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# 1 Solid-state fermentation as a sustainable method for coffee pulp treatment and 2 production of an extract rich in chlorogenic acids

3

4 Jessica Santos da Silveira <sup>a,b</sup>, Noël Durand <sup>a,b</sup>, Stella Lacour <sup>c</sup>, Marie-Pierre Belleville <sup>c</sup>, Ana

5 Perez <sup>d</sup>, Gérard Loiseau <sup>a</sup>, Manuel Dornier <sup>a,\*</sup>

6 <sup>a</sup> QualiSud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ Avignon, Univ Réunion, Montpellier,  
7 France

8 <sup>b</sup> CIRAD, UMR QualiSud, F-34398 Montpellier, France

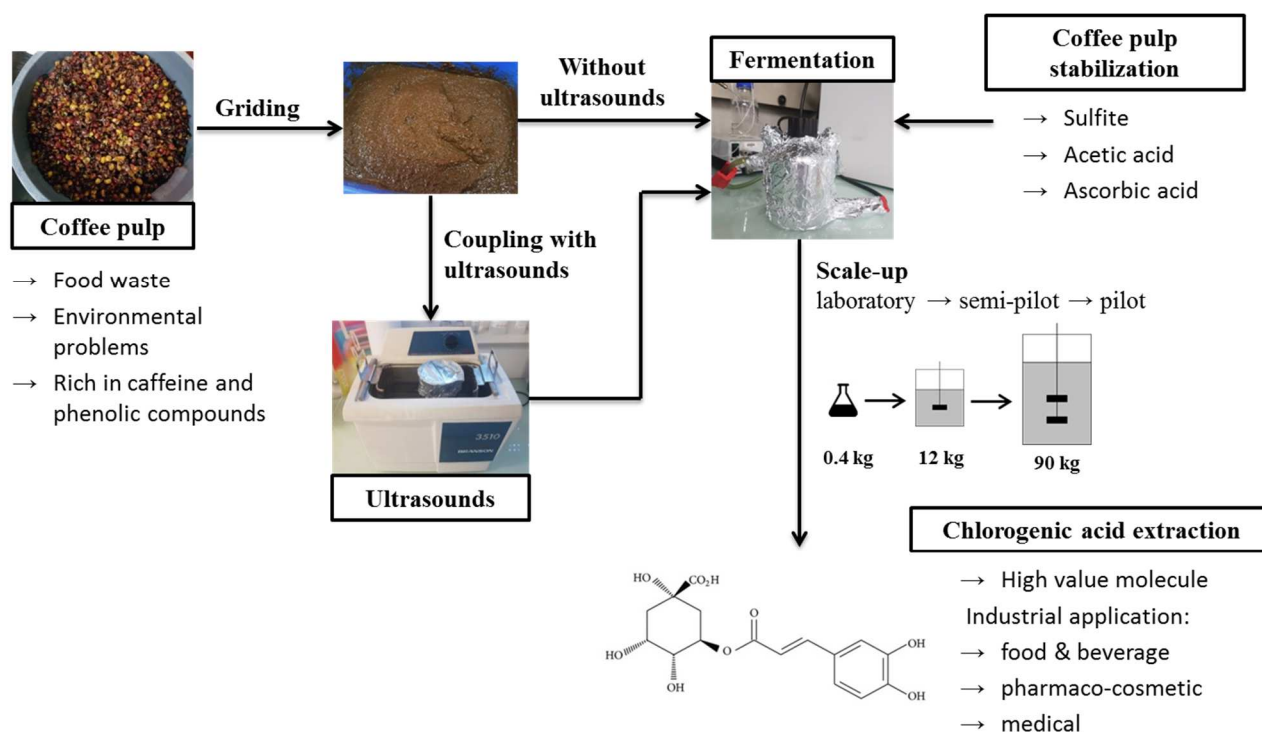
9 <sup>c</sup> Institut Européen des Membranes, IEM – UMR 5635, CNRS, ENSCM, Univ Montpellier, Montpellier,  
10 France

11 <sup>d</sup> Universidad de Costa Rica, CITA, 11501-2060 San José, Costa Rica

12 \* Corresponding author: manuel.dornier@supagro.fr

13

## 14 Graphical Abstract



15

16

17 **Abstract**

18 In this study, coffee pulp was used as carbon source in solid-state fermentation to produce  
19 a phenol-rich extract for industrial applications. Fermentations were carried out at  
20 laboratory (0.4 kg), semi-pilot (12 kg) and pilot (90 kg) scales in presence of 2.5 g kg<sup>-1</sup>  
21 yeast strains and with three different coffee pulp materials. The extract stability was  
22 investigated using different stabilizing agents: SO<sub>2</sub>, ascorbic and acetic acids. Then, a  
23 study was conducted to determine the effect of ultrasound treatment on extraction yields.  
24 Results showed that higher concentrations of chlorogenic acids were obtained with  
25 fermentation without ultrasound treatment and by adding sulfite at 0.5 wt% at 8 h of  
26 fermentation. Despite of variations in coffee pulp composition, the process was validated  
27 at semi-pilot and pilot scales, providing an extract 400% richer in chlorogenic acids (600  
28 mg per kg of coffee pulp) and with lower sugar amounts to separate during the  
29 downstream processing.

30 **Keywords:** coffee pulp, food waste treatment, alcoholic fermentation, phenolic  
31 compounds extraction, chlorogenic acids

32

33 **Highlights**

- 34 • *Saccharomyces cerevisiae* was used to ferment coffee pulp  
35 • Fermentation was investigated at laboratory scale and validated at pilot scale  
36 • Fermentation increased chlorogenic acid content up to 400% in coffee pulp extract  
37 • Sulfite at 0.5 wt% was selected to stabilize phenolics during the fermentation  
38 • Use of ultrasounds was unable to enhance chlorogenic acid extraction

39

40

## 41 **1. Introduction**

42 Worldwide, population growth has accelerated the agro-industrial residues  
43 generation causing major environmental, economic and societal problems (water/soil/air  
44 pollution, toxicity, waste treatment and disposal). The coffee production sector, one of  
45 the most important commodity in the world, generates huge amounts of residues, such as  
46 coffee pulp, husk, skin, among others (Esquivel and Jiménez, 2012), which contribute  
47 about 50% of the total coffee fruit production. According to data from the International  
48 Coffee Organization (“ICO” 2017), global green coffee production in 2017 was estimated  
49 to be around 9.5 million t with over 90% of coffee production taking place in developing  
50 countries (Central and South America, Central and East Africa, Asia), whereas  
51 consumption is mainly in the industrialized economies. Coffee pulp is the main residue  
52 (40% mass of coffee fruit) obtained from the wet processing of coffee cherries and is  
53 considered as both ecotoxic and antinutritional to animals due to its polyphenols, tannins,  
54 and caffeine content (Murthy and Madhava Naidu, 2012). Nevertheless, coffee pulp is  
55 rich in sugars, proteins, minerals, amino-acids and also contains functional molecules of  
56 high industrial interest (phenolic compounds with antioxidative, anti-inflammatory,  
57 antimutagenic, antibacterial, and anticancer properties; caffeine with well-known  
58 psychotropic and diuretic properties) (Shahidi and Chandrasekara, 2010; Arellano-  
59 González et al. 2011).

60 Studies have shown that the data on coffee pulp composition can vary due to the  
61 characteristics of coffee fruits (cultivar, place of production, culture conditions, maturity,  
62 etc.), the postharvest management (methods of depulping, drying, storage, etc.), or even  
63 the analytical methods used (Duangjai et al., 2016; Rios et al., 2014; Rodríguez-Durán et  
64 al., 2014). Four major classes of phenolic compounds were identified in recent studies:

65 flavonols, flavan-3-ols, anthocyanidins, hydroxycinnamic acids and chlorogenic acids as  
66 the predominant phenolic compound (Heeger et al., 2016). These molecules find evident  
67 applications in pharmaco-cosmetic, food and medical industries (Quideau et al., 2011).  
68 Moreover, hydroxycinnamic acids (HA) including chlorogenic acids (CGA) can be  
69 converted into products of even higher added value (vanillin hemisynthesis as an  
70 example) (Di Gioia et al., 2011). However, most of these molecules are found in coffee  
71 pulp conjugated to sugars as glycosides. Compounds such as chlorogenic acids are found  
72 in an ester form bonded to cell wall forming highly complex polysaccharide structures  
73 (Asther et al., 2002).

74 Biotechnological processes such as solid-state fermentation can be used to  
75 enhance the phenolic content in plant extracts through the breakage of ester bonds  
76 between phenolics and the plant cell wall, increasing their concentration and consequently  
77 functional properties (Palmieri et al., 2018; Arellano-González et al. 2011). Solid-state  
78 fermentation is defined as any fermentation process performed on moist solid materials  
79 in the absence of free-flowing water that acts both as physical support and source of  
80 nutrients for microorganisms. Due to this low water availability, a limited number of  
81 microorganisms, mainly yeasts and fungi, can be used for solid-state fermentation  
82 (Thomas et al., 2013).

83 *Saccharomyces cerevisiae* is an important microorganism used worldwide for  
84 producing food and beverages. This yeast has several advantages for ethanol production  
85 from lignocellulosic biomass: efficient ethanol production from simple sugars, it does not  
86 require oxygenation, has a relatively high tolerance to ethanol and inhibitors, has low pH  
87 optimum and is generally recognized as safe (GRAS) as a food additive for human  
88 consumption. The production of ethanol during alcoholic fermentation also presents an

89 advantage for the recovery of phenolic compounds from agri-food solid wastes, as  
90 mixtures of water/ethanol have been shown to enhance the solubilization of phenols  
91 (Benmeziane et al. 2014).

92         Solid-state fermentation offers numerous advantages over other techniques such  
93 as lower energy requirements and the absence of sophisticated and complex machinery  
94 and control systems, providing a low-cost process in a sustainable framework. The main  
95 drawbacks of this method concern the process scale-up (heat transfer and culture  
96 homogeneity) and production yields. Several researchers have studied the application of  
97 ultrasound-assisted extraction to increase the conversion of starch materials to glucose as  
98 well as overall ethanol yield during fermentation processes by destroying plant cell wall  
99 and making it easier to microorganisms to access sugars from plant matrix (Chemat et al.,  
100 2017; Nikolić et al., 2010). Ultrasonication has been applied widely in various biological  
101 and chemical processes. However, the use of ultrasound-assisted extraction coupled to  
102 solid-state fermentation to treat food waste has not been widely investigated. The use of  
103 ultrasonics has the potential to break the pulp cell wall and to release sugars due to  
104 acoustic cavitation, enhancing ethanol production and phenolic compounds recovery.

105         A major concern expressed with regards to the extraction of polyphenols has been  
106 in relation to their stability. Phenolic compounds may undergo degradation due to  
107 temperature, light, oxygen, enzymes, and pH. Light and oxygen in the air are the two  
108 most important factors that facilitate degradation reactions. Enzymes (mainly oxidative  
109 enzymes) already present in the plant material can be released during the extraction  
110 process and promote such degradation reactions (Mäkilä et al., 2016). Therefore, to build  
111 an efficient extraction method, it is crucial to keep the stability of phenolic compounds.  
112 One way to avoid phenol degradation during extraction is by adding a stabilizing agent

113 to the solution. In addition to presenting antifungal and antibacterial properties, sulfur  
114 dioxide (SO<sub>2</sub>) is commonly used as a reducing agent and as an inhibitor of endogenous  
115 oxidases in winemaking (Blouin, 2014; Ribereau-Gayon et al., 2006). Especially under  
116 its bisulfite form (HSO<sub>3</sub><sup>-</sup>), it can efficiently protect phenolics from chemical and  
117 enzymatic oxidations. Another well-known food preservative is ascorbic acid, a water-  
118 soluble antioxidant naturally found in many fruits and vegetables. In industry, ascorbic  
119 acid can be added to plant-based products to preserve its antioxidant capacity and provide  
120 chemical stability. Furthermore, in the presence of oxygen, ascorbic acid tends to oxidize,  
121 removing the environmental resources of oxygen (Varvara et al., 2016).

122         The acidification of the solution presents an alternative to prevent phenol  
123 degradation by adjusting the pH with acidity regulators. The pH optimum of enzymes,  
124 such as polyphenol oxidase (PPO), ranges around pH 6-7 and becomes inactive below pH  
125 4. Hence, the role of acidity regulators is to maintain the pH well below that necessary  
126 for optimal catalytic activity. Moreover, it is well-known that hydroxycinnamic acids are  
127 stable at lower pH (3-5), unlike basic conditions where isomerization and oxidation  
128 reactions can occur (pH≥7.0) (Friedman and Jürgens, 2000; Ma et al., 2011; Narita and  
129 Inouye, 2013). A typical acidity regulator is acetic acid, an organic acid that has  
130 traditionally been used to improve the shelf-life and microbiological safety of food  
131 products. Glacial acetic acid can potentially be used during fermentation processes to  
132 enhance phenolic compounds extraction and stability by acidification of the medium, but  
133 can cause yeast death at certain concentrations (Leão et al., 2001).

134         Based on these premises, the aim of this study was to investigate the extraction  
135 and stabilization of chlorogenic acids from coffee pulp during solid-state fermentation  
136 using commercial yeast strains. First, the process optimization was investigated at

137 laboratory scale under well-controlled conditions. Then, the fermentation process was  
138 applied to larger scales in real condition of production in order to evaluate its interest for  
139 industrial application. The outcomes of this study are expected to contribute to the  
140 development of a sustainable and competitive method for coffee pulp treatment,  
141 applicable to all regions where coffee is produced by the wet processing method (around  
142 50% of the worldwide production).

143

## 144 **2. Materials and Methods**

### 145 **2.1. Raw material**

146 Coffee pulp from the wet depulping and demucilaging process of coffee beans  
147 (*Coffea arabica*) was supplied by the Beneficio Coopeunión, a coffee-producer  
148 cooperative located in Trés Rios (Costa Rica), during the 2015 and 2017 harvests for  
149 assays at laboratory and semi-pilot scale, respectively. As soon as obtained, raw coffee  
150 pulp was split into lots of 3 kg, frozen at -20°C, and shipped to France by aircraft in a  
151 controlled temperature system. Upon arrival, the bags containing frozen coffee pulp were  
152 stored at -20°C. Prior to experiments, batches of 1 kg of coffee pulp were thawed at room  
153 temperature and ground using a mixer Thermomix TM31 (Vorwerk, Wuppertal,  
154 Germany) for 1 min at maximum power (level 10) and without heating. The coffee pulp  
155 was then split into hermetically sealed flasks and stored at -20°C.

156 For the fermentation at pilot scale, coffee pulp was supplied by the Beneficio San  
157 Diego, a coffee-producer cooperative located in San José (Costa Rica), during the 2018  
158 harvest. The night before the experiment, coffee pulp was collected and stored at room  
159 temperature. Prior to the experiment, batches of 10 kg of coffee pulp were ground using  
160 a Bowl Cutter SM 45 (K+G Wetter, Biedenkopf, Germany) for 7 min at maximum power

161 (level 2) and without heating. All batches of coffee pulp were gathered in a sterilized  
162 container and placed in a room at 28°C.

163

## 164 **2.2. Chemicals**

165 Methanol, sulfuric acid, and phosphoric acid were all of analytical grade from  
166 Honeywell (Seelze, Germany). Standard of chlorogenic acids containing a mix of 3-  
167 Caffeoylquinic acid (3-CQA), 5-Caffeoylquinic acid (5-CQA), 4-Caffeoylquinic acid (4-  
168 CQA), 4-Feruloylquinic acid (4-FQA), 5-Feruloylquinic acid (5-FQA), 3,4-  
169 Dicafeoylquinic acid (3,4-diCQA), 3,5-Dicafeoylquinic acid (3,5-diCQA), and 4,5-  
170 Dicafeoylquinic acid (4,5-diCQA), was purchased from International Development and  
171 Manufacturing (New Jersey, USA). Standard of glucose, fructose and ethanol were  
172 purchased from Sigma Aldrich (Steinheim, Germany). L(+)-ascorbic acid, glacial acetic  
173 acid, and sodium metabisulfite, used to stabilize the chlorogenic acids during the assays,  
174 were all of analytical grade purchased from Sigma Aldrich (Steinheim, Germany).

175

## 176 **2.3. Solid-state fermentation**

177 Two *Saccharomyces cerevisiae* strains used for solid-state fermentation, with a  
178 population of  $10^7$  CFU g<sup>-1</sup>, were kindly provided by Lallemand (Toulouse, France). The  
179 yeast strains were dried and stored at 4°C.

180 Fermentations at laboratory scale were carried out using a double-walled glass  
181 reactor (Legallais, Montferrier-sur-Lez, France) with a **working** volume of 400 mL. The  
182 day prior to experiments, samples of 400 g of coffee pulp were thawed at room  
183 temperature under darkness. The yeast strain was re-activated and multiplied using  
184 deionized water at 35°C for 30 min, at a ratio of 10 mL of water per g dried yeast. During

185 this time, the coffee pulp was maintained at 28°C in the fermentation vessels. The  
186 temperature of the outer jacket was controlled with a thermostatic water bath. Coffee pulp  
187 was inoculated at a ratio of 2.5 g yeast/kg coffee pulp, and solid-state fermentation was  
188 carried out at a fixed temperature of 28°C, in darkness and without stirring to maintain  
189 anaerobic conditions during the fermentation. Prior to sampling, coffee pulp was gently  
190 mixed using a sterilized laboratory tool to ensure homogeneity of the mixture. Samples  
191 were clarified by centrifugation at 8603 x g for 15 min.

192         Semi-pilot and pilot scale fermentations were carried out respectively in France  
193 in a 20 L stainless steel tank (Artame, Baguim do Monte, Portugal) containing 12 kg of  
194 coffee pulp and in Costa Rica in a 200 L sterilized polypropylene drum (Lacoplast S.A.,  
195 Guatemala) containing 90 kg of coffee pulp. The tanks were placed in climate rooms set  
196 and preheated at 28°C for temperature control. Fermentations were carried out at 28°C,  
197 without stirring and under darkness. Coffee pulp was inoculated, sampled and clarified  
198 as described for laboratory scale. The coffee pulp extract was separated from the mash by  
199 pressing using a hydraulic press (Stossier LI P MO, Simaco, Bouzonville, France or TC  
200 Y125, Owatonna Tool Company, Minnesota, USA) at 50-60 bar for 30 min.

201

#### 202 **2.4. Stabilization procedures for chlorogenic acid stability**

203         In order to prevent chlorogenic acid (CGA) degradation during the fermentation  
204 process, the performance of different stabilizing agents was studied at laboratory scale.  
205 Sulfur dioxide was used as sodium metabisulfite at 3 concentrations: 30 mg kg<sup>-1</sup> according  
206 to data from the yeast supplier as the limit sulfite concentration supported by their  
207 *Saccharomyces cerevisiae* strains, 500 mg kg<sup>-1</sup> a concentration used for sulfitic  
208 maceration of musts in winemaking that favors polyphenol extraction rate (Blouin, 2014),

209 and 0.5 wt% a very high concentration which guarantees total protection against  
210 oxidation. Glacial acetic acid was chosen as an acidity regulator at two concentrations:  
211 1 wt% to stabilize CGA and allow yeast growth, and at 10 wt% to stabilize CGA and  
212 inhibit yeast activity. Finally, ascorbic acid at 1 wt% was used as an antioxidant (Narita  
213 and Inouye, 2013). The stabilizing agent was directly mixed to the coffee pulp before  
214 inoculation or at a certain time during the fermentation to study its effect on the  
215 stabilization of CGA and on yeast activity.

216

## 217 **2.5. Ultrasound-assisted extraction**

218 For particular experiments, the fermentation at laboratory scale was coupled to  
219 ultrasound-assisted extraction in order to evaluate the possible enhancement of  
220 chlorogenic acid extraction. The ultrasound treatment was carried out using a Branson  
221 model 3510E-MT 100 W ultrasound equipment (Danbury, USA) using 42 kHz as  
222 frequency. Ultrasounds were applied during 10 min (1) prior to inoculation or (2) at a  
223 selected time during fermentation where the maximum amount of CGA was extracted.  
224 Total power used was 250 W/kg of coffee pulp.

225

## 226 **2.6. Analytical methods**

227 The moisture of the raw coffee pulp was determined by gravimetric method in  
228 triplicate at 100°C after 48 h. The pH and temperature were obtained using an ALMEMO  
229 measuring instrument (Ahlborn, Ilmenau, Germany). The concentration of sugars and  
230 ethanol was determined by High-Performance Liquid Chromatography (HPLC) using a  
231 Dionex Ultimate 3000 chromatograph (Thermo Scientific) that was equipped with a  
232 refractive index detector (RID-10A Shimadzu), a UV detector (210 nm), and an Aminex

233 HPX87H column that was operated at a temperature of 35°C using 5 mM sulfuric acid as  
234 the eluent at a flow rate of de 0.6 mL min<sup>-1</sup>.

235 The concentration of chlorogenic acid isomers was determined by HPLC on a  
236 system consisting of Shimadzu (Kyoto, Japan) Model LC 20AD pumping units, an  
237 automated sample injector (Shimadzu SIL 20 AXR), a variable-wavelength UV detector  
238 (Shimadzu SPD20A), column Uptisphere type ODB 5 (5 µm particle size, 250 x 4.6 mm),  
239 with identical pre-column, thermostatically controlled at 30°C. The elution program used  
240 two solvents, A and B. Solvent A was 4 mM phosphoric acid and solvent B was methanol.  
241 The following elution program was used: A—B mixture 95/5 v/v from min 0 to 35, to  
242 A—B mixture 25/75 v/v from min 35 to 40, then pure solvent B from min 40 to 50, to  
243 A—B mixture 95/5 v/v from min 50 to 55. Flow rate was 1 mL min<sup>-1</sup>. UV detection was  
244 at 327 nm, which corresponds to maximum CGA absorption.

245 Samples were filtered through 0.45 µm pore size filter before HPLC injection.  
246 The compounds (sugars, ethanol, CGA) were identified by comparison of their retention  
247 times with the retention times of certified standards. The quantification of compounds  
248 was performed using calibration curves with 5 different concentrations of standard  
249 solutions ( $R^2 > 0.99$ ).

250

### 251 **3. Results and Discussion**

#### 252 **3.1. Coffee pulp**

253 This study explored the fermentation of coffee pulp at three different scales:  
254 laboratory, semi-pilot, and pilot. Different batches of coffee pulp with different  
255 pretreatment conditions were used during the experiments, which resulted in a variability  
256 of the raw material composition. A chemical characterization of coffee pulp was carried

257 out to determine its initial composition in terms of fermentable sugars, ethanol and  
 258 chlorogenic acid 5-CQA (Table 1).

259 **Table 1**

260 Characterization of raw coffee pulp from different batches in terms of origin, pretreatment steps,  
 261 local and year of assays, pH, moisture, sugar, ethanol and 5-CQA content.

262

Laboratory scale					
Harvest year	2015	Supplier	Coopéunion	Pretreatment steps	Freezing, grinding and freezing
Year of assay(s)	2016-2017	Local of assay(s)	France		
		Moisture [%]	85 ± 0.6		
		pH	4.4		
	Glucose [g L <sup>-1</sup> ]	Fructose [g L <sup>-1</sup> ]	Ethanol [g L <sup>-1</sup> ]	5-CQA [mg L <sup>-1</sup> ]	
Upper value	35.16	48.35	0.85	12.7	
Lower value	28.93	37.13	0.36	4.3	
Average( $\sigma$ ) <sub>n</sub>	31.73(1.84) <sub>8</sub>	42.97(4.17) <sub>8</sub>	0.65(0.15) <sub>8</sub>	7.93(3.22) <sub>8</sub>	
Semi-pilot scale					
Harvest year	2017	Supplier	Coopéunion	Pretreatment steps	Freezing, grinding and freezing
Year of assay(s)	2018	Local of assay(s)	France		
		Moisture [%]	85 ± 0.8		
		pH	4.3		
	Glucose [g L <sup>-1</sup> ]	Fructose [g L <sup>-1</sup> ]	Ethanol [g L <sup>-1</sup> ]	5-CQA [mg L <sup>-1</sup> ]	
	14.39	21.42	0.6	54.91	
Pilot scale					
Harvest year	2018	Supplier	Beneficio San Diego	Pretreatment steps	Grinding
Year of assay(s)	2018	Local of assay(s)	Costa Rica		
		Moisture [%]	85 ± 0.8		
		pH	4.3		
	Glucose [g L <sup>-1</sup> ]	Fructose [g L <sup>-1</sup> ]	Ethanol [g L <sup>-1</sup> ]	5-CQA [mg L <sup>-1</sup> ]	
	19.47	28.44	0.65	198.66	

263

264 s

265

266           The chlorogenic acid 5-CQA was reported as being the most abundant simple  
267 polyphenol present in fresh coffee pulp (Martínez and Clifford, 2000) and its  
268 concentration was followed in all assays of this work. For laboratory scale experiments,  
269 eight samples of coffee pulp from the 2015 batch were used. Fermentation at semi-pilot  
270 and pilot scales was carried out only once due to limitations in coffee pulp supply.  
271 According to results, we observed a difference in coffee pulp composition between  
272 samples of the same batch (2015) as well as significant differences between the three  
273 batches. Glucose and fructose contents varied by less than 10 % within the laboratory  
274 scale samples whereas changed by 41% (glucose) and 36% (fructose) between batches at  
275 different scales. Traces of ethanol were found in coffee pulp in concentrations inferior to  
276 1 g L<sup>-1</sup>, probably due to a natural (but very limited) fermentation of coffee pulp during  
277 storage. The biggest difference was found in terms of the amount of 5-CQA. We observed  
278 that the highest concentration of 5-CQA, 198 mg L<sup>-1</sup>, was found in the 2018 batch that  
279 was only stored for one night prior to the experiment. Much lower concentrations were  
280 found in the coffee pulp batches of 2015 that were milled, frozen and stored for several  
281 months. An intermediate value was obtained for the batch of 2017 that was stored for a  
282 shorter period than the 2015 batch. This can be explained by the high sensitivity of  
283 phenolic compounds, such as chlorogenic acid 5-CQA, to isomerization and/or oxidation  
284 by external factors (temperature, light, oxygen) (Narita and Inouye, 2013; Xie et al., 2011)  
285 that can occur during the pretreatment and storage steps, leading to CGA degradation.

286           This finding illustrates the importance of studying the initial coffee pulp  
287 composition. Even though the same biomass is used as a substrate for fermentation,  
288 quality of the raw material may lead to different ethanol production and chlorogenic acid

289 extraction during fermentation, and this can be a challenge for industrial scale  
290 productions.

291

### 292 **3.2. Study of the fermentation process at laboratory-scale**

#### 293 **3.2.1. Selection of yeast strains and kinetics of CGA extraction**

294 A preliminary study was carried out to select a *Saccharomyces cerevisiae* strain  
295 for coffee pulp fermentation with the view to enhance chlorogenic acid extraction. Two  
296 strains were tested, Yeast A and Yeast B, which differed in terms of sulfite production  
297 during fermentation (Yeast A presenting lower SO<sub>2</sub> production). Table 2 presents the  
298 relative concentration (ratio between the final and initial concentrations of a compound)  
299 for the glucose, fructose, ethanol and 5-CQA after 24 h of fermentation.

300 **Table 2**

301 Relative concentration C/C<sub>0</sub> (ratio between the final and initial concentrations) of glucose,  
302 fructose and ethanol after 24 h of fermentation and final concentration of 5-CQA obtained at  
303 laboratory scale for both yeast strains tested (Yeast A and Yeast B) and for a control of coffee  
304 pulp non-inoculated.

305

	Relative concentration (C/C <sub>0</sub> ) after 24 h			Final concentration of 5-CQA after 24 h [mg L <sup>-1</sup> ]
	Glucose	Fructose	Ethanol	
<b>Control</b>	1.00	1.00	0.98	8
<b>Yeast A</b>	0.01	0.11	42.80	30
<b>Yeast B</b>	0.01	0.11	42.60	4

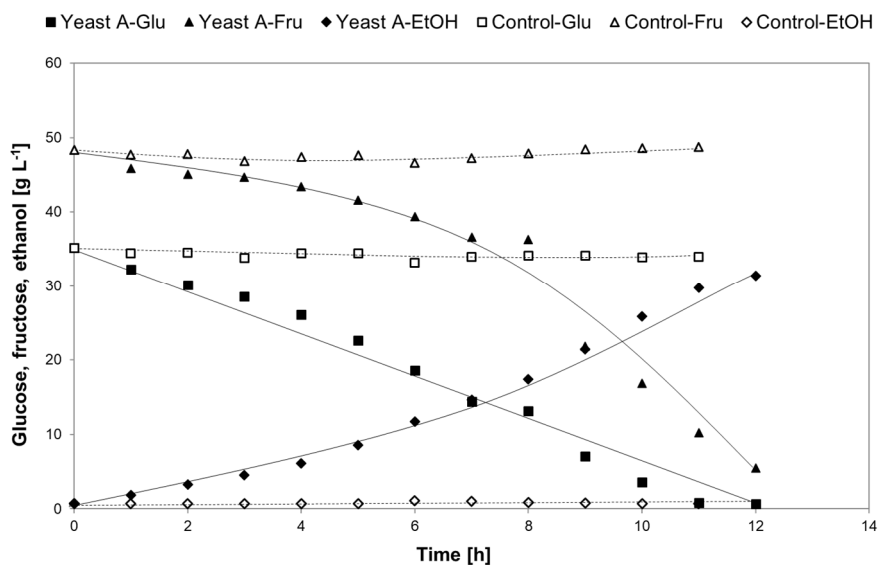
306

307 Results from the control (non-inoculated coffee pulp) show that no alcoholic  
308 fermentation occurred within the 24 h period and that the final coffee pulp extract  
309 contained 8.0 mg L<sup>-1</sup> of 5-CQA. Stability of the raw coffee pulp even with its endogenous  
310 flora is probably correlated with its high phenolic content that inhibits microorganism  
311 growth. Both yeasts were able to ferment coffee pulp and lead to very similar

312 performances in terms of sugar consumption and ethanol production. Nevertheless, only  
313 one strain was capable of increasing the concentration of 5-CQA in the extract from  
314 coffee pulp. While the fermentation using Yeast A increased by almost 4 times the  
315 concentration of 5-CQA compared to the control, results with Yeast B showed the  
316 degradation of this molecule during the fermentation process. This result is probably  
317 correlated to a difference in the production of pectinases,  $\beta$ -glucanases and other  
318 hydrolytic enzymes that are able to facilitate the release of the 5-CQA out of plant cells  
319 (Alimardani-Theuil et al., 2011; Pinelo et al., 2006). This shows that only specific strains  
320 can be used for the extraction of chlorogenic acids from coffee pulp. The Yeast A was  
321 then selected for all the experiments presented in this work.

322         To better understand the fermentation and extraction processes, a kinetic study  
323 was carried out with coffee pulp inoculated with Yeast A and the results were compared  
324 to a control experiment (non-inoculated coffee pulp). Figure 1 presents the evolution of  
325 glucose, fructose and ethanol concentrations during the solid-state fermentation for both  
326 samples.

327



328

329 **Fig. 1.** Substrate consumption (glucose, fructose) and ethanol production during solid-state  
 330 fermentation of coffee pulp carried out at lab scale with Yeast A, compared to a control non-  
 331 inoculated.

332

333 Results showed that glucose was the preferred carbon source, followed by fructose  
 334 as usual. Both sugars were almost completely consumed after 12 h of fermentation. The  
 335 production of ethanol was correlated to the consumption of sugars, increasing during  
 336 fermentation and levelling off to a final concentration of 31.4 g L<sup>-1</sup> (around 4% vol.) at t  
 337 = 12 h, when sugar stocks reached a very low level (glucose < 1 g L<sup>-1</sup>; fructose < 6 g L<sup>-1</sup>).  
 338 No fermentation occurred in the control.

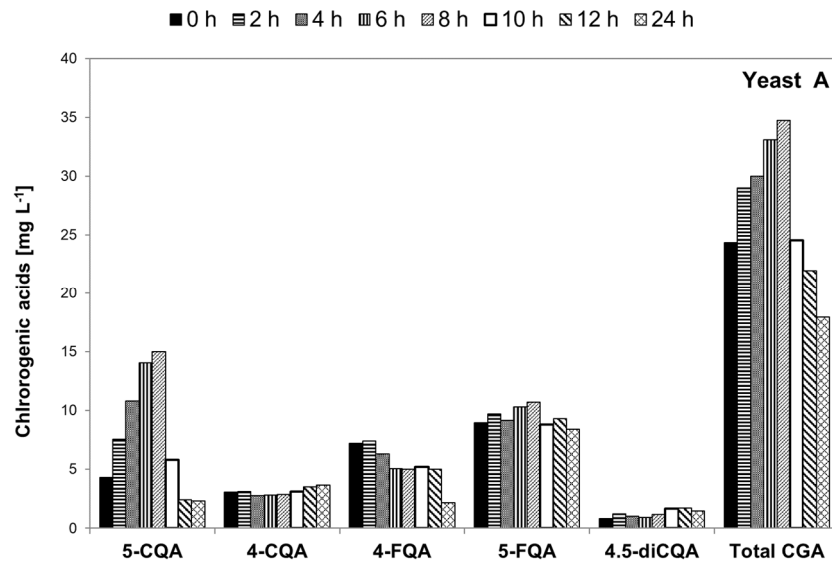
339

Figure 2 presents the evolution of chlorogenic acids (CGA) extraction in the  
 340 inoculated coffee pulp compared to the control. The CGA identified were: 5-CQA, 4-  
 341 CQA, 4-FQA, 5-FQA, and 4.5-diCQA. The evolution of the total amount of these five  
 342 chlorogenic acids is also presented (total CGA).

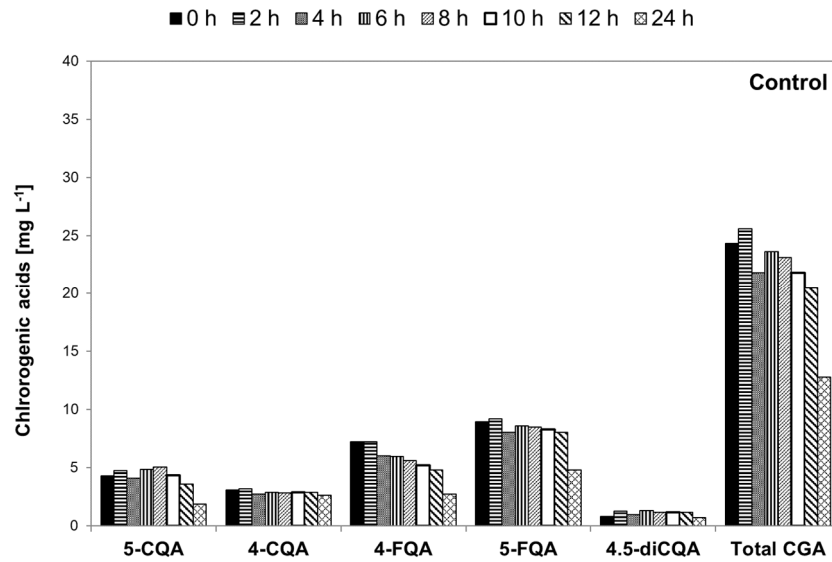
343

Results from the inoculated pulp (Yeast A) show that 5-CQA was the only isomer  
 344 that was significantly extracted during the fermentation process. Its concentration  
 345 increased over time reaching a maximum at t = 8 h and decreasing afterward, probably

346 due to a higher degradation rate of this compound compared to the extraction. The  
347 concentration of 4-CQA, 5-FQA, and 4.5-diCQA varied very little over time, while the  
348 concentration of 4-FQA slowly decreased. The control showed similar behavior for the  
349 4-CQA, 5-FQA, and 4.5-diCQA, with their concentrations staying relatively stable over  
350 time. On the contrary the concentrations of 5-CQA and 4-FQA decreased over time.  
351 During fermentation, different behaviors were observed. Surprisingly for 5-CQA the  
352 concentration increased over time reaching a maximum at 8 h and then decreased  
353 drastically. It was already reported that the hydroxycinnamic acids are mostly present in  
354 the coffee pulp under linked-form with cell walls whereas the soluble compounds could  
355 be mainly located inside the plant vacuoles (Rodríguez-Durán et al., 2014). The  
356 enzymatic pool of the yeast which is responsible for the cell wall weakening could favor  
357 the wall-bonded 5-CQA release as well as the increasing ethanol concentration could  
358 favor the solubility of this compound and its extraction. Losses of 5-CQA become  
359 predominant after 8 h of fermentation through oxidation reactions by chemical, enzymatic  
360 and even fermentation pathways or through adsorption by the yeasts themselves (Mazauric  
361 and Salmon, 2005). Furthermore, with lower sugar stocks in the media, chlorogenic acids  
362 could become the carbon source in its turn. Figure 3 summarizes the possible pathways  
363 of extraction and degradation of CGA during coffee pulp fermentation.



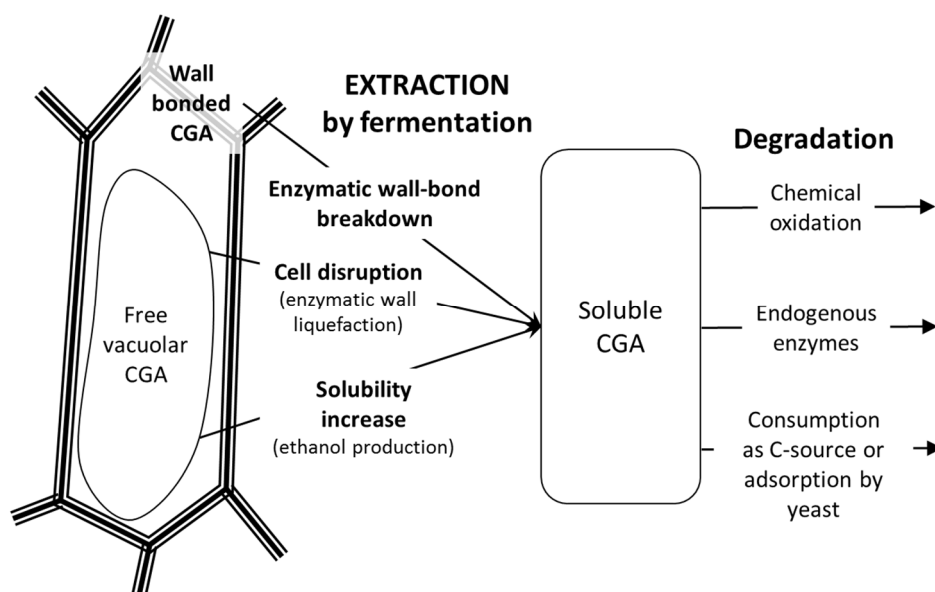
364



365

366 **Fig. 2.** Extraction of chlorogenic acids from coffee pulp during fermentation at lab scale with  
 367 yeast A compared to control non-inoculated.

368



369

370 **Fig. 3.** Suggested competing pathways of the extraction and degradation of chlorogenic acids  
 371 (CGA) during fermentation of coffee pulp.

372

373 Finally, chlorogenic acids may also undergo various isomerization reactions  
 374 (Liang and Kitts, 2015; Moon et al., 2009) and could explain the surprising stability  
 375 observed in Figure 2 for 4-CQA, 5-FQA and 4.5-diCQA, compared to 5-CQA and 4-  
 376 CQA, during fermentation (as an example, 5-CQA could be partially converted into 4-  
 377 CQA and 5-FQA into 4-FQA).

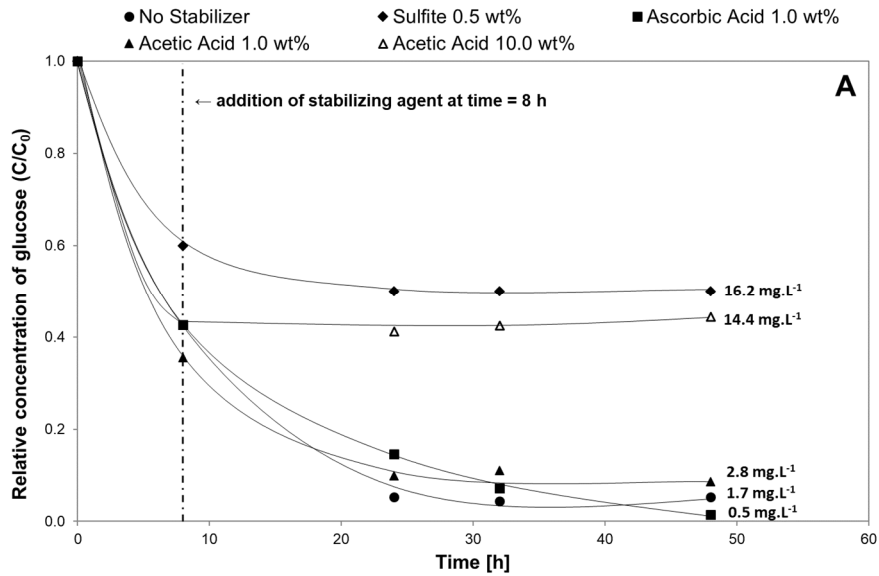
378 To conclude this part of the study, these results showed that it was possible to  
 379 significantly extract the 5-CQA using solid-state fermentation. In fact, alcoholic  
 380 fermentation with Yeast A allowed an increase of 3.5 times of 5-CQA initial  
 381 concentration, from 4.3 mg L<sup>-1</sup> to 15 mg L<sup>-1</sup>, at only 8 h of fermentation. However, in  
 382 order to preserve the 5-CQA extracted during the fermentation process, it is important to  
 383 develop a stabilization method for chlorogenic acids.

384

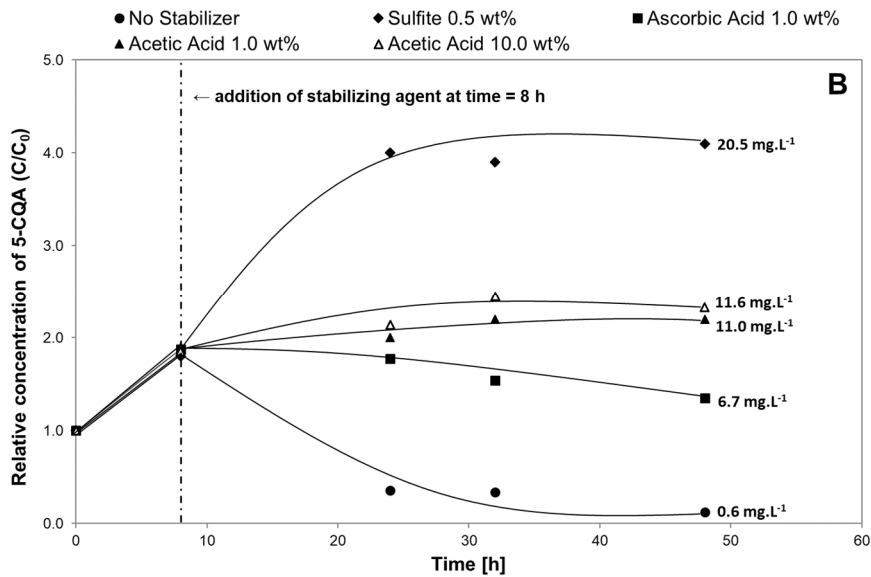
### 385 3.2.2. Stabilization of chlorogenic acids

386 A study was carried out to compare different strategies for the stabilization of  
387 chlorogenic acids in the coffee pulp. Initially, two concentrations of sulfite were chosen  
388 to stabilize coffee pulp prior to inoculation: 30 and 500 mg kg<sup>-1</sup>. It was found that when  
389 adding this agent at the beginning of the fermentation, there were either complete yeast  
390 inactivation resulting in no chlorogenic acid extraction (500 mg kg<sup>-1</sup> of SO<sub>2</sub>) or coffee  
391 pulp fermentation but no chlorogenic acid stabilization (30 mg kg<sup>-1</sup> of SO<sub>2</sub>). So, under  
392 those tested conditions, the stabilization step applied to the coffee pulp before the  
393 fermentation process was not suitable. A complementary study was then performed  
394 comparing 3 different stabilizing agents (sulfite 0.5 wt%, ascorbic acid 1 wt%, and acetic  
395 acid 1 wt% and 10 wt%) added into the culture medium at the optimal time of 5-CQA  
396 extraction, i.e. at 8 h of fermentation (as previously shown in Figure 2). This new strategy  
397 aimed to stabilize the 5-CQA at its highest concentration in the extract and to study the  
398 effect of the addition of stabilizing agent on the yeast activity.

399 Figure 4 presents the evolution of glucose and 5-CQA concentrations for the  
400 different assays. Since 5 coffee pulp samples from batch 2015 were used during the  
401 experiments, the concentrations were expressed in terms of the relative concentration of  
402 each compound ( $C/C_0$ ) due to raw material variability. Indeed, it is worth noting that,  
403 irrespective to the fermentation trials, fructose consumption and ethanol production  
404 showed the same evolution as previously described that confirmed the good  
405 reproducibility of the fermentation of the pulp by yeast. For this reason, the evolution of  
406 glucose concentration was used as the fermentation indicator in all the following results.



407



408

409 **Fig. 4.** Effect of different stabilizing agents on the (A) glucose consumption and (B) 5-CQA  
 410 extraction during solid-state fermentation at lab scale with yeast A (glucose:  $C_0 = 32.5 \pm 1.5 \text{ g L}^{-1}$ ;  
 411 5-CQA:  $C_0 = 5 \pm 2 \text{ mg L}^{-1}$ ).

412

413 From Fig.4, the addition of ascorbic acid at 1 wt% or acetic acid at 1 wt% didn't  
 414 affect yeast activity; the concentration of glucose continued to decrease over time as it  
 415 was observed in the experiment done without any stabilizing agent. As expected, the  
 416 addition of sulfite at 0.5 wt% or acetic acid at 10 wt% stopped the fermentation process,  
 417 which indicates that, at the given concentration, sulfite and acetic acid inhibited yeast

418 activity. Both of these stabilizing agents are known for acting through a strong  
419 acidification of intracellular content and, also by an intrinsic toxic activity like in the case  
420 of  $\text{HSO}_3^-$ , by blocking many carbonyl functions that disrupt the cell metabolism (Blouin,  
421 2014). Concerning the extraction of 5-CQA, the addition of ascorbic acid tended to  
422 stabilize the 5-CQA until 24 h of fermentation. Afterward, due to a gradually oxidation  
423 of ascorbic acid, the 5-CQA started to degrade faster. The acetic acid, in both  
424 concentrations tested, was successful to stabilize the 5-CQA. Surprisingly, the addition  
425 of sulfite not only stabilized the 5-CQA, but also increased its concentration over time,  
426 reaching a maximum at 24 h of fermentation and representing an enhancement of 400%  
427 of 5-CQA concentration compared to its initial concentration. This can be explained first  
428 by the fact that 5-CQA can undergo reversible electrochemical reactions (Tomac and  
429 Šeruga, 2016), that is to say, oxidized forms of 5-CQA can be reversibly reduced by the  
430 redox action of sulfite, unlike the other stabilizing agents tested in the study. Second, this  
431 result could also be due to a faster destruction of the plant cells resulting in higher  
432 amounts of 5-CQA being released. This well-known phenomenon in winemaking is often  
433 called the “dissolvent activity” of sulfites (Ribereau-Gayon et al., 2006).

434         Combining the results in terms of glucose consumption and 5-CQA extraction,  
435 two stabilizing agents could be used for the fermentation of coffee pulp: acetic acid at  
436 1 wt% that allows yeast growth while stabilizing the 5-CQA gradually extracted, and the  
437 sulfite at 0.5 wt% that stops fermentation, stabilizes and enhances 5-CQA extraction. For  
438 acetic acid, the advantage is a complete consumption of sugars during the fermentation.  
439 So, the low sugar content of the final extract should considerably simplify the further  
440 purification steps during downstream processing. The use of sulfite will provide an  
441 extract richer in chlorogenic acids but less pure (with more sugars), and the use of acetic

442 acid at 1wt% will provide a purer extract, but with lower antioxidant capacity. Therefore,  
443 a compromise must be found when choosing a stabilizing agent for this process.

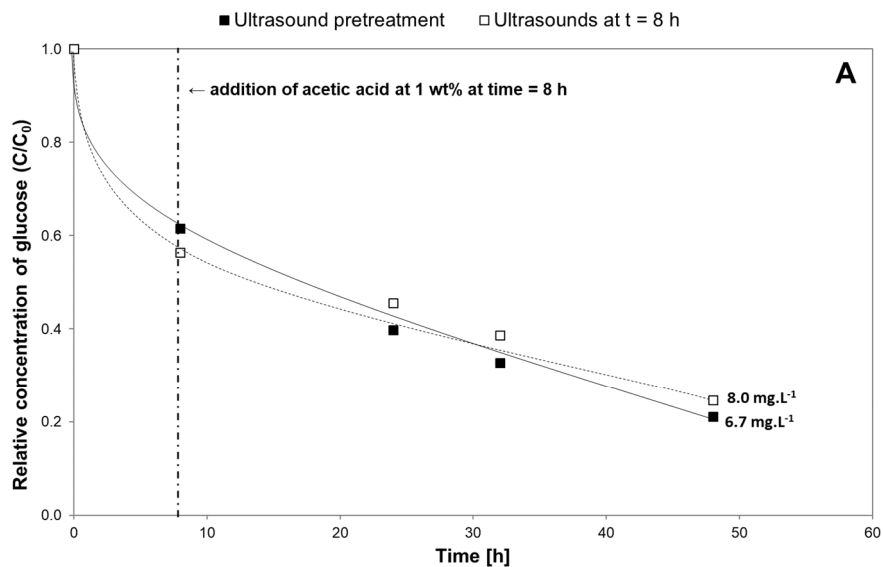
444

### 445 **3.2.3. Study of the process optimization using ultrasound-assisted extraction**

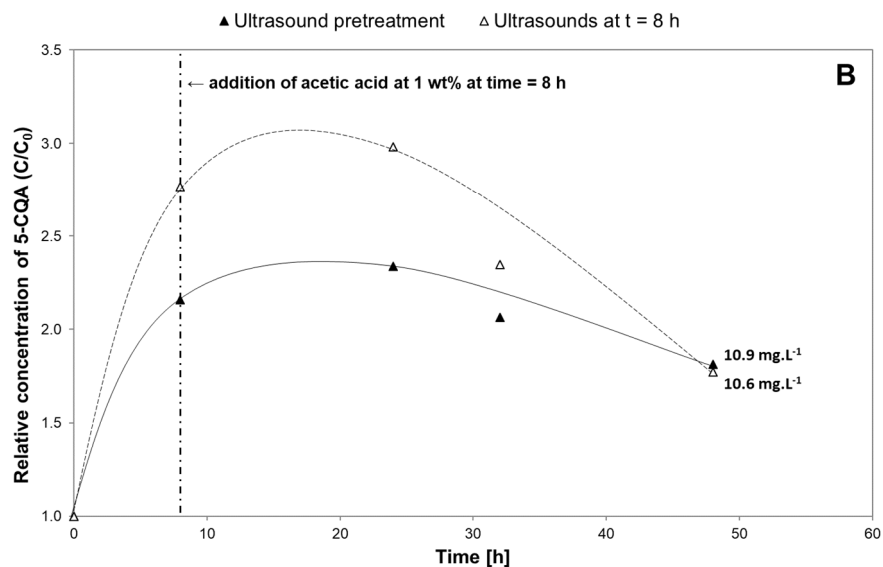
446 Two strategies were chosen to combine ultrasound-assisted extraction to the  
447 fermentation process: prior to fermentation (i.e. before addition of yeasts) or after 8 h of  
448 fermentation when the highest concentration of 5-CQA was achieved (according to  
449 results in Section 3.2.2). Even if ultrasounds can disrupt also the yeast cells causing yeast  
450 death and thus stop the fermentation as it was recently reported (Chemat et al., 2017), the  
451 fermentation during the first hours could potentiate ultrasound effects and thus favor  
452 CGAs' extraction. Since the main purpose of our process was to enhance the amount of  
453 CGA in the coffee pulp extract, the use of ultrasounds during the fermentation was an  
454 interesting strategy to explore. Figure 5 presents the results for glucose consumption and  
455 5-CQA extraction during the fermentation process. In that case, coffee pulp was stabilized  
456 using acetic acid at 1 wt% in order to preserve the 5-CQA while allowing the fermentation  
457 process to continue.

458 Results showed that glucose consumption was similar for both systems.  
459 Surprisingly, the glucose was not completely consumed even after 48 h of fermentation,  
460 compared to previous results that showed the glucose being mainly consumed at 24 h of  
461 fermentation (Figure 4). A moderate 5-CQA extraction optimization could be obtained  
462 with ultrasound-assisted extraction, as the content of 5-CQA at 8 h of fermentation was  
463 only increased by around 280% (US at 8 h) or 220% (US prior to fermentation) compared  
464 to 200% (fermentation without ultrasounds). Moreover, 5-CQA became unstable after  
465 24 h of fermentation, which could be explained by the possible release of endogenous

466 oxidative enzymes due to the ultrasound treatment. Another proposed explanation for the  
467 5-CQA instability relies on the fact that the ultrasound treatment could release other  
468 compounds that could interact with the 5-CQA throughout chemical reactions causing its  
469 degradation. Additionally, ultrasound treatment could enhance the extraction of many  
470 phenolic compounds from the coffee pulp that in turn could create an inhibitory  
471 environment for yeast growth, leading to the decrease of fermentation activity. Under the  
472 experimental conditions applied in this work, the coupling of ultrasounds prior or during  
473 the fermentation process is not an interesting method to improve the extraction yields of  
474 5-CQA from coffee pulp. It didn't improve the 5-CQA extraction yield as expected, and  
475 even slowed down the fermentation process. Therefore, no ultrasound treatment was  
476 applied on further studies.



477



478

479

**Fig. 5.** Effect of ultrasounds on glucose consumption (A) and 5-CQA extraction (B) during fermentation at laboratory-scale. (Glucose:  $C_0 = 31.9 \pm 1.8 \text{ g L}^{-1}$ ; 5-CQA: ( $C_0 = 6 \pm 2 \text{ mg L}^{-1}$ ).

480

### 482 3.3. Application of the fermentation process at larger scales

483

484

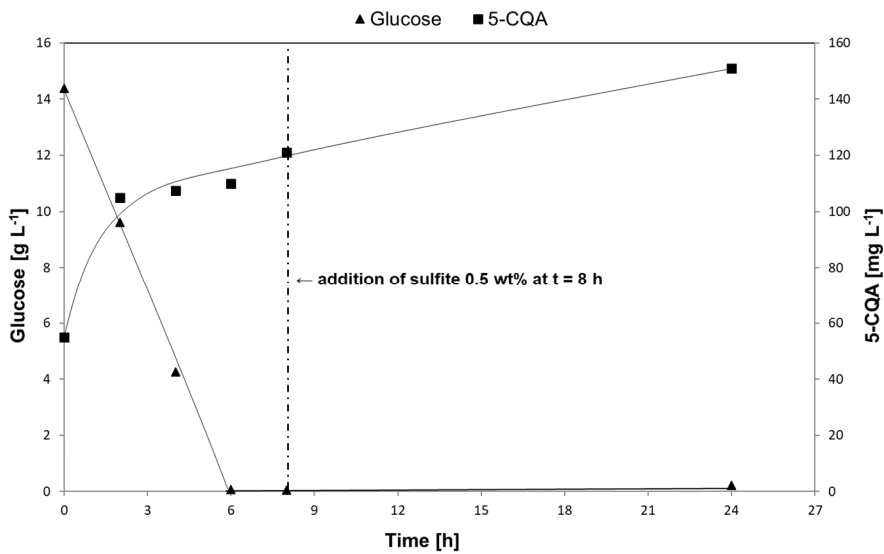
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486

487

To evaluate the validity of the results obtained at laboratory scale, fermentation at semi-pilot scale was carried out and monitored during 24 h. Sulfite at 0.5 wt% was chosen as the best stabilizing agent for the coffee pulp stabilization after a first step of fermentation. As mentioned in Section 3.1, a different batch of coffee pulp was used for this assay (batch of 2017), which resulted in differences on the initial concentrations of

488 sugars and chlorogenic acids compared to the coffee pulp used for laboratory scale  
 489 experiments (batch of 2015). The batch of 2017, used for this study, contained lower  
 490 concentrations of sugars and a higher initial amount of 5-CQA, as presented in the Table  
 491 1 and discussed in Section 3.1. Figure 6 presents the results for the glucose consumption  
 492 and 5-CQA extraction during the fermentation process.  
 493



494 **Fig. 6.** Glucose consumption and 5-CQA extraction during fermentation at semi-pilot scale.  
 495

496

497 The glucose was completely consumed after only 6 h of fermentation, which can  
 498 be explained by the lower initial amount of sugars in the coffee pulp from batch 2017  
 499 (14.39 g L<sup>-1</sup> of glucose and 21.42 g L<sup>-1</sup> of fructose) compared to the coffee pulp from  
 500 batch 2015 (31.73 g L<sup>-1</sup> of glucose and 42.97 g L<sup>-1</sup> of fructose). With most of the  
 501 fermentable sugars being consumed before 8 h of fermentation, the addition of sulfite at  
 502 t = 8 h did not disturb the yeast activity since the fermentation was already completed at  
 503 this time. The concentration of 5-CQA increased over time as previously observed at  
 504 laboratory scale, even after the addition of the sulfite (as explained in Section 3.3), up to

505 a final concentration of 151 mg L<sup>-1</sup> of 5-CQA at t 24 h, 275% higher than the initial  
506 concentration (55 mg L<sup>-1</sup> of 5-CQA). When the fermentation was carried out at laboratory  
507 scale, using sulfite at 0.5 wt% at t = 8 h as the stabilizing agent, the content of 5-CQA in  
508 the coffee pulp extract was increased by 400% at t 24h, against 275% at semi-pilot scale.  
509 Nevertheless, it was shown that the fermentation of coffee pulp and the extraction of  
510 chlorogenic acids were successful at semi-pilot scale, despite of the differences in coffee  
511 pulp composition (Table 1) and of the limitations encountered (reduced mass and heat  
512 transfers during the process scale-up).

513 Finally, to evaluate the process in a coffee producing country, fermentation was  
514 carried out at pilot-scale in Costa Rica with 90 kg of fresh coffee pulp nearby the green  
515 coffee production area. In order to compare the results from the semi-pilot fermentation,  
516 sulfite at a concentration of 0.5 wt% was used to stabilize the chlorogenic acids extracted  
517 during the fermentation. Table 3 summarizes the results for the fermentation performed  
518 at different scales. As mentioned in Section 3.1, the coffee pulp composition varied due  
519 to the year of harvest and the storage time, so the relative concentration ( $C/C_0$ ) of each  
520 compound (glucose, fructose, ethanol and CGA) was chosen instead of direct  
521 concentrations (C) values.

522 **Table 3**

523 Overview of the year of coffee pulp batches, the average temperature during the fermentation  
 524 process (T) and the relative concentration (ratio between the final C and initial C<sub>0</sub> concentrations)  
 525 of glucose, fructose, ethanol and 5-CQA after 24 h of fermentation at laboratory, semi-pilot and  
 526 pilot scales.

527

Scale	Batch	T [°C]	Relative concentration (C/C <sub>0</sub> ) after 24 h			
			Glucose	Fructose	Ethanol	5-CQA
<b>Laboratory</b>	2015	28	0.50	0.72	11.67	4.00
<b>Semi-pilot</b>	2017	26	0.02	0.10	24.00	2.75
<b>Pilot</b>	2018	22	0.14	0.22	22.86	3.09

528

529 For all three scales, sulfite at 0.5 wt% was used to stabilize the 5-CQA extracted,  
 530 added at 8 h of fermentation. At laboratory scale, the fermentation was immediately  
 531 stopped when sulfite was mixed to coffee pulp (Figure 4), which explains the higher  
 532 relative concentrations of glucose and fructose in the product after 24 h. This was not the  
 533 case for the 2017 and 2018 batches, which had lower initial concentrations of sugars,  
 534 allowing the almost complete consumption of glucose and fructose prior to the addition  
 535 of sulfite. In that cases, ethanol production yields reached a quite high level between 0.37  
 536 and 0.41 g ethanol / g sugar which corresponds to 72 and 80% of the maximal theoretical  
 537 conversion yield (0.51 g ethanol / g sugar). Nevertheless, the extraction of 5-CQA was  
 538 very similar for all three scales, with an augmentation of around 300% of its initial  
 539 concentration in less than 1 day of fermentation.

540 Although the operating temperature was set at 28 °C, the average temperature of  
 541 coffee pulp varied with the scale of the process. Obviously the bigger the amount of pulp  
 542 used as substrate, the harder the temperature control of the coffee pulp. Since no stirring  
 543 was applied during the fermentation, heat transfer became an important problem for the  
 544 process. However, even at lower temperatures, solid-state fermentation using a

545 commercial yeast (Yeast A) was able to ferment coffee pulp and to produce a rich phenol-  
546 extract containing more than 600 mg of 5-CQA per kg of coffee pulp and purer in terms  
547 of the lower concentration of sugars (5 g L<sup>-1</sup> of glucose and 12 g L<sup>-1</sup> of fructose). We  
548 showed that the fermentation of coffee pulp at a pre-industrial (pilot) scale with very  
549 simple equipment can be used to obtain a valuable product. It was important to develop a  
550 process that could be easily implemented in the coffee producing countries, without big  
551 financial investment and operating costs, lower environmental impact and that could  
552 generate a new source of income for the small coffee producers.

553

## 554 **Conclusions**

555 Solid-state fermentation was used to produce a valuable product from coffee pulp  
556 at laboratory, semi-pilot and pilot scales. *Saccharomyces cerevisiae* consumed the sugars  
557 releasing chlorogenic acids that are found linked to the plant cell wall probably as  
558 glycosides. Investigation of the extract stability and the effect of ultrasounds revealed that  
559 higher extraction yields were obtained when fermentation was carried out without  
560 ultrasound treatment and by using sulfite at 0.5 wt% as the stabilizing agent. Irrespective  
561 of the scales, a phenol-rich extract was obtained containing 300-400% more chlorogenic  
562 acids than its initial concentration, within less than 24 h of fermentation. After  
563 fermentation and solid/liquid separation steps, the quantity of the remaining solid residue  
564 was considerably reduced. This final by-product will contain much less caffeine and  
565 phenolics (compounds considered as anti-nutritional and eco-toxic), which could  
566 facilitate its reuse as animal feeding, fertilizer or composting substrate.

567

568 For further studies, it could be of great interest to test other yeast strains and to  
569 optimize fermentation conditions for even increasing the extraction yield. In order to find  
570 the best stabilizing method, in terms of (1) CGA preservation, (2) effect on the  
571 fermentation activity and (3) cost and environmental impact, new stabilizing agents must  
572 be tested at larger scales. To obtain a ready-to-market product, the concentration and the  
573 purification of the extract need to be performed and investigated using sustainable  
574 technologies. Finally, the characterization of the final product will have to be carried out,  
575 e.g., in terms of antioxidant activity, to better determine its market added value and to  
576 enlighten about the economic feasibility of the process.

577

578

579

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586 (Toulouse, France) for providing *Saccharomyces cerevisiae* strains.

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