Alcoholic fermentation as a potential tool for coffee pulp detoxification and reuse: analysis of phenolic composition and caffeine content by HPLC-DAD-MS/MS

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Abstract

High-performance liquid chromatography with diode array (HPLC-DAD) and liquid chromatograph triple quadrupole mass spectrometry (HPLC-MS/MS) were used to characterize raw and fermented coffee pulps in terms of their phenolic composition and caffeine content. The qualitative analysis showed no significant differences between the raw and the fermented pulps. Free hydroxycinnamic acids (HAs) were mainly chlorogenic acids, with 5-caffeoylquinic acid as the major compound. Bound HAs released caffeic acids during alkaline hydrolysis, and no bound ferulic and p-coumaric acids were detected. The fermentation process allowed the detoxification of the pulp from caffeine by 50%, while significantly reducing the amounts of residue by 64%. Moreover, the fermented products could be further processed to provide high added-value molecules with potential industrial applications, providing a new source of income for the small coffee producers.

Keywords: coffee byproduct, *Saccharomyces cerevisiae*, chlorogenic acids, methylxanthine, eco-toxicity

37 **1. Introduction**

38 Coffee pulp is the main by-product from the wet processing of coffee cherries and it represents
39 around 40% of the weight of the fresh fruit. With an average annual production of ten million tons
40 of coffee beans ("ICO," 2019), a huge amount of pulp is generated in the coffee producing countries
41 (Central and South America, Central and East Africa, Asia). Most of this residue is disposed by
42 dumping into the nature causing serious environmental problems (contamination of soil and
43 groundwater due to the leachate toxicity). Traditional applications of coffee pulp (as fertilizer,
44 livestock feed, compost, etc.) only use a fraction of the available quantity due to its high content of
45 caffeine and polyphenols, considered anti-nutritional to animals and toxic to nature (Murthy &
46 Madhava Naidu, 2012). Recent attempts have been made to detoxify coffee pulp for improved
47 application in agriculture, and to use it for producing several added-value- products such as
48 enzymes, flavor, aroma compounds, organic acids, etc. (Murthy & Madhava Naidu, 2010).

49 Coffee pulp contains, in a dry weight basis, about 50% of carbohydrates, 20% fibers, 10%
50 protein, 2.5% fat, 1.3% caffeine, and it also contains tannins and other phenolic compounds
51 (Pandey *et al.*, 2000). Four major classes of polyphenols have been identified in coffee pulp: flavan-
52 3-ols, flavonols, anthocyanidins, and hydroxycinnamic acids (HAs). Among them, HAs represent
53 from 39 to 49% of total phenolic compounds, with chlorogenic acid 5-caffeoylquinic acid (5-CQA)
54 as the predominant compound (Rodríguez-Durán *et al.*, 2014). Torres-Mancera *et al.* (2011) studied
55 the content of free and bound HAs in coffee pulp and have shown that most of these compounds
56 were found to be covalently bound to the cell wall (about 74–97%) and that they could not be
57 removed by solvent extraction. Food processes such as fermentation, enzymatic extraction, alkaline
58 and acid hydrolyzes occasionally assisted by ultrasound or microwave have the potential to release
59 phenolics bound to plant cell walls (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014).
60 Alkaline and acidic hydrolyzes are the most common means of releasing phenolic compounds,
61 although these molecules are better released with alkaline hydrolysis than in acid hydrolysis
62 conditions (Kim, Tsao, Yang, & Cui, 2006).

We have previously reported that alcoholic fermentation using *Saccharomyces cerevisiae* can be used to enhance the HAs content in coffee pulp extracts through the breakage of ester bonds between these molecules and the pulp cell wall, increasing their concentration and consequently functional properties (Da Silveira *et al.*, 2019). This extract could potentially be marketed to the food and pharmaco-cosmetics industries, generating a new source of income for the small coffee producers. Moreover, the remaining solid is expected to contain less phenolics and caffeine, a well-known methylxanthine, which could facilitate its reuse as animal feed, fertilizer or composting substrate, minimizing the waste disposal in landfills without proper processing, and improving the environmental impact and economic sustainability of the coffee sector. The aim of this study was to analyze the composition of raw and fermented coffee pulps using chromatographic methods (HPLC-DAD-MS/MS) to evaluate the use of alcoholic fermentation for coffee pulp detoxification.

2. Material and Methods

2.1. Raw Material

Fresh coffee pulp from the wet depulping and demucilaging process of coffee beans (*Coffea arabica* L.) was supplied by the Beneficio Coopeunión, a coffee-producer cooperative located in Trés Rios (Costa Rica) during the 2015 harvest. As soon as obtained, the fresh coffee pulp (Supplementary material, Figure 1a) was frozen at -20°C and shipped to France by aircraft in a controlled temperature system. Upon arrival, batches of 1 kg of coffee pulp were thawed at room temperature and ground using a mixer Thermomix TM31 (Vorwerk, Wuppertal, Germany) for 1 min at maximum power (level 10) and without heating. The ground coffee pulp (Supplementary material, Figure 1b) was then split into hermetically sealed flasks and stored at - 20°C until use.

2.2. Chemicals

Methanol, hexane, acetone, and acetonitrile were of HPLC-grade from Sigma Aldrich (Steinheim, Germany). Deionized water was obtained with a Milli-Q Waters system (Millipore, Germany). Ammonium acetate, formic, hydrochloric, glacial acetic and ascorbic acids, were all of analytical grade from Sigma Aldrich (Steinheim, Germany). Sodium hydroxide was purchased from Honeywell (Seelie, Germany) and ethylenediaminetetraacetic acid (EDTA) was purchased from Fisher Scientific Labosi (Paris, France). Standards of caffeic, p-coumaric, and ferulic acids, (-)-epicatechin, caffeine, and phloroglucinol were all of analytical grade purchased from Sigma Aldrich (Steinheim, Germany). Standard of chlorogenic acids was purchased from International Development and Manufacturing (New Jersey, USA), which contained a mixture of: 3-caffeoylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 4-feruloylquinic acid, 5-feruloylquinic acid, 3.4-dicaffeoylquinic acid, 3.5-dicaffeoylquinic acid, and 4.5-dicaffeoylquinic acid.

2.3. Coffee pulp samples

98 The raw coffee pulp sample was prepared by lyophilization during 48 h to obtain a powder that
99 was kept dry and under darkness until use. The fermented pulp samples were obtained after
100 alcoholic fermentation as we previously reported (Da Silveira *et al.*, 2019) using a commercial
101 strain of *Saccharomyces cerevisiae* (10^7 CFU g^{-1}) kindly provided by Lallemand (Toulouse,
102 France). First, the yeast was re-activated and multiplied using sterilized deionized water at 35°C for
103 30 min, at a ratio of 10 mL of water per g dried yeast, then the coffee pulp was inoculated at a ratio
104 of 2.5 g yeast kg^{-1} pulp. Alcoholic fermentation was carried out in the dark without aeration or
105 agitation, in a 600 mL double-walled glass fermenter (useful volume of 400 mL, previously
106 sterilized) for 24 h (Supplementary material, Figure 2). The temperature was fixed at 28°C, the
107 typical ambient temperature in coffee production areas, thanks to a thermostatic water bath. The pH
108 was chosen as the natural pH of the pulp and was monitored using an Almemo measuring
109 instrument (Ahlborn, Ilmenau, Germany). We verified the inoculated yeasts were the
110 microorganisms that acted in the fermentation process thanks to a not inoculated control where no
111 spontaneous fermentation occurred. Glacial acetic acid at 1.0% and sodium metabisulfite at 0.5%
112 were used to stabilize HAs in the coffee pulp during (at t = 8 h) and after the fermentation,
113 respectively. At the end of the fermentation, the pulp was pressed using a hydraulic press (Stossier
114 LI P MO, Samarco, Bouzonville, France) at 50 bar for 30 min. Two products were obtained after
115 the fermentation process: pulp juice and pulp press cake (Supplementary material, Figure 3). The
116 juice was clarified by centrifugation at 8600 g for 15 min and preserved at 4°C until use, while the
117 press cake was ground into a fine powder and kept dried until use. Both samples were kept under
118 darkness.

119 **2.4. Evaluation of moisture content**

120 The moisture content of samples (raw pulp, press cake and juice) was evaluated by gravimetric
121 method after 48 h at 100°C.

122 **2.5. Extraction of free hydroxycinnamic acids**

123 The extraction of free HAs was performed by successive extractions with hexane and methanol
124 as previously described by Rodríguez-Durán *et al.* (2014). First, 8 g of raw pulp (or press cake)
125 were extracted 3 times with 80 mL of hexane for 15 min under agitation (150 rpm) at room
126 temperature to eliminate lipophilic compounds. Hexane extracts were discarded, and the remaining
127 solid was extracted 3 times with 80 mL of aqueous methanol (20:80, v/v) acidified with 2% (v/v)
128 formic acid. Methanolic extraction was carried out for 15 min under agitation (100 rpm) at room
129 temperature. The methanolic extracts were combined, concentrated under vacuum, and the final
130 volume was adjusted to 10 mL with pure methanol. For HPLC analysis, the extract was filtered
131 through a 0.45 µm pore size filter. The HAs extracted under these conditions were considered as
132 free (not linked to the pulp cell wall). All extractions were carried out in triplicate.
133 The free HAs in the pulp juice were analyzed by filtering the juice through a 0.45 µm pore size
134 filter prior to HPLC analysis.
135
136

137 **2.6. Extraction of bound hydroxycinnamic acids**

138 Bound HAs were extracted by alkaline hydrolysis following the extraction of free HAs (Section
139 2.5) according to Rodríguez-Durán *et al.* (2014). First, 600 mg of the remaining solid obtained after
140 methanolic extraction of free HAs were hydrolyzed using a solution of 10 mL of NaOH at 1.0 or 2.0
141 M, 10 mM of EDTA and ascorbic acid at 1.0%. The mixture was incubated for 30 to 180 min under
142 darkness at room temperature and under constant agitation (150 rpm). After the reaction, the
143 mixture was filtered under vacuum and its pH was adjusted to 3.0 ± 0.2 with 12 M HCl. Prior to
144 HPLC analysis, the mixture was filtered through a 0.45 µm pore size filter. All extractions were
145 carried out in triplicate.

146 **2.7. Extraction of caffeine**

147 First, 1 g of raw pulp (or press cake) was extracted under agitation (150 rpm) at room
148 temperature with hexane (3 x 10 mL, 5 min each), then with aqueous methanol (40:60, v/v)
149 acidified with 2% (v/v) formic acid (4 x 10 mL, 15 min each). The methanolic extracts were
150 combined and concentrated under vacuum. Then, the volume of the extract was adjusted to 30 mL
151 with pure methanol. For HPLC analysis, the extract was filtered through a 0.45 µm pore size filter.
152 All extractions were carried out in triplicate.

153 The caffeine in the pulp juice was analyzed by filtering the juice through a 0.45 µm pore size
154 filter prior to HPLC analysis.

155 **2.8. Extraction of proanthocyanidins**

156 For the extraction of proanthocyanidins, 8 g of raw pulp (or press cake) was successively
157 extracted with hexane (3 x 80 mL, 5 min each), then with aqueous methanol (40:60, v/v) acidified
158 with 2% (v/v) formic acid (3 x 80 mL, 15 min each), then with aqueous acetone (30:70, v/v)(3 x 80
159 mL, 15 min each) according to a method adapted from Ramirez-Coronel *et al.* (2004). All
160 extractions were carried out under agitation (150 rpm) at room temperature. Hexane extracts were
161 discarded, while methanol and acetone extracts were recovered and then lyophilized. The solid
162 residue, obtained after the extraction with aqueous acetone, was recovered and kept dry until use.
163 The juice sample was prepared directly by lyophilization.

164 For the reaction with phloroglucinol, 10 mg of dried extract (or 100 mg of solid residue) were
165 used as starting material. A solution of 0.1 N HCl in methanol containing 50 g L^{-1} phloroglucinol
166 and 10 g L^{-1} ascorbic acid was prepared. Phloroglucinolysis reaction was carried out using 2 mL of
167 this solution, under darkness at 50°C for 30 min (or 60 min for the solid residue). After the reaction,
168 the mixture was put on ice-bath and neutralized with 2 mL of 300 mM aqueous sodium acetate. The
169 extract was filtered through a 0.45 µm pore size filter and analyzed by HPLC-DAD-MS/MS.

170 To calculate the apparent mean degree of polymerization (DPn) of proanthocyanidins, the sum
171 of all subunits (flavan-3-ol monomers and phloroglucinol adducts, in moles) was divided by the
172 sum of all flavan-3-ol monomers (in moles).

173 **2.9. Qualitative analysis by HPLC-MS/MS**

174 The identification of phenolic compounds was carried out using an HPLC chain SURVEYOR,
175 equipped with a diode array detector model UV6000LP, a quaternary pump P4000, an auto sampler
176 AS3000 and coupled to a mass spectrometer LCQ equipped with an electro-spray ionization source
177 (THERMO FINNIGAN, San Jose, USA). A column ACEC18 (250 mm × 4.6 mm, 5 µm, AIT,
178 France) thermostated at 30°C was used. The solvents were a mixture of water/formic acid (99.8/0.2
179 v/v; solvent A) and acetonitrile (solvent B). The gradient elution was from 5 to 35% B in 45 min,
180 and from 35 to 100% B in 5 min, after which the column was washed during 10 min with 100% B,
181 then equilibrated for 15 min with 5% B. The flow rate was 0.7 mL min^{-1}, the injection volume was
182 10 µL, and the detection was carried out between 200 and 600 nm. Experiments were performed in
183 negative mode. Scan range was 100–2000 and scan rate, 1 scan s^{-1}. The desolvation temperature
184 was 350°C. High spray voltage was set at 3600 V. Nitrogen was used as the dry gas at a flow rate of
185 75 mL min^{-1}. MS^{-} and MS^{2} were carried out using He as the target gas, with a collision energy of
186 35%. Identification was achieved on the basis of the ion molecular mass and UV–visible spectra.

187 **2.10. Quantitative analysis by HPLC-DAD**

188 The quantification of phenolic compounds and caffeine was performed by High-Performance
189 Liquid Chromatography (HPLC) using an Agilent Technologies 1200 chromatograph (Santa Clara,
190 USA) equipped with a diode-array detector DAD, a vacuum degasser, quaternary pumps, an
191 automated sample injector, column ACE C18 (250 mm x 4,6 mm, 5 µm, AIT France), with
192 identical pre-column, thermostatically controlled at 30°C. The injection volume was 20 µL. The
193 solvents were a mixture of water/formic acid (98/2 v/v; solvent A) and methanol (solvent B). The
194 gradient elution was as follows: from 5 to 35% B in 20 min, from 35 to 55% B in 15 min, from 55

195 to 70% B in 15 min, and finally from 70 to 100% B in 2 min, at a flow rate of 0.7 mL min^{-1}. The
196 column was washed with 100% B for 5 min, then equilibrated for 15 min with 5% B. Quantification
197 was carried out by external calibration using calibration curves of standard solutions (R^2 > 0.99) at
198 330 nm for HAs, and at 280 nm for epicatechin and caffeine.

199 **3. Results and discussion**

200 **3.1. Identification of free hydroxycinnamic acids**

201 Identification of free HAs was carried out on the basis of UV-visible spectra, mass molecular
202 ions and fragment ions and compared to literature data. The UV-visible characteristics and the mass
203 data obtained by HPLC-MS/MS as well as the chromatogram at 330 nm are presented in Table 1.
204 The numbers of the peaks were assigned according to the order of appearance in the
205 chromatographic run.

206 According to the results, the main HAs identified in the pulp samples were chlorogenic acids,
207 including caffeoylquinic acid isomers (peaks 1-3), 3 dicaffeoylquinic acid isomers (peaks 11, 12,
208 and 15), and feruloylquinic acid (peak 7). The identity of these compounds was confirmed by co-
209 injection with a commercial standard. Two co-eluted compounds, 4-caffeoylquinic acid and
210 caffeoyl hexose, were detected in all three samples (peak 3). Other compounds identified were
211 caffeic acid (peak 5) and a derivate of p-coumaric acid (peak 6). These HAs have been previously
212 identified in coffee pulp by several authors (Heeger, Kosińska-Cagnazzo, Cantergiani, & Andlauer,
213 2016; Duangjai *et al.*, 2016; Rodríguez-Durán *et al.*, 2014; Rios *et al.*, 2014; Martínez & Clifford,
214 2000; Ramirez-Martinez, 1988).

215 Some compounds were not detected by mass spectrometry and could not be identified.
216 Nevertheless, based on their maximum spectral absorptions, it was possible to determine their
217 polyphenol family. Peaks 8, 9 and 10 display the characteristics of HAs, while peaks 14 and 16
218 display the characteristics of flavonoid compounds. Peaks 4 and 13 could not be identified since it
219 was not possible to determine their λ_{max} and mass spectra. In a qualitative point of view, no

220 significant differences were observed between the raw and the fermented pulp samples. Peak 8 was
221 only present in the press cake, and peak 10 was only detected in the fermented pulp samples (press
222 cake and juice). These two compounds were probably formed during the fermentation process,
223 which could explain their absence in the raw pulp. Peaks 11-15 were either not detected or present
224 in traces in the juice, possibly due to a lower solubility of these compounds in the juice, or due to
225 their degradation.

226 The use of HPLC-MS/MS allowed to not only verify the phenolic profile of the coffee pulp but
227 also to identify new phenolic structures formed during the fermentation process. Further studies
228 need to be carried out in order to complete the identification of the unknown compounds as well as
229 to analyze minor compounds that were not studied in this work.

230 **3.2. Mean degree of polymerization of proanthocyanidins**

231 The different pulp extracts and solids were analyzed for proanthocyanidins and characterized in
232 terms of their mean degree of polymerization (DPn). Due to problems encountered during the
233 lyophilization step of the fermented pulp samples and the methanolic extract from the raw pulp,
234 only the acetonic extract and the solid residues from the raw pulp were analyzed.

235 After phloroglucinolysis, only one phloglucinol adduct was detected (m/z 413, fragment ion at
236 m/z 289) and only epicatechin was detected as flavan-3-ol monomer terminal subunit. Results show
237 that proanthocyanidins with higher DPn were solubilized in the aqueous acetone extract (DPn 9.2)
238 as compared to the solid residue (DPn 5.6). Ramirez-Coronel *et al.* (2004) also studied the
239 characteristics of coffee pulp proanthocyanidins and found values of DPn 7.6 in the acetone extract
240 and DPn 5.7 in the solid residue.

241 By using a method based on phloroglucinolysis in conjunction with reversed-phase HPLC
242 analysis, it was possible to characterize the raw pulp proanthocyanidins on a molecular weight basis
243 giving information on their nature, proportion and distribution. For further studies, it could be of
244 great interest to characterize the fermented pulp in order to verify any changes in the polymerization

245 degree of proanthocyanidins, which could possibly have a strong impact on the nutritional and
246 ecological qualities of the treated pulp.
247

248 **3.3. Optimization of extraction conditions for bound hydroxycinnamic acids**

249 A preliminary study was carried out to investigate the optimum conditions of alkaline hydrolysis for
250 the recove ry of bound HAs from coffee pulp. The effect of the NaOH concentration (in the
251 presence of ascorbic acid and EDTA) and the incubation time was evaluated using the raw pulp.
252 Figure 1 shows the time course of caffeic acid extracted from the sample during the alkaline
253 hydrolysis using two different NaOH concentrations (1.0 and 2.0 M).

254 In Figure 1, we observe a continuous increase in the amount of caffeic acid with the incubation
255 time up to 2 h, followed by a decrease of this compound for longer incubation times, irrespective to
256 the NaOH concentration. This observation can be explained by the coexistence of at least two
257 reactions: First, the hydrolysis of the HAs covalently bound to the pulp cell wall that releases
258 caffeic acid, and then a second reaction of degradation by oxidation (auto-oxidation, dimerization,
259 dismutation) in such basic medium, that transforms the caffeic acid into other compounds (quinonic
260 species, dimers, etc.), thus decreasing their content in the solution (Farah & Donangelo, 2006).

261 Caffeic acid was the only compound detected after the hydrolysis reaction, which indicates that
262 the bound HAs are caffeic acid derivates, like caffeoylquinic acids, that release caffeic acid when
263 hydrolyzed. Bound ferulic and p-coumaric acids were not detected in the coffee pulp. These
264 observations are in accordance with studies carried out by Rodríguez-Durán *et al.* (2014).

265 Give the above, the optimum conditions for the extraction of bound HAs from coffee pulp are
266 achieved with a solution of 2.0 M NaOH and an incubation time of 2 h at room temperature.
267 Therefore, we used these conditions for the analytical procedure.

268 **3.4. Effect of alcoholic fermentation on coffee pulp composition and ecotoxicity**

269 Figure 2 shows the concentration of each compound, in a dry weigh basis (dw), in the different
270 pulp samples.

271 The most abundant compound is 5-CQA, which was found at 290 mg kg^{-1} dw in the raw pulp,
272 followed by 3,5-DiCQA (104 mg kg^{-1}) and 5-FQA (58 mg kg^{-1}). CQA isomers account for about
273 50% of total free HAs, and DiCQA isomers for about 25%, in the raw pulp. Data on coffee pulp
274 chlorogenic acids (CGA) is usually given in total CGA content, which makes it difficult to compare
275 values. Moreover, studies have shown that coffee pulp composition can vary due to the
276 characteristics of coffee fruits (cultivar, place of production, culture conditions, maturity, etc.), the
277 postharvest management (methods of depulping, drying, storage, etc.), or even the analytical
278 methods used (Duangjai *et al*., 2016; Rios *et al*., 2014; Rodríguez-Durán *et al*., 2014).

279 Caffeic acid was found in concentrations 4.1 times higher in the press cake and 6.6 times higher
280 in the juice as compared to the raw pulp, which could indicate either the release of this compound
281 from the pulp cell walls or the conversion of other compounds into caffeic acid during the
282 fermentation process. Most of the free HAs identified were recovered in the juice, with the
283 exception of the dicaffeoylquinic acids, although higher amounts of HAs can be expected in the
284 juice if the stabilization of these compounds during the fermentation process is achieved (Da
285 Silveira *et al.*, 2019).

286 Table 2 shows the content of free and bound HAs, 5-CQA and caffeine in the different products.
287 The content of free HAs was estimated as the sum of the compounds completely identified by
288 HPLC-MS/MS (Section 3.1), i.e.: 3-caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA),
289 4-caffeoylquinic acid (4-CQA), caffeic acid (CA), p-coumaric acid derivate (PCA), 5-feruloylquinic
290 acid (5-FQA), 3.4-dicaffeoylquinic acid (3.4-diCQA), 3.5-dicaffeoylquinic acid (3.5-diCQA), and
291 4.5-dicaffeoylquinic acid (4.5-diCQA).

292 Caffeine was found at 5.62 g kg^{-1} dw in the raw pulp, which is in accordance with literature data
293 (Juliastuti *et al*., 2018; Rios *et al*., 2014; Braham & Bressani, 1979), although some authors have
294 reported higher values of caffeine in coffee pulp up to 18 g kg^{-1} dw (Ulloa Rojas, Verreth, Amato,

295 & Huisman, 2003; Mazzafera, 2002; Pandey *et al.*, 2000). The analysis of free and bound HAs
296 showed that about 55% of these compounds were found covalently bound to the pulp cell wall, in
297 agreement with reports by Torres-Mancera *et al.* (2011) who found even higher portions of bound
298 HAs, above 70% of total content. This ratio of bound HAs was also found in the press cake, which
299 indicates that the fermentation process impacted preferably the free HAs trapped into the pulp cells
300 and not so much the covalently bound HAs.

301 The fermentation process reduced the amount of caffeine in the press cake by 47% in a dry
302 basis, and by 49% of 5-CQA. Since these compounds are considered as toxic to nature ("ecotoxic")
303 and are present in important concentrations in the pulp, alcoholic fermentation could be used as a
304 pretreatment to detoxify the coffee pulp prior to its reuse in agriculture. Moreover, these compounds
305 could be recovered from the juice and marked to several industries due to their functional
306 properties.

307 Table 3 summarizes the mass balance of the fermentation process in terms of the total amounts
308 of compounds for 100 kg of raw pulp (64% of recovery yield for the pressing step and no mass
309 losses during the fermentation process). Considering the amounts of each compound recovered in
310 both press cake and juice, as compared to the raw pulp, we observed losses of 32% of HAs, 47% of
311 5-CQA, and 32% of caffeine that could be related to their degradation due to oxidation, light,
312 enzymes, despite the use of stabilizing agents (glacial acetic and sulfite) during the fermentation
313 process, or caused by biotransformation through yeast activity (Mäkilä *et al.*, 2016; Mazauric &
314 Salmon, 2005). Further investigations are needed to better understand phenomena that are involved
315 in these HA losses. The ratio of free/total HAs slightly increased from 0.46 in the raw pulp to 0.51
316 in the fermented one, but considering the losses mentioned above, this ratio difference could have
317 been much greater if the HAs extracted were successfully stabilized. The positive effect of the
318 process was also noticeable in terms of the amounts of residue to dispose, 64% less pulp than
319 without pretreatment, and with 50% less caffeine in the pulp residue (press cake). This remaining
320 residue could be potentially used for obtaining organic carbon, applicable in agriculture as a

321 fertilizer or as a precursor for biofuel, and as a raw material in a flash pyrolysis to obtain a rich bio-
322 oil containing valuable molecules applicable to industry (Ruiz Bailon, 2018). The juice, formed
323 mainly by water (93%), can be further fractionated and purified to provide a natural extract rich in
324 high added-value molecules (polyphenols and caffeine), while the water can be recovered during
325 the downstream processing and reused in the process to obtain green coffee.

326 The overall environmental impact of the different products can be evaluated in terms of their
327 ecotoxicity (content of total HAs and caffeine) and the amounts of residue generated (Figure 3). As
328 we can observe, the fermentation process transformed the raw pulp into two products of inferior
329 ecotoxicity, which could increase the opportunities for their reuse. Furthermore, the important
330 decrease in pulp residue generated after the pretreatment would strongly contribute to the
331 sustainable management of the coffee sector, considering the huge amounts of pulp generated every
332 year worldwide (around ten million tons). Finally, we showed that with a simple fermentation
333 process which addresses the goal of green chemistry, it is possible to detoxify the coffee pulp from
334 caffeine, while proving a providing a new source of income for the small coffee producers. The
335 pretreatment process could be improved by studying new stabilizing agents to preserve the
336 compounds extracted during the fermentation and by optimizing the operating conditions. It could
337 also be relevant to characterize both raw and fermented pulps as soon as they are obtained to avoid
338 losses of phenolic compounds caused by environmental stressors (light, temperature, oxygen) and
339 storage methods, proving more precise values of coffee pulp composition. Other analytical methods
340 could be tested to further characterize the products, giving more information on the potential
341 industrial applications.

342

343 **4. Conclusion**

344 This study allowed us to characterize a raw coffee pulp and to evaluate the effect of a new
345 pretreatment process combining alcoholic fermentation and pressing on the composition of the pulp

346 products in terms of the hydroxycinnamic acids (HAs) and caffeine contents using chromatographic
347 methods (HPLC-DAD-MS/MS). Chlorogenic acids were the main compounds identified in the free
348 form of HAs, with 5-caffeoylquinic acid as the major compound. The alcoholic fermentation
349 released bound HAs linked to the pulp cell wall, providing a natural extract with valuable
350 molecules, and decreased by half the amount of caffeine in the residue, contributing for its reuse in
351 agricultural applications. The pretreatment significantly reduced the amounts of pulp to dispose,
352 which would reduce the waste disposal and environmental impact of the coffee sector. However,
353 further characterization studies are needed to gain more insight in the polyphenol content of both
354 raw and fermented products. The fermentation process should continue to be optimized in order to
355 achieve higher recovery yields and to avoid losses of valuable compounds.

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362 **Conflict of interest**

363 The authors declare that they have no conflict of interest. The authors declare that they have no
364 known competing financial interests or personal relationships that could have appeared to influence
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366

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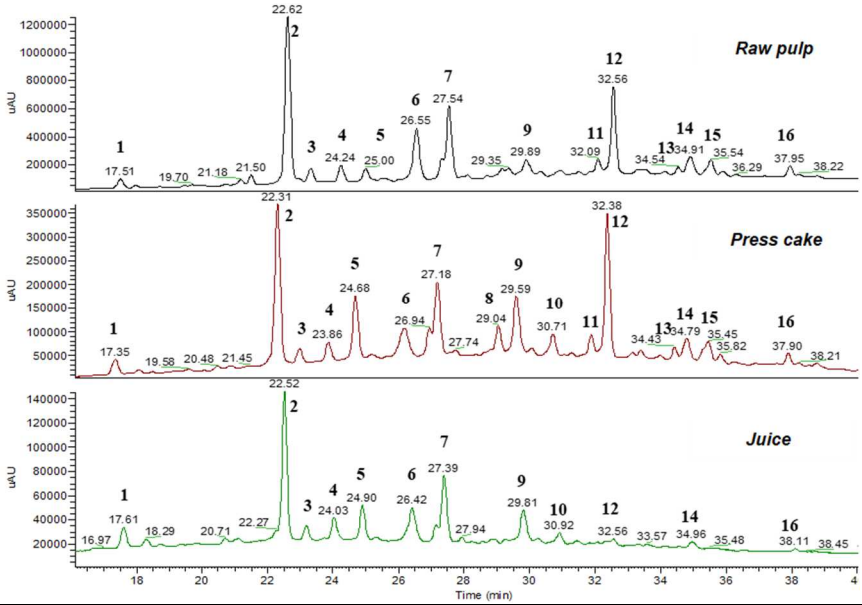
452 **Table 1.** Free hydroxycinnamic acids identified by HPLC-MS/MS in the raw pulp, press cake, and
453 juice (chromatograms at 330 nm).



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Peak nr.	R_t (min)	λ_{max} (nm)	MS^- (m/z)	MS^2 (m/z)	Tentative identification	Sample		
						Raw pulp	Press cake	Juice
1	17.3-17.6	300sh, 328	353	191 (100%), 179 (50%)	3-caffeoylquinic acid	X	X	X
2	22.3-22.6	300sh, 329	353	191 (100%), 179 (10%)	5-caffeoylquinic acid	X	X	X
3	23.0-23.5	300sh, 327	353	173	4-caffeoylquinic acid	X	trace	X
		300sh, 327	341	-	caffeoyl hexose	X	trace	X
4	23.8-24.2	un.	nd.	-	ni.	X	X	X
5	24.7-25.0	300sh,327	179	-	caffeic acid	X	X	X
6	26.2-26.5	316	337	-	p-coumaric acid derivate	X	X	X
7	27.2-27.5	300sh, 324	367	193	5-feruloylquinic acid	X	X	X
8	29.0	300sh, 313	nd.	-	ni. (HA)	-	X	-
9	29.6-29.9	300sh, 313	nd.	-	ni. (HA)	X	X	X
10	30.7-30.9	300sh, 326	nd.	-	ni. (HA)	-	X	X
11	31.8-32.1	300sh, 330	515	179	3.4-dicaffeoylquinic acid	X	X	-
12	32.3-32.6	300sh, 331	515	179	3.5-dicaffeoylquinic acid	X	X	trace
13	34.4-34.5	un.	nd.	-	ni.	X	X	-
14	34.8-34.9	256, 360	nd.	-	ni. (flavonoid)	X	X	trace
15	35.4-35.5	300sh, 332	515	179	4.5-dicaffeoylquinic acid	X	X	-
16	37.9	256, 360	nd.	-	ni. (flavonoid)	X	X	trace

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457 **Table 2.** Characteristics of the raw pulp, press cake, and juice in terms of dry matter,
458 hydroxycinnamic acids and caffeine.

Characteristic	Raw pulp		Press cake		Juice	
	wet basis	*dry basis*	*wet basis*	*dry basis*	*wet basis*	*dry basis*
Dry matter [kg kg^{-1}]	0.16		0.24		0.07	
Free HA [mmol kg^{-1}]	0.25	1.51	0.30	1.24	0.12	1.68
Bound HA [mmol kg^{-1}]	0.30	1.80	0.50	1.95	0.00	0.00
5-CQA [mg kg-1]	47	288	34	141	20	271
Caffeine [g kg^{-1}]	0.9	5.6	0.6	2.6	0.6	8.6

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479 **Table 3:** Mass balance through the process for 100 kg raw pulp.

	Raw pulp	Fermented Pulp	
		Press cake	Juice
Total mass [kg]	100	36	64
Dry matter [kg]	16.3	8.7	4.7
Free HA [mmol]	24.7	10.7	8.0
Bound HA [mmol]	29.5	18.0	0.0
Total HA [mmol]	54.3	28.7	8.0
5-CQA [g]	4.7	1.2	1.3
Caffeine [g]	92.2	22.6	40.5

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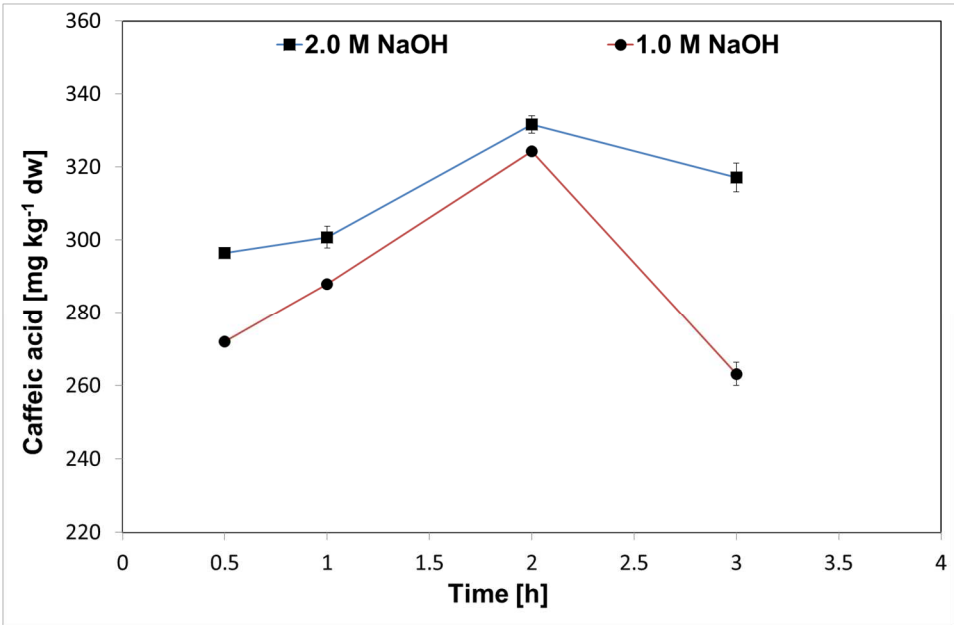
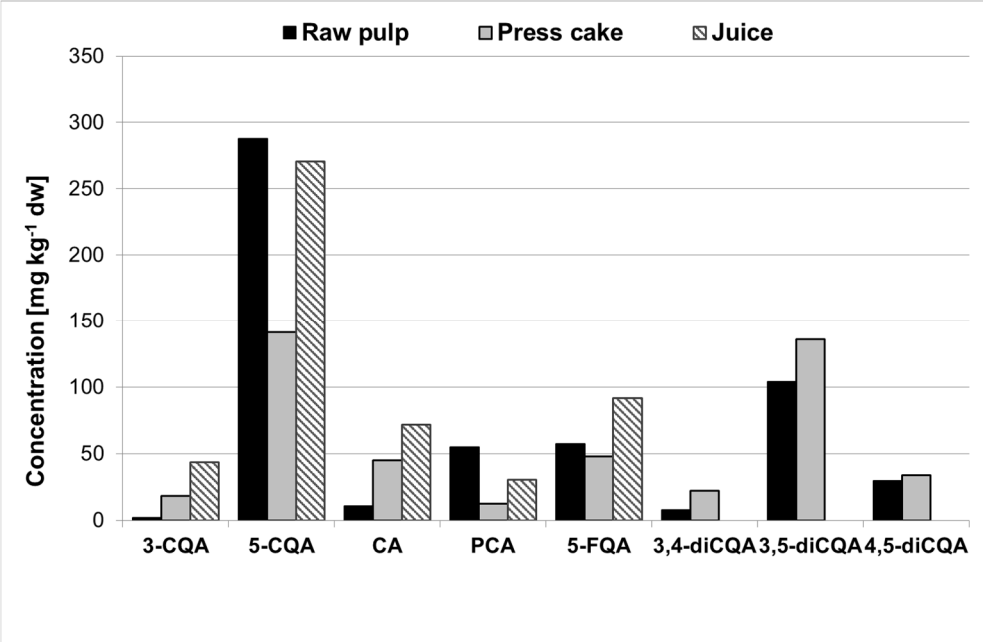
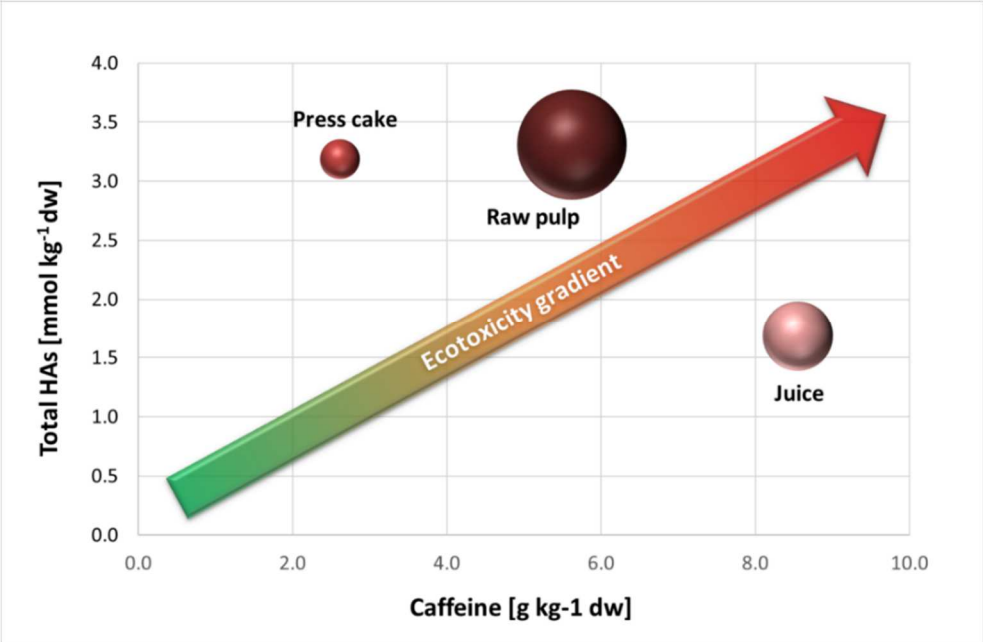
Figure 1. Time course of caffeic acid concentration during alkaline hydrolysis of raw coffee pulp with two different concentrations of NaOH (in the presence of 1% ascorbic acid and 10 mM EDTA).

Figure 2. Concentration of free HAs per compound in the raw pulp, press cake and juice (dw = dry weight).

Figure 3. Comparison between the raw coffee pulp, the press cake and the juice obtained after fermentation, in terms of ecotoxicity indicators (caffeine and hydroxycinnamic acid loads in dry matter) and of quantity of generated byproduct (ball size proportional to the mass of byproduct).


2.0 M NaOH
1.0 M NaOH
Caffeic acid [mg kg-1 dw]
360
340
320
300
280
260
240
220
0
0.5
1
1.5
2
2.5
3
3.5
4
Time [h]


Raw pulp
Press cake
Juice
Concentration [mg kg-1 dw]
350
300
250
200
150
100
50
0
3-CQA
5-CQA
CA
PCA
5-FQA
3,4-diCQA
3,5-diCQA
4,5-diCQA


Press cake
Raw pulp
Juice
Ecotoxicity gradient
Total HAs [mmol kg-1 dw]
Caffeine [g kg-1 dw]
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
2.0
4.0
6.0
8.0
10.0