples were irregularly supplied by producers but a minimum of two samples was
164 analyzed in duplicate each month for the duration of the tested shelf-life. A principal component
165 analysis (PCA) was carried out on the data using open-source data-mining software Tanagra
166 1.4.²⁷ For each PCA, the two first PC were retained for interpretations. Correlations with
167 principal components resulting from analysis determined which volatile compounds best
168 characterized each group, as visualized by PCA.

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

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

181 **RESULTS**

182 **Selection of the volatile compounds for PCA analysis**

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

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

190 **Volatile compounds in marinated samples**

191 Integration failed to retrieve all the volatile compounds for each sample. Compounds or sample
192 duplicates with missing values were removed from the dataset. Thus, on the 36 compounds,
193 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.

218 Volatile compounds in salted samples

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

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

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

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

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

250 **Changes in biogenic amines**

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

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

266 **DISCUSSION**

267 Principal component analysis helped to visualize a significant proportion of the variance
268 contained in the datasets analyzed. For each analysis, principal components explained at least
269 64% of the variability in the whole dataset, which is an acceptable level (**Figure 1 & Figure 2**).

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

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

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

290 The results of salted samples corroborate those of a study carried out on the selection of sensory
291 attributes for the evaluation of the maturation of processed anchovies: the smell of Iberian ham
292 is a reliable marker of maturation of salted anchovies.²² SPME-GC/MS studies have specifically
293 shown the presence of heptanal, with a maximum at the sixth month of storage. This compound
294 is described in the literature as having a characteristic odor of ham, fat, even rancidity.^{29,30}
295 Moreover, higher proportions of 2,3-octanedione, 2-nonanone, (Z)-4-heptenal, (E)-2-heptenal,
296 benzaldehyde, hexanal, benzeneacetaldehyde, (E,E)-2,4-heptadienal and (E,E)-3,5-octadien-2-
297 one were recorded for these samples. After six months, the proportions of all compounds
298 decreased except for benzaldehyde. Among the above-mentioned compounds, only 2,3-
299 octanedione and 2-nonanone have never been described in the total volatile profile, in previous
300 studies on ripened anchovy maturation.¹⁹⁻²¹ Based on the aromatic qualifiers defined by Leduc
301 *et al.*¹⁶, odors can be divided into the following classes: earthy/woody smell, vegetable odor and
302 floral smell.³⁰⁻³² Moreover, anchovy samples from the tenth to eighteenth month are
303 characterized by higher proportions of TMA compared to the eighth month. This suggests that
304 the increase in total volatile basic nitrogen (TVB-N), the chemical class to which TMA
305 belongs³³ and recorded during the anchovy maturation process,³ continues during storage.

306 Regarding marinated anchovies, the fifth month appears to mark the volatilome optimum of the
307 product. These samples were characterized by a high proportion of benzeneacetaldehyde, (E,E)-
308 2,4-heptadienal, (E)-2-heptenal, benzaldehyde, (E,Z)-2,6-nonadienal and 2,3-octanedione
309 compared with other months. Only 2,3-octanedione has never been described in other studies on
310 salted anchovies¹⁹⁻²¹ (there are no published studies on the volatilome of marinated anchovies).
311 The proportion of the above-mentioned compounds decreased except benzaldehyde. These
312 compounds are characterized by respective odors of moss/solvent,³² fat/soap,^{34,35}
313 cardboard/fat,^{30,31} almond/woody^{29,30} and cucumber for the latter two.^{31,34}

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

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

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

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

337 The present work helps to visualize the changes in the profile of volatile compounds and safety
338 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: Gas 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	benzaldehyde (0.95)	(E,E)-2,4-heptadienal [2] (0.96)
		2,3-octanedione (0.88)	(E)-2-heptenal (0.93)	heptanal (0.95)
		(E,E)-2,4-heptadienal [2] ^b (0.86)	2,3-octanedione (0.9)	hexanal (0.83)
		(E)-2-heptenal (0.85)	(E,Z)-2,6-nonadienal (0.86)	(E)-2-heptenal (0.83)
		benzeneacetaldehyde (0.83)	(E,E)-2,4-heptadienal [2] (0.81)	(E)-2-hexenal (0.83)
		heptanal (0.82)	benzeneacetaldehyde (0.78)	(E)-2-pentenal (0.8)
		(E,Z)-2,6-nonadienal (0.75)	(E,E)-2,4-heptadienal [1] (0.75)	2,3-octanedione (0.76)
	hexanal (0.71)		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)	(E)-2-hexenal (-0.95)	(E,Z)-2,6-nonadienal (-0.9)
		2-ethyl-furan (0.92)	(E)-2-pentenal (-0.91)	
		(E)-2-hexenal (0.84)	2-ethyl-furan (-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)	(E,E)-3,5-octadien-2-one [1] (0.98)	(E,E)-3,5-octadien-2-one [2] (0.95)
		(Z)-4-heptenal (0.96)	heptanal (0.97)	(E,E)-3,5-octadien-2-one [1] (0.93)
		(E,E)-3,5-octadien-2-one [2] (0.95)	2-nonanone (0.97)	(E,E)-2,4-heptadienal [1] (0.91)
		heptanal (0.95)	(Z)-4-heptenal (0.97)	(E,E)-2,4-heptadienal [2] (0.91)
		(E)-2-heptenal (0.94)	(E,E)-3,5-octadien-2-one [2] (0.97)	(Z)-4-heptenal (0.89)
		hexanal (0.94)	(E)-2-heptenal (0.95)	(E)-2-heptenal (0.85)
		2-nonanone (0.92)	hexanal (0.95)	(E)-2-pentenal (0.82)
		(E,E)-2,4-heptadienal [2] (0.92)	benzaldehyde (0.92)	benzeneacetaldehyde (0.78)
		2,3-octanedione (0.87)	(E,E)-2,4-heptadienal [2] (0.9)	hexanal (0.75)
		benzaldehyde (0.85)	benzeneacetaldehyde (0.9)	heptanal (0.73)
		(E,E)-2,4-heptadienal [1] (0.79)	2,3-octanedione (0.87)	2,3-octanedione (0.71)
	benzeneacetaldehyde (0.77)	(E,E)-2,4-heptadienal [1] (0.72)	trimethylamine (-0.84)	
		1-penten-3-ol (-0.7)		
(Inertia)	(52.9%)		(57.7%)	(45.4%)
PC 2		(E)-2-pentenal (-0.83)	3-methyl-butanal (-0.78)	3-methyl-butanal (0.85)
			2-methyl-butanal (-0.74)	2-nonanone (0.76)
			(E)-2-hexenal (-0.72)	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
<i>p</i> ^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
<i>p</i>	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
<i>p</i>	< 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 -3 fatty acid oxidation (ALA, EPA, DHA) ⁵¹

FIGURES

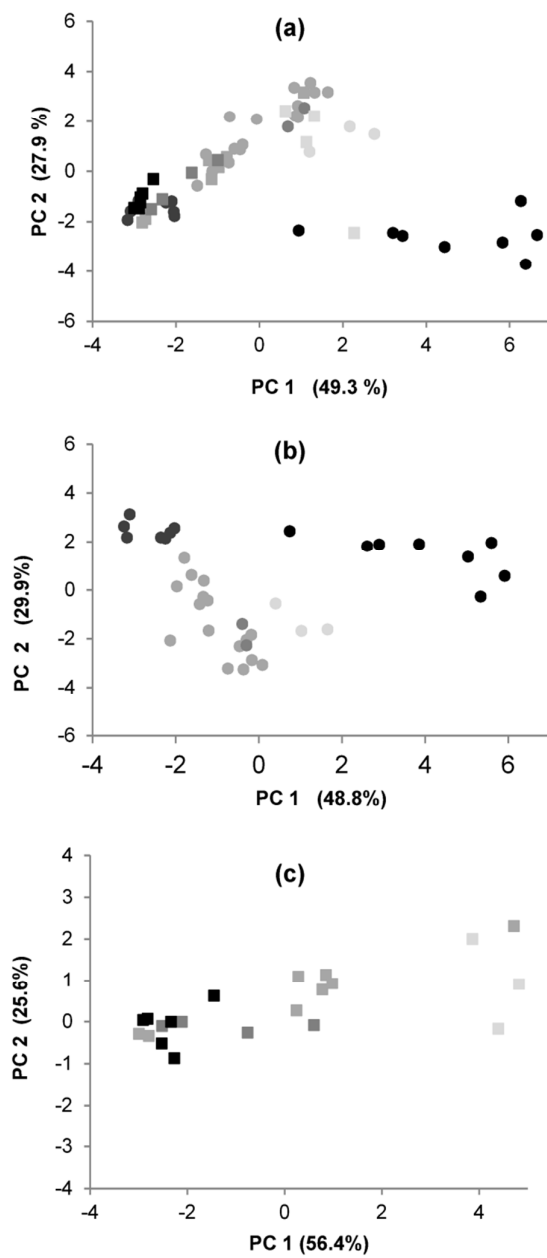


Figure 1

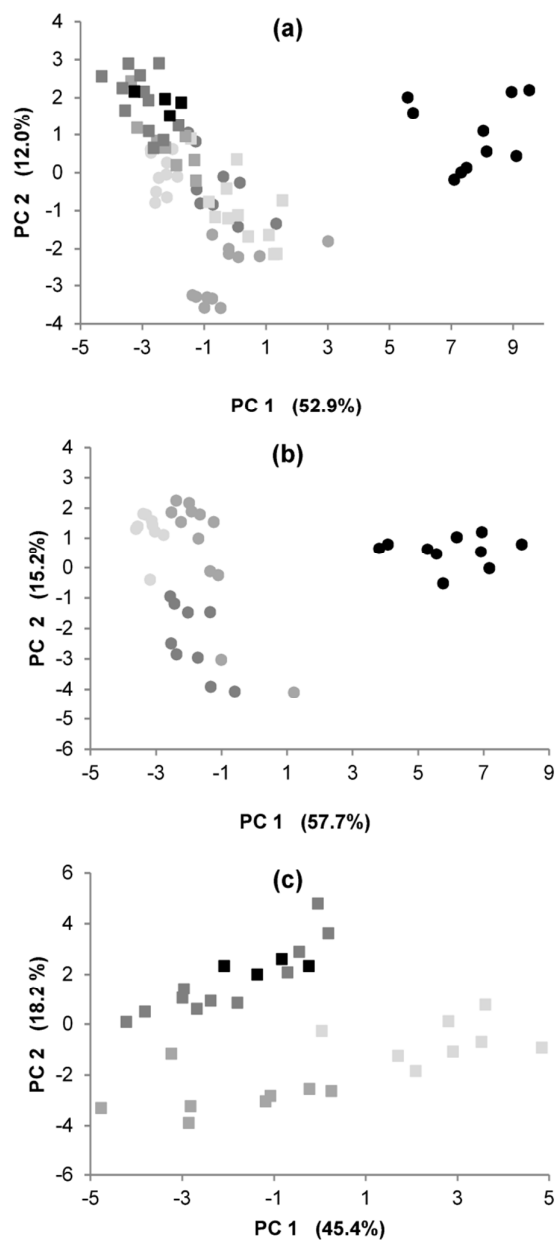


Figure 2

TOC GRAPHIC

