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Volatile fingerprint of unroasted and roasted cocoa beans (*Theobroma cacao* L.) from different geographical origins

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27 **ABSTRACT**

28 The aroma characterization of 59 fermented and dried cocoa beans from 22 different geographical
29 origin, covering the whole world cocoa production (America, Africa and Asia), was performed by
30 HS/SPME/GC/MS analyses. Analysis of cocoa beans before and after roasting were performed to
31 follow the aroma modification with the aim to achieve a volatile fingerprinting and a discrimination
32 model based on beans origin.

33 A total of 62 volatiles was identified in fermented and dried beans, while 76 volatiles were identified
34 in roasted cocoa beans. The compounds belong to several chemical groups including esters, alcohols,
35 organic acids, aldehydes, ketones and pyrazines. Datasets were submitted to multivariate statistical
36 analysis (Principal Component Analysis, PCA). Results allowed to discriminate not roasted cocoa
37 beans based on their geographical origin; in particular, samples coming from African countries were
38 separated from samples of Caribbean regions, whereas samples from Asia and Oceania were not
39 discriminated. Principal component analysis, applied on the corresponding roasted samples showed
40 that although the same roasting treatment has been applied to all the samples, the differences among
41 the fermented samples are also maintained in the aromatic profile resulting after roasting confirming
42 the discrimination model.

43 The volatile fingerprint showed interesting potential as authentication tools of raw and roasted cocoa
44 beans, and from a technological point of view to give information in order to pilot the secondary
45 processing steps of cocoa towards the final product designing.

46

47

48 **Keywords**

49 Fermented cocoa beans, roasted cocoa beans, flavour, fingerprint, geographical origin, PCA

50

51

52 **INTRODUCTION**

53 Cocoa authenticity is becoming an important issue due to the growing market of high quality cocoa
54 of mono-origin. The geographic traceability of cocoa is important for both cocoa transforming
55 industries and consumers. Food authenticity resides in food molecular composition and in the case of
56 cocoa only recently studies have been focused on different classes of molecules trying to link them
57 to cocoa geographic origin.

58 Cocoa of different geographical origins have different organoleptic characteristics and influence the
59 final quality of chocolate (Cambrai et al., 2010). In particular, chocolate and cocoa aroma resides in
60 large part in the corresponding volatile fraction, which is composed of a complex mixture of over 500
61 compounds and this complexity is demonstrated by the inability to reproduce in the laboratory cocoa
62 typical flavor (Dimick, Hoskin, 2003). The cocoa aroma development is the result of many different
63 technological processes applied to cocoa beans but it also depends on the cocoa variety and origin.
64 Fermentation and roasting are the two crucial steps for aroma development during cocoa processing.

65
66 During fermentation numberless volatile compounds and flavor precursors were produced by
67 microbial processes, which involves several biochemical reaction on carbohydrates, proteins and
68 polyphenols already present in the beans. Several microorganisms have been reported as producers
69 of volatile compounds during cocoa fermentation (Thompson, Miller, & Lopez, 2001). Alcohols,
70 aldehydes and ketones have been reported as the major groups of compounds found in raw cocoa
71 beans at the beginning of the fermentation (1 or 2 days). However, alcohols, esters and acids (acetic
72 acid mainly) were developed in the middle of fermentation (3–5 days); finally acids, esters and
73 alcohols were the most important groups of volatile compounds at the end fermentation (6–8 days)
74 (Rodriguez-Campos, Escalona-Buendía, Orozco-Avila, Lugo-Cervantes, Jaramillo-Flores, 2011).

75 Carbohydrates are mainly present in the cocoa beans as simple sugars like sucrose, fructose and
76 glucose; during fermentation, sucrose is almost completely hydrolyzed to fructose and glucose by the
77 invertase enzyme.

78 During the fermentation of the beans the proteolysis, catalysed by aspartic endoprotease and
79 carboxypeptidase, leads to the releasing of amino acids and oligopeptides. (Voigt & Biehl, 1995).

80 Although it is well known that relevant differences in enzyme activities exist between cocoa
81 genotypes, simple and general relationships have not been yet established between genotype flavour
82 and key enzyme activities in unfermented beans. Furthermore, how enzymatic processes are
83 regulated, which substrates and products are related to desirable flavours, and which are the limiting
84 factors for the enzymatic contribution to fermentation processes remain unclear.

85

86 During roasting process, flavor precursors generated during the fermentation step are transformed
87 into flavor compounds through the Maillard reaction. Kirchoff et al., (1989) observed a correlation
88 between free amino acid accumulation and generation of specific aroma precursors.

89 The main aim of fermented beans roasting is to obtain two results: the elimination of undesirable
90 compounds with low boiling point, such as acetic acid, and the formation of the typical flavor of
91 roasted beans. The heat treatment activates reactions between reducing sugars and free amino acids or
92 short-chain peptides, so leading to a significant reduction in the concentration of free amino acids and
93 reducing sugars.

94 The compounds that originate during roasting are alcohols, ethers, furans, pyranes, thiazoles, acids,
95 esters, imines, amines, oxazoles and pyrroles. Nevertheless, the most important class of compounds
96 are pyrazines and Strecker aldehydes. While pyrazines are not present in unfermented beans,
97 fermented and dried beans contain low concentrations of them. During roasting, depending on time
98 and temperature of treatment, the concentration of pyrazines further increases, giving rise to
99 increasingly substituted pyrazines. Specific compounds recognized as cocoa/ nutty notes were: 2,3-
100 dimethylpyrazine, trimethylpyrazine, tetramethylpyrazine, 3(or 2),5-dimethyl-2(or 3)-ethylpyrazine,
101 3,5(or 6)-diethyl-2-methylpyrazine. Three Strecker aldehydes present a strong chocolate flavor: 2-
102 ethylpropanal, 2-methylbutanal, and 3-methylbutanal (Caligiani, Marseglia & Palla, 2015).

103

104 Flavor quality of chocolate usually depends on the cocoa beans source; beans from different origins
105 can show distinct flavor characteristics such as acidic, hammy or smokey notes (Powell, 1983).
106 Some authors have studied the influence of geographical origin of cocoa on the composition of the
107 volatile fraction. Most studies concern the fermented, dried, and, most often, roasted beans
108 (Hernandez & Rutledge, 1994; Muggler-Chavan & Reymond, 1967). The profile comparison and
109 multivariate analyses allowed to distinguish beans (roasted or not) from various geographical origins
110 (Cambrai et al., 2010).

111 Most of studies therefore are focused on a limited dataset, few are focused on fermented cocoa beans
112 and rare are the works focused on fermented cocoa bean from a significant numbers of different
113 countries representative of the world production, as instead considered in the present work.

114 SPME has been used to determine the volatile fingerprint of cocoa bean (Humston, Zhang, Brabeck,
115 McShea, & Synovec, 2009). This technique has been reported to be cheap, solventless, fast and also
116 with high reproducibility, low limits of detection and high sensitivity (Balasubramanian & Panigrahi,
117 2011; Ducki, Miralles-Garcia, Zumbé, Tornero, & Storey, 2008).

118 Cocoa aroma is generally studied after roasting, however, also fermented cocoa beans present a
119 specific aroma that could be linked to the geographic origin and as a consequence to the
120 spontaneous fermentation.

121

122 In this paper, aroma characterization of 59 fermented and dried cocoa beans samples from 22
123 different geographical origins was performed by HS/SPME/GC/MS analyses. Sampling is
124 representative of the average world production (America, Africa, Asia).

125 Analysis of cocoa beans before and after roasting were performed to follow the aroma modifications,
126 with the aim to better understand the effect of the geographical origin on the intrinsic aroma of
127 fermented cocoa and the related aroma potential after roasting. The goal of this work is to see how
128 identical roasting condition results in different volatile fingerprinting profiles of cocoa beans from
129 different geographic origins. Another goal was to check if volatile profile can be used to trace the

130 cocoa origin, both before and after roasting, in order to achieve elements useful to both cocoa
131 transforming industries and consumers.

132

133 **Materials and Methods**

134 *2.1 Chemicals*

135 The compounds used as standards such as 3-Methylbutanal, 2,3-Butanedione, 2-Pentanol, 3-Methyl-
136 1-butanol, Acetoin, 2,3-Dimethylpyrazine, Trimethylpyrazine, Tetramethylpyrazine, Benzaldehyde,
137 2,3-Butanediol, 1-Phenylethanol, 1H-Pyrrole-2-carboxaldehyde, toluene, n-alkanes (C8-C17) were
138 all obtained from Sigma-Aldrich.

139 *2.2 Samples*

140 59 fermented and dried cocoa beans samples from 22 different geographical origins (Mexico, Cuba,
141 Santo Domingo, Grenada, Sulawesi, Trinidad, Venezuela, Ecuador, Perù, Brazil, Sierra Leone, Ivory
142 Coast, Ghana, Nigeria, São Tomé, Uganda, Tanzania, Madagascar, Malaysia, Java, Papua Guinea
143 New, Flores) were investigated. Samples were provided by Barry Callebaut, Belgium. All fruits were
144 harvested in 2012, and were fermented and dried in the countries of origin according to traditional
145 procedures. The samples analysed were of Forastero variety, except one sample of Criollo variety
146 coming from Mexico. The countries of origin and the numbers of cocoa beans samples collected were
147 shown in Table 1.

148 The samples of fermented cocoa beans were analysed before and after roasting. Roasting of the beans
149 was performed in a laboratory scale with a coffee-roaster (Probat BRZ 4, Emmerich, Germany).
150 Cocoa beans were freshly roasted for each experiment, and roasting conditions were optimized by
151 variation of roasting time and temperature. A temperature of 140 °C applied for 30 min was finally
152 chosen to simulate the industrial process generally adopted by Barry Callebaut, Belgium.

153 For chemical analysis, the samples were frozen with liquid nitrogen, ground with a coffee mill to
154 obtain a powder and immediately analysed.

155 *2.3 Extraction of volatile compounds*

156 The volatile compounds from cocoa beans sample were extracted using the head space solid phase
157 microextraction (HS-SPME). The cocoa samples were analysed according to the method described
158 by Rodriguez-Campos et al. (2011) with minor modifications.

159 A DVB/CAR/PDMS, 50/30 μm fiber was used. Both SPME manual holder and fibers were purchased
160 from Supelco (Bellefonte, PA, USA).

161 Before use, the fiber was conditioned following the instructions of the manufacturer. The
162 experimental conditions were as follows: 1.5 g of cocoa powder was placed in a 30-mL vial, added
163 to 10 μL of a toluene solution 250 mg/kg in water (2.5 μg) as internal standard and sealed with Black
164 Viton septa (Supelco, Bellefonte, PA, USA).

165 The SPME fibre was then exposed to the headspace of the sample. During the equilibration step (10
166 min) and the extraction step (20 min), the vial was maintained at 60 $^{\circ}\text{C}$ under magnetic stirring. After
167 sampling, the extracted volatile compounds were thermally desorbed in the GC injector (splitless
168 mode).

169

170 *2.4. Separation and identification of volatile compounds*

171 A Trace 1300 GC Thermo scientific (Thermo Fisher Scientific Inc.) system coupled to an ISQ mass
172 spectrometer was used to perform the analyses. The capillary column used in the GC-MS was a
173 SUPELCOWAX 10 capillary column (Supelco, 30 m \times 0.25 mm, f.t. 0.25 μm). Helium at 15 psi was
174 used as carrier gas with a flow rate of 1.0 ml min⁻¹.

175 Thermal desorption of the compounds from the fiber coating took place in the GC injector at 250 $^{\circ}\text{C}$
176 for 3 min. Analyses were performed at programmed temperature starting from 40 $^{\circ}\text{C}$ for 3 min,
177 increasing of 5 $^{\circ}\text{C}$ per minute to 200 $^{\circ}\text{C}$ and maintaining this final temperature for 5 min.

178 The identification of compounds was based on three criteria: (1) by comparing the mass spectra with
179 the NIST 14 of mass spectra (2) by comparing the retention index with literature data, and (3)
180 whenever possible, the identification was confirmed by using pure standards of the components.

181 Retention indices were calculated using n-alkanes (C8-C17) as reference compounds according to
182 Bianchi et al., (2007).

183 Semiquantitative analysis was carried out by comparing the peak area of each compound with that of
184 the internal standard. Because of the non-availability of standards for all the detected substances, the
185 response factor was considered as one for all the identified compounds.

186 **Results and Discussion**

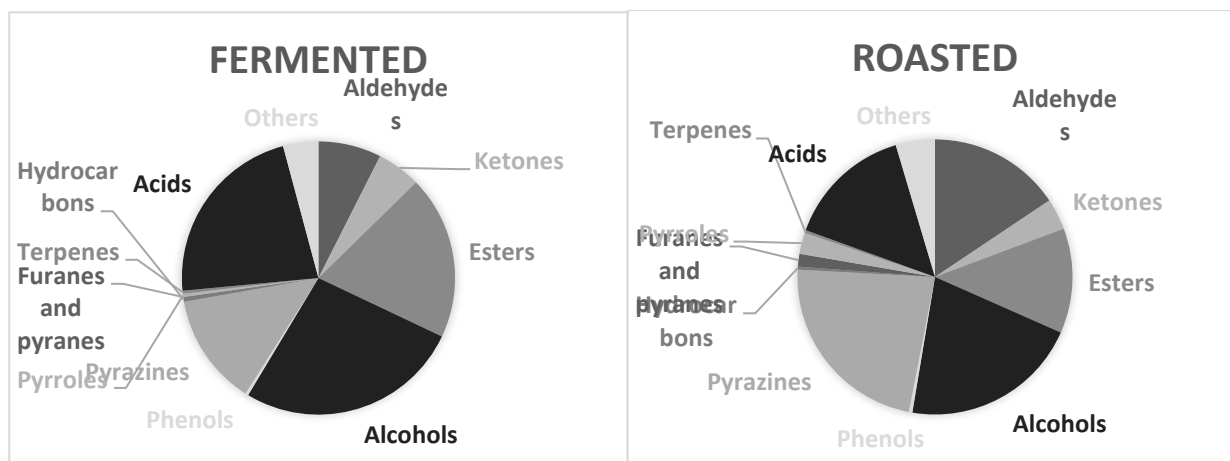
187 In this research 59 samples of fermented cocoa beans were analyzed by HS-SPME GC-MS before and
188 after roasting. A total of 76 aroma compounds were determined and grouped into different chemical
189 classes (Tab 2, 3). The concentrations of each compound in each sample analysed are reported in
190 supplementary materials..

191

	Fermented	Roasted	Fermented	Roasted
	mg/kg	mg/kg	%	%
Aldehydes	1.62	7.98	7.46	15.62
Ketones	1.13	1.88	5.22	3.68
Esters	4.21	6.30	19.37	12.33
Alcohols	5.78	10.74	26.57	21.01
Phenols	0.08	0.21	0.36	0.42
Pyrazines	2.88	11.65	13.24	22.79
Hydrocarbons	0.12	0.18	0.57	0.35
Furanes and pyranes	0.00	0.77	0.00	1.50
Pyrroles	0.08	1.27	0.37	2.49
Terpenes	0.08	0.17	0.36	0.34
Acids	4.85	7.60	22.31	14.87
Others	0.91	2.35	4.18	4.60
Total sum	21.75	51.12		

192 Tab. 2 *Mean total sum of volatile compounds (mg/kg) determined on all cocoa samples before and*
193 *after roasting divided by chemical classes*

194



195

196 *Figure 1: Mean distribution of the main classes of aroma compounds in b) fermented unroasted cocoa*
 197 *beans and c) roasted cocoa beans.*

198

199 The total sum of volatiles in cocoa samples is highly variable, both in fermented and roasted beans,
 200 as demonstrated by data reported in tab 1. Roasting had a pronounced impact on the concentration of
 201 the volatiles, giving origin to total values of volatile about doubled respect to the fermented beans, from
 202 21.75 to 51.12 mg/kg respectively (tab 2).

203 To better understand the cocoa aroma modification after roasting, figure 1a and 1b report, respectively,
 204 the mean distribution in classes of aroma compounds before and after roasting. In unroasted beans, the
 205 total relative concentrations of esters (4.21/19.37) and alcohols (5.78/26.57) were higher than the
 206 concentrations of aldehydes (1.62/7.46) and ketones (1.13/5.22), pyrazines (2.88/13.24) and other
 207 compounds (0.91/4.18), as shown in Figure 1. After roasting, the distribution of the different classes of
 208 compounds changes; pyrazines (11.65/22.79) and aldehydes (7.98/15.62) increased and new compounds
 209 were formed from the Maillard reactions, as pyrroles, furanes and pyranes.

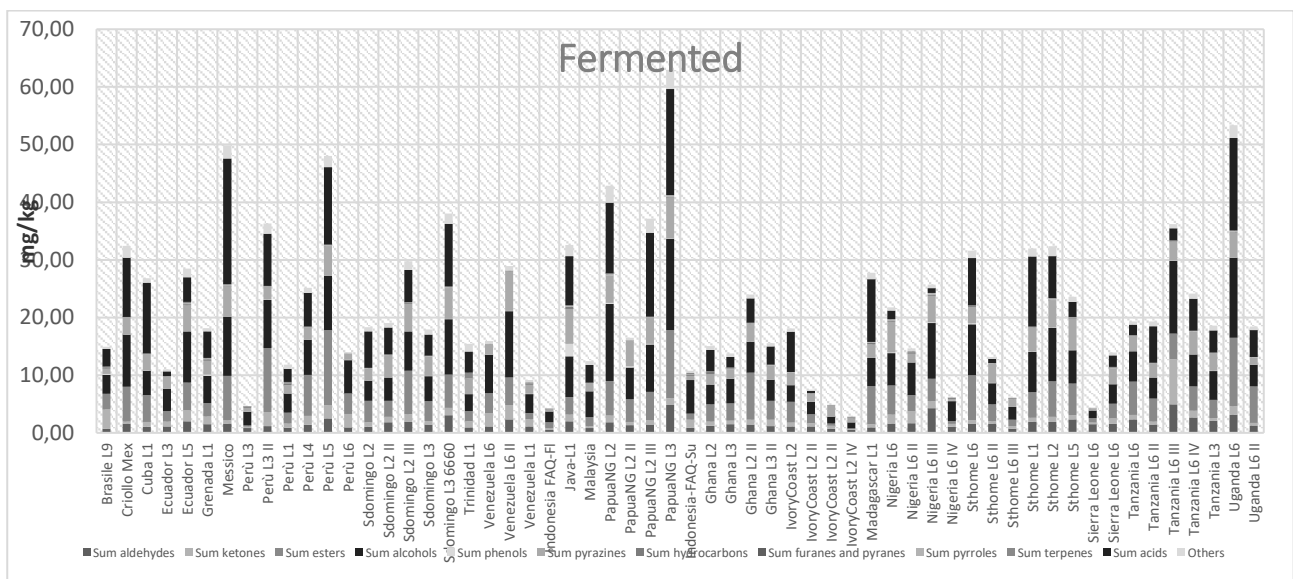
210 Aroma compounds before roasting are mainly of fermentative origin, even if a slight effect of drying
 211 procedures has been also reported, so in principle their amount is not strictly linked to the development
 212 of aroma after roasting. However, a positive correlation was observed between total aroma before and
 213 after roasting (correlation coefficient: 0.76). This was tentatively explained by the fact that the most
 214 aromatic beans before roasting ex mexico correspond to the most fermented ones, which are richer of

215 amino acids of proteolytic origin and so they are also able to develop more aromatic compounds during
216 roasting.

217

218 *Composition of volatile fraction of fermented cocoa beans*

219 The sum of each main classes of compounds for each sample is reported in Figure 2, showing a large
220 variability in the aromatic compounds in fermented cocoa beans of different origin.



221

222 Figure 2: Total amounts of a) esters, b) aldehydes/ketones, c) pyrazines and d) alcohols in each cocoa
223 sample of different geographical origin.

224

225 Esters. Esters of acetic acid are predominant. 3-methyl-1-butanol acetate, 2-phenylethyl acetate,
226 methyl acetate, 1-butanol-3-methylbenzoate, and butyrolactone were the major esters found in the
227 samples analyzed, as reported in Table 1 supplementary data. Ester concentration is quite variable in
228 fermented cocoa beans of different origin, ranging from minimum value of 0.13 mg/kg for one sample
229 from Ivory Coast, to 13.01 mg/kg for one sample from Peru.

230 The concentration of esters compounds depends on the duration of the fermentation process, in fact
231 it decreases as fermentation time increases (Rodriguez-Campos et al., 2012).

232 To get a cocoa flavor of high quality is desirable the presence of 2-phenylethyl acetate and
233 ethylphenyl acetate in high concentrations, as they are considered essential for the formation of the

234 typical cocoa aroma. The production of these esters can be a result of yeast metabolism during the
235 fermentation process, which produces key cocoa aromas such as flowery and honey flavour notes
236 (Aculey et al., 2010; Frauendorfer & Schieberle, 2008). Samples containing the higher amounts of 2-
237 phenylethyl acetate (> 1mg/kg) are Criollo variety (considered a fine cocoa) and mainly American
238 cocoa beans (Mexico, Perù, S.Domingo), Uganda, S.Thome and samples from Papua New Guinea,
239 the last containing also the higher amount (0.39 mg/kg) of ethylphenyl acetate (see supplementary
240 materials S1).

241 Aldehydes and ketones. A total of 13 compounds belonging to the class of aldehyde and ketones, were
242 detected, ordered by the most abundant to the least: benzaldehyde, 2-nonanone, 2-heptanone,
243 acetophenone, acetoin, 3-methylbutanal, 2,3-butanedione, phenylacetaldehyde, 2-methylbutanal,
244 nonanal, 1H-pyrrole-2-carboxaldehyde and 2-phenyl-2-butenal. A high concentration of aldehydes and
245 ketones is desired in cocoa beans as it helps to impart a fruity and flowery notes (Serra-Bonvehi 2005),
246 total concentration in the samples analyzed ranging from 0.13 to 7.82 mg/kg.

247 The presence of 2-and 3-methylbutanal is appreciated because these aldehydes exhibit notes of malt and
248 chocolate. The formation of these two aldehydes can be attributed to degradation of isoleucine and leucine
249 by lactic acid bacteria during fermentation (Jinap et al., 1994, Frauendorfer & Schieberle, 2008). Their
250 contents reaches values of 0.17 mg/Kg and 0.46 mg/Kg respectively in one sample from Tanzania, and
251 values of 0.14 and 0.43 in one sample of Nigeria.

252 2-nonanone, acetophenone, acetoin and diacetyl were the major ketones identified. Diacetyl is a well-
253 known secondary metabolite of yeasts and bacteria and imparts a buttery aroma to many foodstuffs, it
254 ranging from minimum value of 0.01 mg/kg for Perù and Ivory Coast samples to 0.33 for one samples of
255 Nigeria. Acetoin can be produced from pyruvate and butanediol during alcoholic fermentation (Pretorius,
256 2000) and has been reported as a precursor of tetramethylpyrazine (Hashim, Jinap, Muhammad & Ali,
257 1999). Samples containing the lowest levels of acetoin are costa avorio 0.01 mg/kg malesia 0.04 mg/kg
258 sulawesi 0.03 mg/kg, samples containing the higher amount are venezuela 0.52 mg/kg perù 0.40 mg/kg s
259 domingo 0.33 mg/kg.

260 Alcohol. The concentration of alcohols increases in the beans that undergo long fermentation processes,
261 but it decreases during the drying process (Rodriguez-Campos et al., 2012). High alcohol contents are
262 desirable to obtain cocoa products with flowery and candy notes (Aculey et al., 2010; Frauendorfer &
263 Schieberle, 2008), total concentration founded in the samples analyzed ranging from 1.05 to 15.86 mg/kg.
264 Several alcohols were detected in the samples under study: 2 phenylethanol, 2,3-butanediol, 2-heptanol,
265 3-methyl-1-butanol, 2-nonanol, 2-pentanol, 2-ethyl-1-hexanol, benzyl alcohol, phenol, 1-phenylethanol,
266 4-methyl phenol, 3-methyl phenol. Some identified alcohols are responsible for producing desirable
267 flavour notes, i.e. 3-methyl-1-butanol, 2-heptanol and 2-phenylethanol (Ducki et al., 2008; Jinap, Wan-
268 Rosli, Russly, & Nordin, 1998).

269 2,3-butanediol is normally considered as a fermentation product (Owusu et al., 2011), it ranging from a
270 minimum value of 0.15 mg/Kg in one sample from sierra leone to 4.46 mg/Kg in a sample from papua.

271 The benzyl alcohol and 2-phenylethanol are known products of yeast metabolism (Delfini, Gaia, Bardi &
272 Mariscalco, 1991; Nykanen, 1986) providing flowery flavor notes (Jinap et al., 1998) and increase with
273 an increase of fermentation time (Crafack et al., 2014). Samples containing the higher amounts of 2-
274 phenyethanol are Tanzania 0.24 3.03 papua 0.18 4.21 Nigeria 0.14 2.96, while peru 0.02 0.25 costa av
275 0.02 0.17 are the samples containing the lowest amounts (see supplementary materials S1).

276

277 Pyrazines. Pyrazines are considered the most important of the volatile components in cocoa beans. They
278 originate in part by microbiological processes during fermentation but especially by Strecker degradation
279 that accompanies the Maillard reaction during roasting. Pyrazines are substances with low molecular
280 weight and high volatility and constitute the fundamental part of the cocoa aroma together with esters,
281 alcohols, aldehydes and hydrocarbons.

282 Five pyrazynes, namely tetramethylpyrazine, trimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethyl-6-
283 ethyl pyrazine and 2,3-dimethyl-5-ethyl pyrazine were present in all the fermented samples but in different
284 proportions ranging from 0.21 to 7.08 mg/kg. A major quantitative difference involved primarily the
285 dimethyl, trimethyl and tetramethyl pyrazine peaks.

286 Tetramethylpyrazine, the most abundant, reaches the highest values in American/Asiatic samples from
287 Venezuela (6.02 mg/Kg), Papua New Guinea (5.04 mg/Kg) and Java (4.41 mg/Kg) while the lowest
288 values in African samples from Sierra Leone 0.15 (mg/Kg) and Nigeria (0.30 mg/Kg).
289 Tetramethylpyrazine is known to be a metabolic product of *Bacillus subtilis*, and its presence is an
290 indication of *B. subtilis* activity during the fermentation of cocoa beans (Gill, MacLeod & Moreau,
291 1984). This compound is one of the important components of cocoa flavor that can be used as cocoa
292 flavor enhancer (Rohan & Stewart 1965; Nebesny & Rutkowski 1998).

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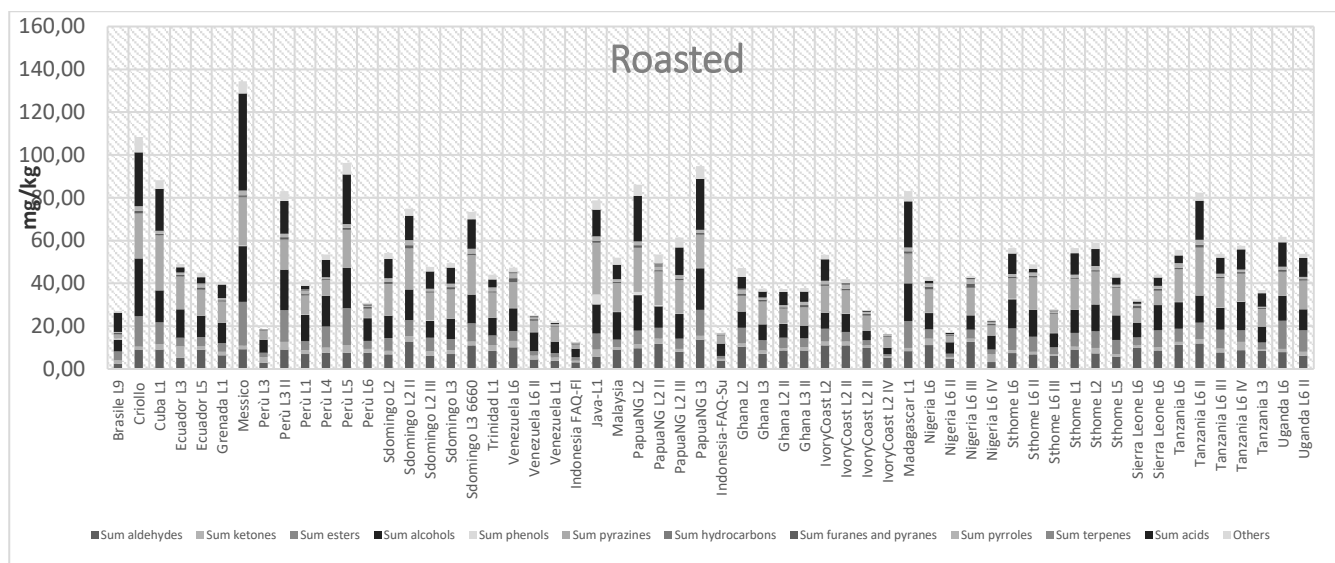
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295

296 PCA

297 In order to obtain an easily interpretable quali-quantitative description of the eventual differences existent
298 in the aroma profile of cocoa beans, a PCA was performed on all the bean samples of different geographic
299 origins. PCA was performed on the matrix of 62 volatiles for 59 fermented cocoa samples, and the
300 principal components were constructed with the correlation method. Four Components were extracted,
301 accounting for 57 % of the variation in the volatile fingerprint. PC1, PC2 and PC4, accounting for 50.5 %
302 of total variance explained, were plotted in a 3D score plot (Fig. 2a), which shows a clustering of cocoas
303 according to their geographical macro areas of origin. Plotting the first two components only, no specific
304 grouping was evidenced, so PC4 was also considered, because it appears the component mainly related
305 to the origin of cocoa. PC3 was not included in the score plot because it contributes only to a large
306 separation of a single sample from Java. Variables with high positive value on PC3, related to Java sample,
307 correspond to phenol and methyl phenols (smoky note), which have unusual high values only in Java
308 cocoa.

331 The 59 samples of spontaneously fermented and dried cocoa beans were analyzed by HS-SPME GC-MS
 332 after roasting. Roasting conditions were the equivalent to industrial processes, 140°C for 30 minutes.
 333 A total of 76 aroma compounds (Table 2) was detected. Roasting had a pronounced impact on the
 334 composition and relative concentration of the volatiles identified in cocoa beans showing a general
 335 increasing (Figure 2b, 4); in fact, 14 novel compounds were detected after roasting: 1,3-Propanediolo
 336 diacetate, 2-Methylpropanal, 2-Isopropyl-5-methyl-2-hexenal, 5-Methyl-2-phenyl-2-hexenal, 2-Ethyl
 337 pyrazine, 2,3-Dimethylpyrazine, 2-Ethyl-6-Methyl pyrazine, 3-Ethyl-2,5-Dimethyl pyrazine, n-
 338 Butylbenzene, 3-Furfuryl alcohol, 4-hydroxy-2,5-dimethyl 3(2H)furanone, 4H-pyran-4-one-2,3-dihydro-
 339 3,5-dihydroxy-6-methyl (dihydroxy-maltol), (1-ethyl-2-pyrrolidinyl)-methanol, Propanoic acid-2-metil.
 340
 341



342
 343 Fig 4

344
 345 The global mean variation in cocoa specific aroma compounds before and after roasting is evidenced
 346 in Table 1, sub-divided in classes of compounds.
 347 Whilst the total concentration of ketones, terpenes and terpene alcohols changed only slightly or remained
 348 at the same level, the concentration of aldehydes, alcohols and pyrazines increased remarkably upon
 349 roasting and were the classes that undergo major modification.

350

351

352 *Figure 5:*

353 Aldehydes. In the samples analysed total sum of aldehydes ranging from 2.49 to 13.75 mg/Kg.

354 Pronounced increases of the Strecker aldehydes, 2-methylbutanal, 3-methylbutanal, benzaldehyde and

355 benzenacetaldehyde were observed during roasting, whilst 2-methylpropanal, 2-isopropyl-5-methyl-

356 2-hexenal and 5-methyl-2-phenyl-2-hexenal, not present in the un-roasted beans, were identified in

357 the roasted ones.

358 5-methyl-2-phenyl-2-hexenal is formed through aldol condensation of phenylacetaldehyde with

359 acetaldehyde and 2-methylpropanal (Ziegler, 2009), and is reported as being a key constituent of

360 chocolate aroma (Bonvehí, 2005; Counet & Callemien, 2002; Owusu et al., 2011) which possesses a

361 deep bitter, persistent cocoa note (Van Prang et al. 1968).

362

363 Alcohol. Total sum of alcohol ranging from 2.76 to 27.10 mg/Kg. The most abundant alcohols

364 identified in the roasted beans were 2,3-butanediol, 2-phenylethanol and 2-heptanol, although they are

365 normally considered as fermentation products.

366 2-heptanol is also important to cocoa flavor, which can be used as cocoa flavoring materials. This

367 compound has been identified as volatile compounds of thermally degradable amino acids and has a

368 strong green flavor and sweet aroma, which could contribute to the cocoa bean flavor.

369

370 Pyrazines. The group of pyrazines is one of the most important volatile compounds in roasted cocoa, in

371 samples analysed in the present work total sum ranging from 1.78 to 25.38 mg/Kg. Serra-Bonvehí (2005)

372 mentioned that pyrazines represented 40% of the aroma in roasted cocoa, in our samples they represent

373 about 23% of the aroma in roasted beans, showing an increasing of the mean total sum of about 70% after

374 roasting.

375 Besides the 2,6-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazines, 2,3-dimethyl-5-ethylpyrazine
376 and 2,3,5-trimethyl-6-ethylpyrazine identified in the un-roasted beans, four new pyrazines were formed as a
377 result of roasting: 2-ethyl pyrazine, 2,3 dimethylpyrazine, 2-ethyl-6-methylpyrazine, 3-ethyl-2,5-
378 dimethylpyrazine.

379 Important increases of 2,3-dimethyl-, 2,3,5-trimethyl- and 2,3,5,6-tetramethylpyrazine were observed
380 upon roasting with the latter being the most abundant pyrazine.

381 Tetramethylpyrazine is one of the important components of cocoa flavor that can be used as cocoa flavour
382 enhancer (Rohan and Stewart 1965; Nebesny and Rutkowski 1998). From organoleptic descriptions,
383 trimethyl- and tetramethylpyrazine possess a nutty, grassy and pungent persistent cocoa note (Van Praag
384 et al. 1968). Samples containing the higher amounts of tetramethylpyrazine are Cuba (16.16 mg/Kg)
385 Java (14.08 mg/Kg) and Tanzania (11.84 mg/Kg), while Flores (0.97 mg/Kg) Nigeria (0.99 mg/Kg) and
386 Brazil (1.11 mg/Kg) are the samples containing the lowest amounts (see supplementary materials S1).
387 The second most represented pyrazine is 2,3,5-trimethylpyrazine, which accounted for a minimum of 0.37
388 mg/Kg (in sample Flores) to a maximum of 6.39 (in sample Mexico); its aroma contribution has been
389 described as green and earthy, cocoa- and roasted nuts-like [16,21,17]

390

391 **PCA**

392 Data obtained were submitted to multivariate chemometric analysis (PCA).

393 PCA was performed on the matrix of 76 volatiles for 59 fermented cocoa samples, and the principal
394 components were constructed with the correlation method. Two Components were extracted, accounting
395 for 38 % of the variation in the volatile fingerprint.

396 PC2, PC3 and PC4, accounting for 51 % of total variance explained, were plotted in a 3D score plot
397 (Fig. 2a), which shows a clustering of cocoa beans according to their origin. Plotting the first two
398 components only, it was not possible to obtain a clear separation of cocoa beans origin, so PC4 was
399 also considered to improve the differentiation of origin (Fig. 2c).

400 PC1 is not specifically linked to the geographical origin because essentially separates criollo (higher
401 positive values on PC1) from the others. Criollo is a different variety known to be a fine flavor cocoa.

402 On the opposite side of PC1 samples from sulawesi are found that are generally poor fermented and
403 with low aroma potential. Therefore PC1 is the component explaining mainly the system variability
404 linked to the different fermentation level or different variety.

405 To gain insight in the observed spectral clustering, the PCA loadings were inspected (Fig. 3).

406 PC1 is the component mainly related to the differentiation based on fermentation level or different
407 variety; it explains 27 % of the total variance in the samples, and the highest positive component
408 loadings were Etanone-1-(1H-pirrol-2-il), Trimethylpyrazine, 1,3-Butanediol, Benzyl acetate; the
409 signals 2,3-Dimethylheptane 2-Heptanone 2-Pentanol 2-Nonanone were those with higher negative
410 loadings on PC1.

411 PC2 explains 38% of the variation and it is important for the separation of american and asiatic
412 samples. PC2 is able to separate a small group of caribbean cocoa beans and the highest positive
413 loadings related to asiatic samples were the aldehydes Nonanal, 2-Isopropyl-5-methyl-2-hexenal, 2-
414 Methylpropanal, 3-Methylbutanal and 2,6-Dimethyl pyrazine; the highest negative related to
415 caribbean samples loadings on PC2 were the esters 2-Phenylethyl acetate, Isobutyl acetate and 3-
416 Methyl-1-butanol acetate.

417 PC3 (46%) contributes only to grouping a small group of Asiatic samples characterized by high negative
418 value on PC3 correspond to phenol and methyl phenols (smoky note), variables with high positive value
419 were 2 heptanone 2 heptanol acetato 2 nonanone.

420 PC4 explains 51% of the variance and is important for the differentiation between africa and
421 america (see Fig. 2c). The highest positive loadings that characterize samples from africa were the
422 alcohols 2-pentanol, 1-phenilethanol, 3-Methyl-1-butanol and ketons 2,3-butanedione,
423 acetophenone. The highest negative loading related to american beans were tetramethylpyrazine,
424 phenol and methyl phenols (smoky note).

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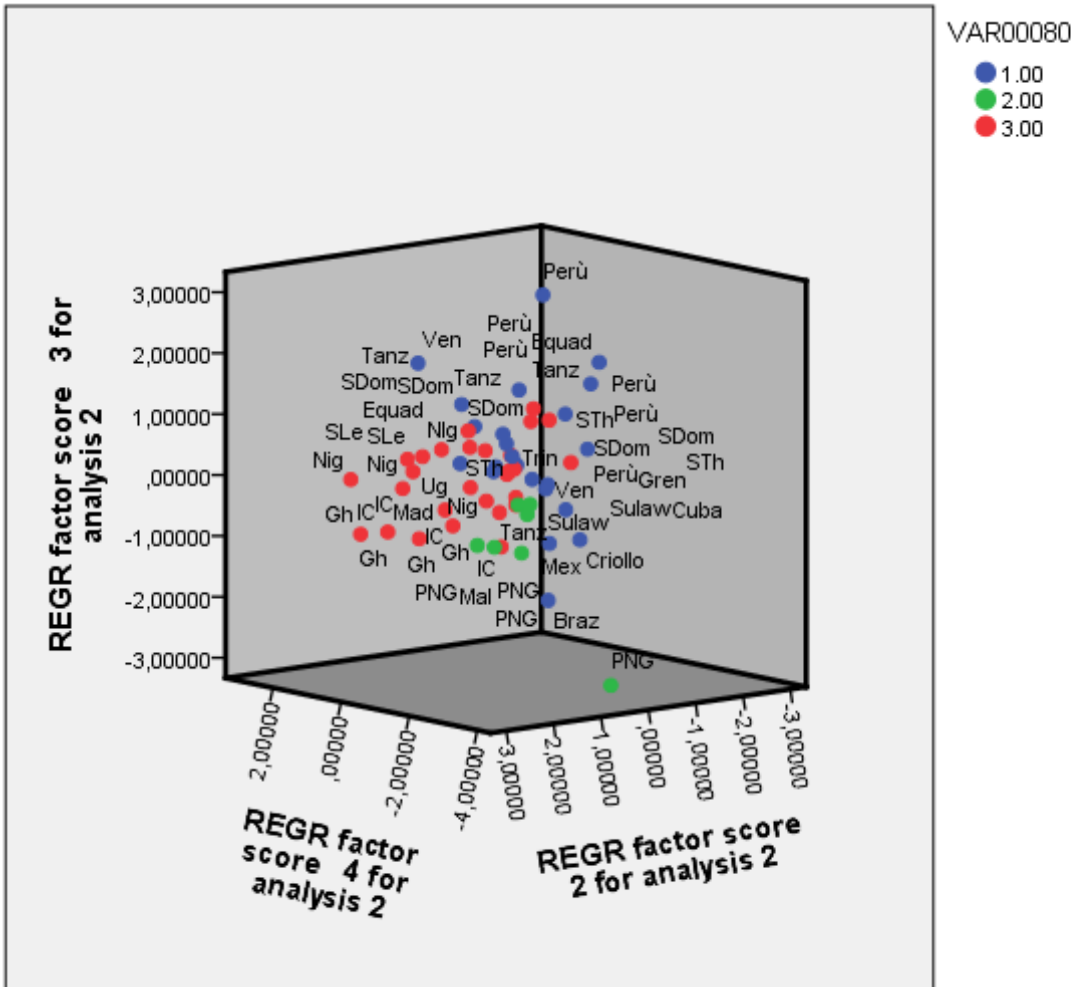
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437 *Figure 5: Principal component analysis: 3d-score plot of all fermented and not-roasted cocoa bean*
 438 *samples on PC2, PC3, PC4*

439 Results showed that although the same roasting treatment has been applied to all the samples, the
 440 differences among the fermented samples are also maintained in the aromatic profile resulting after
 441 roasting. As reported for not roasted cocoa beans, the volatile fingerprint showed interesting potential

442 for authentication of both raw and roasted cocoa beans suggesting that the native aromatic profile of
443 fermented beans continues to differentiate cocoa showing a pronounced impact on the characteristics
444 of the final product.

445 **Conclusions**

446 Only few studies are present in literature about the analytical methods to determine the metabolic
447 differences of cocoa composition related to cocoa beans origin. Most of studies are focused on a
448 limited dataset, few are focused on raw cocoa beans and rare are the works focused on raw cocoa
449 bean from a significant numbers of different countries. One of the main strength point of the samples
450 considered in this study is that they are representative of the entire world cocoa production.

451 HS SPME GC MS analysis of cocoa aroma combined with chemometric tools has been revealed as a
452 promising application for the authentication of high quality cocoa, both in raw and roasted form. A
453 total of 62 volatiles were identified in raw beans while 76 were those identified in roasted cocoa
454 beans. The compounds belong to the following chemical groups: esters, alcohols, organic acids,
455 aldehydes and ketones, pyrazines and other compounds.

456 Data obtained were submitted to multivariate chemometric analysis (PCA). Results showed that
457 cocoa volatile combined with chemometrics are able to discriminate cocoa samples from
458 macroareas as Africa and America. Samples from Asia/Oceania are instead not discriminated. The
459 same statistical analysis applied on the corresponding roasted samples showed that although the same
460 roasting treatment has been applied to all the samples, the differences among the fermented samples
461 are also maintained in the aromatic profile resulting after roasting.

462 As reported for fermented cocoa beans, the volatile fingerprint showed interesting potential also for
463 authentication of roasted cocoa beans, and this could be an advantage from a commercial point of
464 view since it is important to determine the quality of chocolate and the veracity of labeling after
465 technological processing to trace the cocoa production site's country.

466 The latter important practical application could be the improvement of chemical composition
467 knowledge on cocoa raw material to give information to pilot the secondary processing steps of cocoa
468 towards the desired final product characteristics.

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Table 1 Geographical origin of cocoa samples together with the number of samples for single country.

Continent	Country	N° of samples	
América	Mexico (var. Criollo)	1	
	Mexico	1	
	Cuba	1	
	Dominican Republic	5	
	Grenada	1	
	Trinidad	1	
	Venezuela	4	
	Ecuador	2	
	Brazil	1	
	Perú	6	
	África	Sierra Leone	2
		Ivory Coast	4
		Ghana	4
Nigeria		4	
São Tomé		6	
Uganda		2	
Tanzania		5	
Asia	Madagascar	1	
	Java	1	
	Malaysia	1	
	Flores	1	
	Sulawesi	1	
	Papua New Guinea	4	

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580 **Table 3:**

581 *concentration (mg/kg) of cocoa aroma compounds before and after cocoa beans roasting.*

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N°	Compound name	R.T.	RI	Odour Description ^a	Identificatio n ^b	Anova test	Mean in fermented beans	Std dev	Mean in roasted beans	Std dev
Esters										
1	Methyl acetate	2,68	83 2		MS, IK		0,217		0,208	
2	Ethyl acetate	3,35	89 3	Fruity, pineapple	MS, IK		0,105		0,194	
3	Isobutyl acetate	5,81	10 19	Fruity	MS, IK		0,094		0,116	
4	Butyl acetate	7,35	10 77		MS, IK		0,310		0,357	
5	3-Methyl-1-butanol acetate	8,73	11 28	Banana	MS, IK		2,000		2,277	
6	2-Heptanol acetate	12,77	12 69		MS	<0.05	0,294		0,694	
7	Ethyl octanoate	17,48	14 42	Fruity, flowery	MS, IK	<0.05	0,187		0,312	
8	2-Methyl-propanoic acid ethyl ester	22,24	16 32		MS	<0.05	0,035		0,057	
9	Butyrolactone	22,49	16 42	Bready	MS, IK	<0.05	0,082		0,211	
10	Ethyl decanoate	22,56	16 45	Fruity, floral, pear, grape	MS, IK	<0.05	0,035		0,064	
11	1,3-Propanediolo diacetato*	23,04	16 66		MS	<0.05	0,000		0,080	
12	Benzoic acid ethyl ester	23,32	16 78		MS	<0.05	0,063		0,122	
13	Benzyl acetate	24,78	17 41	Flowery, rose, jasmine	MS, IK	<0.05	0,047		0,101	
14	Benzene acetic acid methyl ester	25,47	17 72		MS	<0.05	0,020		0,045	
15	Ethylphenyl acetate	26,03	17 97	Flowery, rose, fruity, sweet	MS, IK	<0.05	0,087		0,186	
16	2-Phenylethyl acetate	26,69	18 27	Honey, flowery	MS, IK	<0.05	0,550		1,078	
17	Ethyl laurate	27,06	18 45	leaf, fruity, floral	MS, IK	<0.05	0,000		0,003	
18	1-Butanol-3- methylbenzoate	27,18	18 50		MS		0,086		0,194	
Aldehydes										
19	2-Methylpropanal*	2,55	82 0	Malty, chocolate	MS, IK	<0.05	0,000		0,259	
20	2-Methylbutanal	3,75	91 7	Malty, chocolate, cocoa	MS, IK	<0.05	0,037		0,500	
21	3-Methylbutanal	3,82	92 0	Malty, chocolate, cocoa	MS, IK, STD	<0.05	0,115		2,929	
22	2-Isopropyl-5-methyl- 2-hexenal*	15,58	13 70		MS	<0.05	0,000		0,953	
23	Nonanal	16,41	14 01	Tallowy, soapy-fruity	MS, IK	<0.05	0,016		0,041	
24	Benzaldehyde	19,85	15 34	Bitter almond, grass, fruity	MS, IK, STD	<0.05	1,369		2,234	
25	Phenylacetaldehyde	22,8	16 56	Green, honey, flowery	MS, IK	<0.05	0,068		0,283	
26	2-Phenyl-2-butenal	29,19	19 46	Flowery, cocoa, roasted, rum	MS, IK	<0.05	0,016		0,408	
27	5-Methyl-2-phenyl-2- hexenal*	32,06	20 86	Sweet, roasted cocoa	MS, IK	<0.05	0,000		0,376	
Ketones										
28	2,3-Butanedione	5,05	98 5	Buttery	MS, IK, STD		0,087		0,077	
29	2-Heptanone	10,43	11 87	Fruity, green, flowery	MS, IK		0,296		0,277	

30	Acetoin	13,43	12 92	Buttery, cream	MS, IK, STD	<0.05	0,128	0,427
31	2-Nonanone	16,27	13 96	Flowery, fatty	MS, IK		0,391	0,611
32	Acetophenone	22,96	16 62	Floral, swet	MS, IK	<0.05	0,233	0,482
33	1H-inden-1-one-2,3-dihydro	30,87	20 28		MS	<0.05	0,000	0,005
Alcohols								
34	2-Pentanol	8,58	11 22	Green	MS, IK, STD		0,162	0,198
35	3-Methyl-1-butanol	11,09	12 10	Malty, chocolate	MS, IK, STD		0,477	0,568
36	2-Heptanol	14,31	13 24	Citrusy, sweet	MS, IK	<0.05	0,527	0,904
37	2-Ethyl-1-hexanol	18,89	14 96		MS, IK	<0.05	0,148	0,279
38	2-Nonanol	19,59	15 24		MS, IK		0,227	0,290
39	2,3-Butanediol	20,16	15 47	Sweet, flowery	MS, IK, STD	<0.05	1,334	2,536
40	1,3-Butanediol	21,07	15 83		MS	<0.05	1,389	2,541
41	1-Phenylethanol	26,59	18 23	Honey, floral	MS, IK, STD		0,049	0,108
42	Benzyl alcohol	27,98	18 87	Sweet, fruity	MS, IK		0,074	0,157
43	2 Phenylethanol	28,71	19 22	Floral	MS, IK	<0.05	1,393	3,158
Phenols								
44	2-methylphenol	30,62	20 16	Smoky	MS, IK		0,000	0,002
45	Phenol	30,71	20 20	Smoky	MS		0,068	0,151
46	4-Methyl phenol	32,21	20 93	Horse stable, phenolic	MS, IK	<0.05	0,004	0,040
47	3-Methyl phenol	32,36	21 00	Smoky	MS, IK		0,004	0,020
Pyrazines								
48	2,6-Dimethyl pyrazine	14,5	13 31	Nutty, coffee, green	MS, IK	<0.05	0,114	0,192
49	2-Ethyl pyrazine*	14,68	13 38	Peanut-butter, musty, nutty	MS, IK	<0.05	0,000	0,027
50	2,3-Dimethylpyrazine*	15	13 49	Caramel, cocoa, sweet, baked	MS, IK, STD	<0.05	0,000	0,463
51	2-Ethyl-6-Methyl pyrazine*	16,08	13 89		MS, IK	<0.05	0,000	0,129
52	Trimethylpyrazine	16,57	14 07	Earthy, cocoa, fried potato, roasted	MS, IK, STD	<0.05	0,389	2,390
53	3-Ethyl-2,5-Dimethyl pyrazine*	17,71	14 51		MS	<0.05	0,000	0,479
54	2,3-Dimethyl-5-ethyl pyrazine	18,11	14 66	Cocoa, chocolate	MS, IK	<0.05	0,003	1,637
55	Tetramethyl pyrazine	18,49	14 80	Chocolate, cocoa, roasted	MS, IK, STD	<0.05	2,336	5,983
56	2,3,5-Trimethyl-6-ethyl pyrazine	19,43	15 17	Candy, sweet	MS, IK	<0.05	0,036	0,349
Hydrocarbons								
57	2,3-Dimethylheptane	2,47	81 3		MS		0,046	0,021
58	1-Ethyl-2-methylbenzene	11,69	12 31		MS	<0.05	0,001	0,005

59	Styrene	12,6	12		MS, IK		0,065	0,060
60	n-Butylbenzene*	20,94	15		MS	<0.05	0,000	0,069
61	Naphtalene	25,04	17		MS, IK	<0.05	0,011	0,025
			53					
Furanes/pyranes								
62	3-Furfuryl alcohol*	23,14	16	Bready	MS, IK	<0.05	0,000	0,367
			70					
63	4-hydroxy-2,5-dimethyl 3(2H)furanone*	31,23	20	Caramel-like- STRAWBERRY	MS, IK		0,000	0,101
			45					
64	4H-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl (dihydroxymaltol)*	35,84	22	caramel?	MS	<0.05	0,000	0,301
			70					
Pyrroles								
65	(1-ethyl-2-pyrrolidiny)-metanol*	14,79	13		MS	<0.05	0,000	0,441
			42					
66	Etanone-1-(1H-pirrol-2-il)	29,96	19		MS		0,064	0,752
			83					
67	1H-Pyrrole-2-carboxaldehyde (2-acetyl-1H-pyrrole)	31,09	20	Chocolate, hazelnut	MS, STD		0,016	0,081
			39					
Terpenes								
68	β -myrcene	9,88	11	Herbaceous, metallic	MS, IK		0,068	0,138
			68					
69	Linalool	20,54	15	Rose, flowery	MS, IK	<0.05	0,011	0,035
			62					
Acids								
70	Acetic acid	17,97	14	Sour, vinegra	MS, IK		4,852	7,042
			61					
71	Propanoic acid-2-metil*	21,21	15		MS		0,000	0,558
			89					
Others								
72	unknown1	11,9	12				0,063	0,000
			40					
73	unknown2	11,95	12				0,079	0,354
			40					
74	unknown3*	14,89	13		MS		0,000	0,363
			45					
75	unknown4	17,66	14		MS		0,048	0,039
			49					
76	unknown5	20,76	15				0,718	1,596
			71					

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Table 4: Loadings values of the variable for the first four principal components

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