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Metabolomics fingerprint of Philippine coffee by SPME-GC-MS for geographical and varietal classification

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1 **Metabolomics fingerprint of Philippine coffee by SPME-GC-MS for geographical and varietal**
2 **classification**

3
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22

24 **Abstract**

25 Volatile metabolites of Philippine Arabica and Robusta coffee beans in the both forms standard (not-
26 eaten by the Asian palm civet) and civet coffee grown in different Philippine regions were identified
27 using the hyphenated technique headspace-solid phase microextraction-gas chromatography-mass
28 spectrometry. A great number of volatile metabolites with a wide variety of functional groups were
29 extracted and forty-seven prominent compounds were identified.

30 The volatile metabolomics (volatilomics) fingerprint of Arabica coffees considerably differed with
31 Robusta coffee and geographical origin slightly altered the fingerprint profile of coffee samples.
32 Chemometric analysis such as principal component analysis (PCA) displayed a good classification
33 between Arabica and Robusta coffee samples. Although, Arabica coffee samples from different
34 geographical origins were clustered separately from each other, the proximity of clusters between
35 Arabica coffee samples which can be classified into one large group, indicated their close similarity of
36 headspace metabolites. PCA also identified several key volatile metabolites for the distinction of this
37 group from Robusta coffees which is attributed to the higher amount of acetic acid, furfural, 5-
38 methylfurfural, 2-formylpyrrole, and maltol, and lower concentration of 4-ethylguaiacol and phenol in
39 all Arabica samples. These discriminating metabolites could be useful quality markers to differentiate
40 Arabica with Robusta coffee. Results revealed that the headspace metabolites in coffee provide
41 significant information on its inherent aroma quality. Also, the findings suggested that the overall
42 quality of Philippine coffee is variety and region specific.

43

44 **Keywords:** Volatile metabolites, Volatilomics, Civet coffee, Asian palm civet, Arabica, Robusta,
45 Geographical origin, HS-SPME-GC-MS, Discriminant markers

46

47 ¹*Abbreviations*

¹ *Abbreviations:* AC, Asipulo Civet; AR, Asipulo Robusta; CA, Cordillera Arabica; CC, Cordillera Civet; GC, Gas chromatography; HS, headspace; i.d., Internal diameter; KC, Kalinga Civet; KR, Kalinga Robusta; MA, Matutum Arabica; MC, Matutum Civet; MS, Mass spectrometry; MW, Molecular weight; PC, Principal Component; PCA, Principal Component Analysis; SPME, Solid phase microextraction

49 1. Introduction

50

51 Coffee aroma is the result of the multiplicity of volatile compounds present in roasted coffee beans
52 (*Coffea* spp.). The complex balance of the most important volatile compounds in coffee has a relative
53 contribution to its overall aroma quality (Bernard, Roberts, & Kraehenbuehl, 2005). So far, more than
54 eight hundred volatile compounds belonging to a wide range of chemical classes have been identified
55 in roasted coffee (Mayer & Grosch, 2001; Rocha, Maetzu, Barros, Cid & Coimbra, 2003), including
56 aliphatic volatile metabolites (carbonyl-containing compounds, sulfur-containing compounds), alicyclic
57 compounds (including several ketones), benzenoids (phenols); heterocyclic compounds (furans,
58 hydrofurans, pyrroles, pyridines, quinolines, pyrazines, quinoxalines, indoles, thiophens, thiophenones,
59 thiazoles, oxazoles) (Clarke, 1986).

60 Nowadays, coffee drinking is the best social lubricant and people are becoming more discriminating in
61 their preference for coffee. The aroma of coffee is one of the most important consumer's preference
62 vectors due to its contribution to the palatability and appreciation of overall coffee quality. This has
63 recently given rise to a fast growing demand for specialty coffee or commonly referred to gourmet or
64 premium coffee produced from special geographic microclimates beans with unique flavor profiles
65 (Teuber, 2019).

66 Among the specialty coffees, civet coffee ranks as the most expensive and best coffee in the world due
67 to its unique aroma and taste (Lee, 2006). It is made from coffee cherries which have been eaten and
68 passed through the digestive tract of the (Asian palm) civet. Civets naturally select and consume the
69 ripest and sweetest coffee cherries, and excrete the undigested inner beans. The passage of the beans
70 through the digestive tract of civet adds flavor to the coffee by partially breaking down the proteins,
71 thus modulating the coffee bitter taste (Marcone, 2004).

72 Civet coffee is produced in only few countries from Far East including Philippines, where it has been
73 recognized as one of the important indigenous export products of the country (Yulia & Suhandy, 2017).
74 Philippine civet coffee is derived mainly from the beans of Arabica and Robusta coffee trees found in
75 the forests where the Asian palm civet thrives, particularly those in the mountains of the Cordillera
76 region, Batangas, Davao, and Cotabato. The different aroma characteristics of Philippine Arabica and
77 Robusta (not eaten and eaten by the Asian palm civet) and their inherent attributes are still a puzzle and
78 require deeper understanding of their chemical nature.

79 The need to identify reliable method that can determine the volatile compounds responsible for the
80 aroma quality of Philippine coffee varieties and geographical origin is therefore of crucial relevance.
81 Some studies have recently used a metabolomic approach to ascertain the authenticity of far Eastern
82 civet coffees. They focused on non-volatile compounds, such as organic and phenolic acids,
83 carbocyclic sugars, and their ratios (Jumhawan, Putri, Marwani, Bamba, & Fukusaki, 2013; Jumhawan,
84 Putri, Bamba, & Fukusaki, 2016). In particular, inositol to pyroglutamic acid ratio was selected as a
85 chemical marker to discriminate the authenticity of civet coffee. This index makes sense, as
86 pyroglutamic acid derives from the degradation of two amino acids, glutamine and glutamic acid
87 (Montevecchi, Masino, & Antonelli, 2010), which could originate from the enzymatic action of Asian
88 palm civet on protein structures of the green coffee.

89 Volatile metabolomics, or volatilomics, is a novel approach and a useful tool for the assessment of food
90 quality and authenticity. It involves separation and detection of volatile metabolites using a
91 multidisciplinary field of science including analytical chemistry, bioinformatics, statistics, and
92 biochemistry (Bouhifd, Hartung, Hogberg, Kleensang, & Zhao, 2013; Lytou, Panagou, & Nychas,
93 2019).

94 The volatilomic analytical platform commonly utilized for the analysis of headspace (HS) metabolites
95 is gas chromatography coupled with mass spectrometry (GC-MS) (Rowan, 2011). Several extraction
96 methods can be employed for HS-GC-MS analysis such as vacuum or steam distillation (Stoffelsma,
97 Sipma, Kettenes, & Pypker, 1968; Kumazawa & Masuda, 2003); purge and trap (Costa Freitas &
98 Mosca, 1999); static headspace (Sanz, Ansorena, Bello, & Cid, 2001; Mayer & Grosch, 2001); sorptive
99 extraction and stir bar sorptive extraction (Bicchi, Iori, Rubiolo, & Sandra, 2002); and finally solid
100 phase extraction (SPE) (Ishikawa et al, 2004). The application of headspace solid phase
101 microextraction (HS-SPME) has been widely recognized because it is a non-destructive and non-
102 invasive method in the determination of volatile and semi-volatile metabolites (Hamm et al., 2003).
103 Also, it is a solvent-free, simple and fast, relatively compact and low cost sampling technique.
104 Moreover, it is highly sensitive, selective and compatible with analytical systems having low detection
105 limits (Pawliszyn, Yang, & Orton, 1997).

106 The general aim of the project is the characterization of Philippine coffees and the safeguard of their
107 authenticity, in the both forms standard (not-eaten by the Asian palm civet) and civet coffee. Also, the
108 present study aims to outline through the hyphenated technique HS-SPME-GC-MS a volatilomic
109 fingerprint of four types of roasted coffee beans coming from different geographical regions of the

110 Philippines. The selected samples belong to the two main species of *Coffea* genus (Arabica and
111 Robusta) in their standard form and in their civet version.

112

113 **2. Materials and methods**

114

115 *2.1. Sampling*

116

117 Samples of *Coffea arabica* (throughout the paper referred to as Arabica) and *C. canephora* (sin. *C.*
118 *robusta*; throughout the paper referred to as Robusta) roasted beans were acquired from different
119 regions of the Philippines. Arabica and Robusta coffee beans eaten and not-eaten by Asian palm civet
120 (*Paradoxurus hermaphroditus*) were included in the samples.

121 Four Robusta coffee beans samples were taken from the northern part of the Philippines (Kalinga
122 province and Asipulo district, located in Ifugao province), while four Arabica coffees were obtained
123 from the southern part (Matutum district located in South Cotabato province) and the northern part
124 (Cordillera, Mountain province) of the country. A map of the Philippines indicating the sites of the
125 geographic origin of the coffee samples is shown in figure 1. Arabica coffee samples, namely Matutum
126 Arabica (MA), Matutum Civet (MC), Cordillera Arabica (CA), and Cordillera Civet (CC) were
127 compared with four Robusta coffee samples, notably Kalinga Robusta (KR), Kalinga Civet (KC),
128 Asipulo Robusta (AR), and Asipulo Civet (AC). All coffee samples are commercially available and
129 dark roasted between 220 °C and up to 230 °C.

130

131 *2.2. Chemicals and standards*

132

133 All high-purity analytical standards were purchased from Sigma-Aldrich (Merck KGaA, Milan, Italy).

134

135 *2.3. Method for the volatiles extraction*

136

137 *2.3.1. Optimization of the method*

138

139 To optimize the protocol of extraction, the effects of sample weight (0.5 g, 1.0 g, and 1.5 g), extraction
140 time (10 min, 20 min, and 30 min) and temperature (60 °C, 70 °C, and 80 °C), desorption time (5 min

141 and 10 min) were assessed based on the highest number of peaks and highest peak areas. All the
142 optimization analysis was carried out on the same sample of Cordillera Arabica coffee.

143

144 2.3.2. *Optimized HS-SPME protocol for the extraction of coffee volatile metabolites*

145

146 The roasted coffee beans (1.0 g) were placed in a 20-mL crimped-top-sealed vial. Each vial was heated
147 at 70 °C for 10 min to reach sample headspace equilibrium. The volatile compounds were extracted
148 using a 50/30 µm divinylbenzene–carboxen-polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco,
149 Merck KGaA, Bellefonte, PA, USA). The fiber was inserted into the vial and exposed to the headspace
150 above the coffee sample for 20 min at 70 °C. After the extraction, the fiber was thermally desorbed into
151 the GC injection port for 5 min. Each coffee sample was analyzed thrice.

152 [Arabica (2 standard + 2 civet) + Robusta (2 standard + 2 civet)] x 3 = 24 samples (total)

153

154 2.4. *GC-MS analysis*

155

156 The analysis was performed using a gas chromatograph Hewlett-Packard (HP) 6890 series instrument
157 (Hewlett-Packard, Waldbronn, Germany) with a split/splitless injection port coupled with a mass
158 spectrometer instrument HP 5973 Mass Selective Detector (Hewlett-Packard, Waldbronn, Germany),
159 equipped with a crossbond acid-deactivated Carbowax-like polyethylene glycol capillary column
160 (Stabilwax-DA 11023, Restek Corporation, Bellefonte, PA, USA), measuring 30 m, having an internal
161 diameter of 0.25 mm and film thickness of 0.25 µm. GC-MS analysis was performed in splitless mode
162 at 250 °C. The oven temperature was set at 60 °C, held for 2 min and increased at 5 °C/min up to
163 240 °C and finally held for 5 min.

164 The molecular fragmentation was obtained by electron ionization (EI). The data were obtained in full-
165 scan mode and the mass/charge ratio (m/z) was recorded between 50 and 550 at 70 eV. Chromatograms
166 were acquired and processed using the software Enhanced Chem Station (G1701AA Version A.03.00,
167 Hewlett Packard).

168 Identification was carried out by comparing retention times and mass spectrum of all the available pure
169 standards. In the absence of pure standards, the volatiles were identified by comparing their mass
170 spectra with those present in the data system libraries (Wiley 7th Edition Library and NIST-14). Only
171 those compounds with match probabilities above 80% (considered a satisfactory match), and those ones

172 for which the same identification was matched across several samples and for which a similar mass
173 spectra spectrum was observed, were identified. In cases in which unacceptable confident matches
174 were found through the libraries, the compounds were individually checked and in cases where the
175 compounds showed the same retention time, molecular ion, base ion, and fragmentation patterns in all
176 samples were taken into account and labeled as ‘*unknown 1-8*’ accordingly. The absence of said
177 compounds was verified in blank injections. Whenever it was possible, the identification of volatiles
178 was also verified based on the presence in the literature. A semi-quantitation was carried out by
179 considering the average values of the absolute peak areas.

180

181 *2.5. Statistical analysis*

182

183 Multivariate analyses, notably principal component analysis (PCA) and cluster analysis, were applied
184 to the whole data set. All tests were performed with Statistica version 8.0 software (Stat Soft Inc.,
185 Tulsa, OK, USA).

186

187 **3. Results and discussion**

188

189 *3.1. Optimization of HS-SPME operating conditions*

190

191 The DVB/CAR/PDMS fiber was chosen for HS-SPME due to its high affinity towards a pool of
192 analytes characterized by a wide-range of polarity, including aromatic heterocycles, benzenoids,
193 aliphatic and alicyclic hydrocarbons. In addition, this fiber has already been successfully applied in
194 previous studies (Bicchi et al., 2002; Mondello et al., 2004; Ryan, Shellie, Tranchida, Casilli,
195 Mondello, & Marriott, 2004; Mondello et al. 2005; Toci & Farah, 2008; Franca, Oliveira, Oliveira,
196 Agresti, & Augusti, 2009).

197 Increasing the sample weight from 0.5 g to 1.0 g, the intensity peaks of most compounds substantially
198 improved. However, 1.5 g of sample did not yield a further increase in the response. This is probably
199 due to a decrease of phase ratio “ β ” (headspace to sample ratio), and in the retention capacity of the
200 fiber (Kolb & Ettre, 2006). For this reason, 1.0 g was used as a standard sample weight.

201 Headspace generation was held at 70 °C for 10 min and the extraction temperature was varied from
202 60 °C to 70 °C and up to 80 °C at the constant extraction time of 20 min. The lowest extraction

203 temperature of 60 °C generated lower peak areas for most of the semi-volatile compounds. Conversely,
204 the highest extraction temperature of 80 °C resulted in an increase of peak areas of the high boiling
205 compounds, but caused the reduction of the areas of the compounds with a high vapor pressure. This
206 was due to a displacement effect that occurred onto the fiber to the detriment of substances with a high
207 vapor pressure. Extraction temperature of 70 °C was therefore deemed the best condition to achieve the
208 maximum extraction efficiency of volatile metabolites and used for the standard protocol.

209 Extraction time depends on factors affecting the mass repartition of the volatile metabolites among
210 sample, headspace, and fiber coating. In order to determine the optimum extraction time, extraction
211 temperature was held constant, without sample agitation, and extraction time varied from 10, 20, and
212 30 min. Results showed that 10-min extraction time yielded high areas of the low boiling volatiles,
213 whereas 30 min were more favorable for some semi-volatile compounds. The finding implied that there
214 was an inverse relationship between the extraction time and the volatility of the analytes. Extraction
215 time of 20 min was considered a good compromise for both volatile and semi-volatile compounds and
216 was adopted as standard procedure.

217 The complete thermal desorption of volatile metabolites from the fiber coating is necessary to improve
218 chromatographic resolution and prevent carry-over of volatile metabolites to the subsequent extraction
219 process. Desorption of volatile metabolites from the fiber coating was carried out at 250 °C based on
220 previous studies (Toci and Farah, 2008; Oliveira, Oliveira, Franca, & Augusti, 2009; Costa Freitas,
221 Parreira, & Vilas-Boas, 2001). Instead, desorption time was established to achieve the complete
222 purging and cleaning of SPME fiber. The fiber was desorbed in the GC injection port for 5 and 10 min
223 and subjected again to desorption in a subsequent blank run. No peaks appeared during the latter run in
224 both cases, thus indicating that 5 min was a suitable time to prevent carry-over effects.

225

226 *3.3. Identification and semi-quantitation of volatile metabolites*

227

228 The list of volatile metabolites extracted and identified is shown in Table 1. IUPAC names are
229 indicated together with the main synonyms. The latter are used throughout the article as they are most
230 commonly used in the literature.

231 Arabica and Robusta coffees showed a high number of volatile metabolites belonging to a wide variety
232 of chemical classes, notably aromatic heterocycles (furans, pyranes, pyrazines, pyridines, pyrroles),
233 aliphatic and alicyclic hydrocarbons, phenols, aldehydes, ketones, alcohols, esters, lactones, and fatty

234 acids. Forty-seven volatile metabolites were considered in total, 27 of which were confirmed using pure
235 reference standards, while other 12 were tentatively identified based on MS-libraries matching. Eight
236 peaks were included in the list as unknown compounds, since their presence was verified in most of the
237 samples.

238 Figure 2 presents the volatiles composition of the complete samples set. The volatile that showed by far
239 the highest concentrations was furfuryl alcohol, followed by furfuryl acetate, 5-methylfurfural, and 3-
240 acetylanisole. Furfuryl alcohol has a very mild, slightly caramel-like, warm-oily smell and is well
241 correlated with the undesirable burnt and bitter note of dark-roasted coffees (Flament, 2002).

242 The comparison between Robusta and Arabica samples showed that the latter had higher amounts of
243 acetic acid, furfural, 2-acetylfuran, 5-methylfurfural, furfuryl alcohol, 3-methylcyclopentane-1,2-dione,
244 maltol, and 2-formylpyrrole. Conversely, Robusta samples showed higher amounts of 3-ethyl-2,5-
245 dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, guaiacol, phenol, 4-ethylguaiacol, and 3-
246 acetylanisole. Pyrrole and 2-ethyl-3,5-dimethylpyrazine were not detected at all in Arabica samples and
247 only found in small concentrations in Robusta samples.

248 In general, the concentrations of furanic compounds in Arabica and of pyrazine compounds in Robusta
249 stood out. A marked prevalence of furanic derivatives in Arabica samples, as well as a concomitant
250 slighter prevalence of pyrazine volatile metabolites in Robusta samples, has already been described
251 (Mondello et al., 2005). Furthermore, Ryan et al. (2004) reported that maltol was significantly higher in
252 Arabica samples, as well as phenol was significantly lower, in comparison with Robusta samples.
253 However, phenol has a medicinal odor and does not contribute to the pleasantness of coffee flavor
254 (Dorfner, Ferge, Kettrup, Zimmermann, & Yeretizian, 2003). Robusta coffees showed also higher
255 content of phenolic compounds. In particular, guaiacol is an important character impact volatile that
256 provides a smoky peaty phenolic note (Semmelroch, Laskawy, Blank, & Grosch, 1995).

257 Acetic acid must be considered separately. Unlike many other volatiles, the concentration of this
258 compound decreases with increasing degree of roasting (Somporn, Kamtuo, Theerakulpisut, &
259 Siriamornpun, 2011). Although acetic acid may represent a valid chemical marker for the degree of
260 roasting, its concentration in Arabica coffee was generally higher than that of Robusta once roasted
261 under the same conditions (Caporaso, Whitworth, Cui, & Fisk, 2018).

262 At least 22 compounds identified in Arabica (coming from El Salvador, Costa Rica, and Brazil) and
263 Robusta (coming from Togo, India, and Vietnam) coffees (Mondello et al., 2005) were also present in
264 Philippine coffees, notably pyridine, pyrazine, 2-methylpyrazine, 2-ethylpyrazine, 2,3-

265 dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, furfural, 2-acetylfuran, pyrrole,
266 furfuryl acetate, 5-methylfurfural, furfuryl alcohol, γ -butyrolactone, furfurylpyrrole, guaiacol, 2-
267 acetylpyrrole, furfuryl ether and 2-formylpyrrole.

268 AR samples showed a lack of volatile substances compared not only to their corresponding civet
269 samples but also to all other samples, being its average volatiles sum from one third to one fifth lower.
270 The cause of it is unknown and might be due to the specific lot of sample. This behavior has drastically
271 affected a correct comparison of this sample within the characterization of all other Philippine coffees.

272 Roasting time and temperature of coffee cause extensive chemical modification on green beans (Franca
273 et al., 2009). Non-enzymatic browning reactions are responsible of the formation of a very high number
274 of volatile compounds, most of them belonging to aromatic heterocycles, such as furans, ketones, and
275 pyrazines. Furans partly come from the dehydration of sugars that occurred during the sugar
276 caramelization (Montevecchi, Masino, Chinnici, & Antonelli, 2008), while ketones and pyrazines were
277 produced through Maillard-like reactions between sugars and amino acids (Knoch & Baltes, 1992).
278 Grinding size and brewing methods are equally relevant in the coffee-flavor expression. However, the
279 quali-quantitative variations in volatile metabolites observed in roasted coffee beans can also be
280 attributed to the specific species/variety. Aside from the genotypic traits, the sensory properties of
281 roasted coffee are particularly affected by other factors, such as growing region, altitude, macro- and
282 micro-climatic conditions, and different cherries-fermentation processes (dry or wet) (Illy & Viani,
283 2005). In addition, for Philippine coffees must be also considered whether or not the cherries were
284 passed through the gastrointestinal apparatus of the Asian palm civet (Ongo et al., 2012; Ongo et al.,
285 2015).

286 Based on the present results, it was not possible to make general observations on the different
287 composition in volatiles between civet and non-civet coffees. As for Robusta civet coffees, KC showed
288 an average increase (ratio 1.4) in volatile amount in comparison to its standard coffee. In particular, the
289 volatiles that showed the highest increase were pyrazines (in particular ethyl and isopropenyl
290 substituted), furanic derivatives, phenolic compounds, maltol, and other minor volatiles.

291 As for the Arabica civet samples, MC showed no difference in the comparison (average ratio 1.0) with
292 its standard MA, while CC has even shown an opposite behavior with an average reduction (ratio 0.7)
293 in volatile amount compared to the CA. The only volatile compound that showed an increase in all the
294 civet samples was furfural, a compound that mainly originates from pentose-sugars degradation during
295 the roasting process. This remark consistently leads to confirm a hydrolytic action that occurs in the

296 digestive tract of the Asian palm civet on polysaccharides rich in pentose sugars, such as
297 arabinogalactan (Bradbury & Halliday, 1990). A similar action on protein constituents with consequent
298 release of amino acids, precursor of nitrogen volatiles, cannot be excluded.

299

300 *3.4. Coffee Classification*

301

302 *3.4.1. Principal Component Analysis*

303

304 Autoscaled data concerning the areas of volatile compounds were chemometrically processed through
305 the principal component analysis (PCA) to evaluate the possibility of discriminating Arabica with
306 Robusta coffee, as well as civet and non-civet coffees, through specific volatilomic fingerprints. The
307 PCA score plot of the whole sample set is shown in Figure 3a.

308 The clustering among all Arabica samples and the clear separation with Robusta samples was mainly
309 evident on the second principal component (PC2), which explained 30.70% of the total variance. In the
310 negative quadrants of the PC2, the proximity of CC, CA, MC and MA, which can be also clustered into
311 one large group, indicated a close similarity of the volatiles composition of Arabica coffee samples.
312 The distinction of this wide group from Robusta samples, which were set on the positive quadrants of
313 the PC2, was mainly due to the higher amount of acetic acid, furfural, maltol, 2-formylpyrrole and the
314 lower concentrations of phenol and 4-ethylguaiacol showed in all Arabica samples.

315 Figure 3b depicts the loading plot. The 47 volatile metabolites (for compounds names see Table 1)
316 were all distributed in the negative quadrants of the PC1, except dodecane. This result confirmed that
317 using this data set PCA could separate the samples on the PC2 more than on PC1. Indeed, due to their
318 general scarcity of volatile substances, AR coffees were completely separated from all the other
319 samples. For this reason, different PCAs were run in order to reduce this effect. In particular, civet
320 coffees were subjected alone to a PCA (Fig. 4a), while Arabica (CA and MA) standard coffee samples
321 were compared individually with Asipulo Robusta (Fig. 4b) and Kalinga Robusta (Fig. 4c) standard
322 coffees in two different PCA analysis.

323 The PCA score plot of all civet coffees successfully discriminated Arabica civet (CC and MC) from
324 Robusta civet (AC and KC) coffees (Fig. 4a). A clear separation between Arabica and Robusta civet
325 coffees was, indeed, observed on PC1, while PC2 discriminated the samples coming from different

326 regions of production. Likewise, a clear discrimination between Arabica and Robusta samples on PC1
327 was showed in the figures 4b and 4c.

328 To determine the volatilomic fingerprints conducive to the discrimination among the different coffees
329 samples, an accurate variable-loading analysis was performed using the loadings with consistent values
330 in the all the three latter PCAs. Variables that exhibit loading values higher than 0.8 (80%) provide a
331 major contribution within each PC and can be considered as discriminating variables. On the contrary,
332 variables associated with very low loading values are considered useless and can be ruled out.

333 The volatile metabolites primarily accountable for this discrimination (Table 2) were acetic acid,
334 furfural, 5-methylfurfural, 2-formylpyrrole, and 4-ethylguaiacol. Furfuryl alcohol, pyrrole, and maltol
335 could be considered potential discriminating volatile metabolites as well, although they presented some
336 loading value lower than 0.8. The high positive loading values of acetic acid, furfural, 5-methylfurfural,
337 and 2-formylpyrrole on PC1 indicated a higher amount of these volatile metabolites in samples with
338 positive scores on PC1, notably Arabica coffees. On the contrary, 4-ethylguaiacol weighed on PC1
339 with a negative loading value, thus indicating that the samples with negative scores, notably Robusta
340 coffees, contained a higher amount of it. Similarly, Robusta samples contained higher concentrations of
341 pyrrole and a lower amount of furfuryl alcohol and maltol than Arabica coffees. Furthermore, these
342 findings are consistent with previous reports showing that the higher amounts of furfural, 5-
343 methylfurfural, maltol, and 2-formylpyrrole and the lower concentrations and 4-ethylguaiacol are
344 characteristics of Arabica samples (Blank, Sen, & Grosch, 1991; Semmelroch & Grosch, 1996; Ryan et
345 al., 2004; Mondello et al., 2005; Caporaso, Whitworth, Cui, & Fisk, 2018).

346 Furfural is produced during the acid hydrolysis or heating of polysaccharides containing pentose (or
347 hexose) sugars (Maarse et al., 1994). It has a characteristic of lightly roasted coffee to give it a flavor
348 similar to that of roasted cereals. Furfural is also described as pungent, but sweet, bread-like, caramel-
349 like, cinnamon-almond-like odor of poor tenacity (Fors, 1983). Maltol is a degradation product of
350 disaccharides (maltose). Its odor is sweet, caramel-like, cotton-candy with fruity overtones (Flament,
351 2002). 2-Formylpyrrole was found as a product of the reaction of glutamine with ribose (Ho & Chen,
352 1999) and has a corny, pungent odor (Shibamoto & Russell, 1977). Finally, 4-ethylguaiacol was
353 identified in the thermal decomposition of ferulic acid. It has a smoky and roasted flavor, burnt taste.
354 Likewise, guaiacol is characterized by a smoky aroma (Flament, 2002).

355

356 *3.4.2. Cluster analysis*

357

358 Cluster analysis (Fig. 5) confirmed the similarity among coffee varieties. The individual spots (n = 24)
359 of samples were arranged along the bottom of the dendrogram. The similar spots were formed into
360 clusters by joining them together. The clusters that were nearer to the bottom of the dendrogram were
361 considered highly correlated. The left sub-branch of the grouped points of the dendrogram was
362 populated by all Arabica coffees (CA, MC, MA and CC), while the right sub-branch was populated by
363 Robusta coffees (AC, KR, KC, AR).

364 MC was closely similar to MA, so that the two samples were connected to CA followed by CC. On the
365 other side, all KC samples were linked with two KR samples. The level of similarity between the two
366 samples was less intense as indicated by the distance connecting the two different samples. AC was
367 more similar to Kalinga coffee samples (KC and KR) than to AR samples. Indeed, AR samples were
368 isolated from all the other samples, as already highlighted through the other statistical analysis.

369

370 **4. Conclusions**

371

372 The classification of volatile metabolites of Philippine Arabica and Robusta coffee roasted beans was
373 successfully carried out using a hyphenated analytical approach to outline specific volatilomic
374 fingerprints through multivariate statistical tools. PCA and cluster analysis allowed the discrimination
375 between Arabica and Robusta samples. The key volatile metabolites responsible for the classification
376 of Arabica and Robusta coffees (both types, standard and civet) were acetic acid, furfural, 5-
377 methylfurfural, 2-formylpyrrole, maltol, phenol and 4-ethylguaiacol. The achieved results suggest that
378 the overall quality of Philippine coffee is variety/species and region specific. The findings revealed that
379 the composition of volatile metabolites in coffee is able to provide significant information on the
380 authenticity like other non-volatile markers already used for the same purpose.

381

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394

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569

571 **Figure captions**

572

573 **Figure 1.**

574 Philippine map showing the site of the geographical origin of coffee samples.

575

576 **Figure 2.**

577 Average amounts of the volatiles of the complete samples set.

578 AC, Asipulo Civet; AR, Asipulo Robusta; KC, Kalinga Civet; KR, Kalinga Robusta; CA, Cordillera
579 Arabica; CC, Cordillera Civet; MA, Matutum Arabica; MC, Matutum Civet.

580

581 **Figure 3. a)** PCA score plot (PC1 vs. PC2) of the complete samples set. Robusta samples are in light
582 grey; **b)** PCA loading plot of PC1 vs. PC2. For compounds names refer to Table 1.

583

584 **Figure 4. a)** PCA plot (PC1 vs. PC2) of Arabica and Robusta civet coffees; **b)** PCA plot (PC1 vs. PC2)
585 of Arabica (MA and CA) vs. Asipulo Robusta standard coffees; **c)** PCA plot (PC1 vs. PC2) of Arabica
586 (MA and CA) vs. Kalinga Robusta standard coffees.

587 All Robusta samples are in light grey.

588

589 **Figure 5.**

590 Cluster Analysis (dendrogram) of the complete samples set.

591

592

593 **Table 1.**594 Volatile compounds detected in Philippine roasted coffee beans and their retention times (t_R).

595

#	t_R (min)	Volatiles (IUPAC name)	Synonyms	#	t_R (min)	Volatiles (IUPAC name)	Synonyms
1	2.14	2-Methylfuran		25	16.29	1-Pyridin-2-ylethanone	2-Acetylpyridine
2	6.20	Pyridine		26	16.42	2-(Furan-2-ylmethyl)furan	2-Furfurylfuran
3	6.30	Dodecane		27	16.60	5-Methyl-6,7-dihydro-5H-cyclopenta[b]pyrazine	
4	6.76	Pyrazine		28	16.74	1-Methylpyrrole-2-carbaldehyde	
5	7.21	<i>Unknown 1</i>		29	16.97	Oxolan-2-one	γ -Butyrolactone
6	8.05	2-Methylpyrazine		30	17.54	Furan-2-ylmethanol	Furfuryl alcohol
7	9.37	2,5-Dimethylpyrazine		31	18.38	1-(6-Methylpyrazin-2-yl)ethanone	2-Acetyl-6-methylpyrazin
8	9.51	2,6-Dimethylpyrazine		32	18.74	<i>Unknown 3</i>	
9	9.66	2-Ethylpyrazine		33	18.98	<i>Unknown 4</i>	
10	9.98	2,3-Dimethylpyrazine		34	20.25	<i>Unknown 5</i>	
11	10.88	2-Ethyl-6-methylpyrazine		35	20.64	<i>Unknown 6</i>	
12	11.04	2-Ethyl-5-methylpyrazine		36	20.71	<i>Unknown 7</i>	
13	11.37	2,3,5-Trimethylpyrazine		37	21.27	3-Methylcyclopentane-1,2-dione	
14	12.35	3-Ethyl-2,5-dimethylpyrazine		38	21.32	1-(Furan-2-ylmethyl)pyrrole	Furfurylpyrrole
15	12.71	Acetic acid		39	21.98	2-Methoxyphenol	Guaiacol
16	12.74	2-Ethyl-3,5-dimethylpyrazine		40	24.13	3-Hydroxy-2-methylpyran-4-one	Maltol
17	12.94	<i>Unknown 2</i>		41	24.24	1-(1H-Pyrrol-2-yl)ethanone	2-Acetylpyrrole
18	12.99	Furan-2-carbaldehyde	Furfural	42	24.52	2-(Furan-2-ylmethoxymethyl)furan	Furfuryl ether
19	13.53	3,5-Diethyl-2-methylpyrazine		43	24.72	<i>Unknown 8</i>	
20	13.98	1-(Furan-2-yl)ethanone	2-Acetylfuran	44	24.95	Phenol	
21	14.18	1H-Pyrrole	Pyrrole	45	25.34	1H-Pyrrole-2-carbaldehyde	2-Formylpyrrole
22	14.66	Acetic acid;furan-2-ylmethanol	Furfuryl acetate	46	25.41	4-Ethyl-2-methoxyphenol	4-Ethylguaiacol
23	15.64	5-Methyl-2-furancarbaldehyde	5-Methylfurfural	47	28.60	1-(3-Methoxyphenyl)ethanone	3-Acetylanisole
24	16.12	2-Prop-1-en-2-ylpyrazine	Isopropenylpyrazine				

596

598 **Table 2.**

599 Loading values of PC1 e PC2 obtained from PCA processings: A) civet coffees alone; Arabica (CA and MA) standard coffee samples
 600 compared individually with Asipulo Robusta (AR) (B) and Kalinga Robusta (KR) (C) standard coffees.

601

#	Volatiles (IUPAC name)	Synonyms	A) Arabica civet (CC, MC) coffees vs. Robusta civet (AC, KC) coffees		B) CA and MA vs. AR		C) CA and MA vs. KR	
			PC1	PC2	PC1	PC2	PC1	PC2
1	2-Methylfuran		-0.84	0.23	0.97	0.21	-0.53	-0.84
2	Pyridine		-0.59	-0.17	0.95	-0.27	0.98	0.15
3	Dodecane		0.58	-0.64	-0.02	-0.99	0.44	0.87
4	Pyrazine		-0.24	0.52	0.99	0.02	-0.56	-0.79
5	<i>Unknown 1</i>		-0.65	0.67	0.38	0.31	-0.14	-0.27
6	2-Methylpyrazine		-0.57	-0.65	1.00	0.04	-0.89	-0.45
7	2,5-Dimethylpyrazine		-0.60	-0.66	0.99	0.06	-0.93	-0.37
8	2,6-Dimethylpyrazine		-0.58	-0.72	1.00	0.02	-0.96	-0.28
9	2-Ethylpyrazine		-0.72	-0.49	1.00	0.09	0.97	-0.24
10	2,3-Dimethylpyrazine		-0.91	-0.36	0.99	0.10	-0.96	-0.28
11	2-Ethyl-6-methylpyrazine		-0.90	-0.24	0.99	-0.05	-0.99	-0.14
12	2-Ethyl-5-methylpyrazine		0.19	-0.93	0.63	-0.77	-0.72	0.66
13	2,3,5-Trimethylpyrazine		-0.80	-0.12	0.98	0.14	-0.96	-0.25
14	3-Ethyl-2,5-dimethylpyrazine		-0.86	0.13	0.96	0.27	-0.94	-0.34
15	Acetic acid		0.89	0.37	0.95	0.29	0.92	-0.35
16	2-Ethyl-3,5-dimethylpyrazine		-0.96	0.05	0.00	0.00	-1.00	-0.08
17	<i>Unknown 2</i>		0.55	-0.81	0.99	-0.11	1.00	-0.01
18	Furan-2-carbaldehyde	Furfural	0.94	0.12	0.96	0.02	0.93	-0.21
19	3,5-Diethyl-2-methylpyrazine		-0.96	-0.03	0.93	0.29	-0.79	-0.55

20	1-(Furan-2-yl)ethanone	2-Acetylfuran	0.75	0.24	1.00	0.09	0.52	-0.85
21	1 <i>H</i> -Pyrrole	Pyrrole	-0.71	0.51	-0.97	0.20	-0.99	-0.08
22	Acetic acid;furan-2-ylmethanol	Furfuryl acetate	-0.53	-0.71	0.99	-0.05	0.84	-0.47
23	5-Methyl-2-furancarbaldehyde	5-Methylfurfural	0.92	-0.18	0.99	0.11	0.95	-0.25
24	2-Prop-1-en-2-ylpyrazine	Isopropenylpyrazine	-0.82	0.04	0.92	-0.21	0.64	-0.02
25	1-Pyridin-2-ylethanone	2-Acetylpyridine	-0.88	-0.01	0.99	0.09	0.73	-0.63
26	2-(Furan-2-ylmethyl)furan	2-Furfurylfuran	-0.72	0.06	0.92	-0.25	0.10	0.17
27	5-Methyl-6,7-dihydro-5 <i>H</i> -cyclopenta[<i>b</i>]pyrazine		-0.98	0.08	0.95	0.27	-0.80	-0.56
28	1-Methylpyrrole-2-carbaldehyde		0.11	-0.69	0.99	-0.15	-0.98	-0.14
29	Oxolan-2-one	γ -Butyrolactone	0.14	-0.75	0.87	0.43	0.96	-0.25
30	Furan-2-ylmethanol	Furfuryl alcohol	0.66	-0.68	1.00	0.01	0.97	-0.20
31	1-(6-Methylpyrazin-2-yl)ethanone	2-Acetyl-6-methylpyrazin	-0.43	-0.83	0.96	0.24	0.52	-0.80
32	<i>Unknown 3</i>		-0.96	0.13	0.99	0.01	-0.97	-0.21
33	<i>Unknown 4</i>		-0.39	-0.87	0.99	0.11	0.94	-0.32
34	<i>Unknown 5</i>		0.05	-0.89	1.00	-0.04	0.97	-0.18
35	<i>Unknown 6</i>		-0.64	-0.58	0.99	0.03	0.94	-0.29
36	<i>Unknown 7</i>		0.33	-0.17	0.99	-0.06	0.79	-0.38
37	3-Methylcyclopentane-1,2-dione		0.46	-0.79	0.99	-0.11	1.00	-0.03
38	1-(Furan-2-ylmethyl)pyrrole	Furfurylpyrrole	-0.94	-0.24	0.99	-0.12	0.98	-0.12
39	2-Methoxyphenol	Guaiacol	-0.91	0.21	0.71	0.19	-0.97	-0.16
40	3-Hydroxy-2-methylpyran-4-one	Maltol	0.77	-0.30	0.91	-0.34	0.94	0.19
41	1-(1 <i>H</i> -Pyrrol-2-yl)ethanone	2-Acetylpyrrole	-0.51	-0.21	0.99	-0.14	0.98	-0.06
42	2-(Furan-2-ylmethoxymethyl)furan	Furfuryl ether	-0.84	0.09	0.96	-0.23	0.94	0.08
43	<i>Unknown 8</i>		-0.70	0.32	0.96	0.12	-0.75	-0.51
44	Phenol		-0.77	0.26	-0.76	0.49	0.54	-0.65
45	1 <i>H</i> -Pyrrole-2-carbaldehyde	2-Formylpyrrole	0.98	-0.11	0.99	0.04	0.97	-0.17
46	4-Ethyl-2-methoxyphenol	4-Ethylguaiacol	-0.90	0.27	-0.97	0.12	-0.99	-0.06
47	1-(3-Methoxyphenyl)ethanone	3-Acetylanisole	-0.66	-0.59	0.98	-0.02	0.56	-0.48