1 **TITLE:**

2 Validation of coffee by-products as novel food ingredients

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27 **Abbreviations**

28 ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ATR,
29 attenuated total reflection; CGA, chlorogenic acid; 5-CQA, 5-caffeoylquinic acid; CS,
30 coffee silverskin; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DDD,
31 dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT,
32 dichlorodiphenyltrichloroethane; DMEM, Dulbecco's Modified Eagle's Medium;
33 DMSO, dimethyl sulfoxide; EFSA, European Food Safety Authority; FOS,
34 fructooligosaccharides; FT, Fourier-Transform; HCH, hexachlorocyclohexane; IDF,
35 insoluble dietary fiber; IR, infrared; MTT, 3-(4,5-dimethylthiazole-y)-2,5-
36 diphenyltetrazolium bromide; OECD, Organization for Economic Co-operation and
37 Development; OTA, ochratoxin A; ROS, reactive oxygen species; RUT, rutin; SDF,
38 soluble dietary fiber; TAC, total antioxidant capacity; tBOOH, tert-butyl hydroperoxide;
39 TDF, total dietary fiber; TFC, total flavonoid content; TPC, total phenolic compounds.

40

41 **Abstract**

42 This research aimed to validate coffee husk, parchment and silverskin as new health-
43 promoting food ingredients. Characterization of the novel ingredients was carried out by
44 Raman and infrared spectroscopy and analysis of total phenolic compounds, chlorogenic
45 acid, caffeine and dietary fiber. Antioxidant properties of the novel ingredients were
46 tested by ABTS and intracellular ROS formation in HepG2 cells. Pesticides, mycotoxins,
47 acrylamide and acute toxicity experiments following OECD Test Guidelines 425 were
48 performed to assess the food safety of extracts, solid residues and raw materials. Husk
49 and silverskin are proposed as a source of two food ingredients: aqueous extracts enriched
50 in phytochemicals and antioxidant dietary fiber while parchment is proposed as a natural
51 source of antioxidant dietary fiber. No lesions were found in selected isolated vital organs
52 from treated animals. Coffee by-products can be converted into safe food ingredients
53 allowing a whole food waste recovery. Analyses of contaminants is essential for
54 achieving this goal.

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57 **Keywords:** Antioxidants, coffee by-products, dietary fiber, food safety, novel food
58 ingredient, sustainability.

59

1. Introduction

Coffee is a cherry formed by various anatomic parts with distinct morphologies (Figure 1) and unique chemical compositions. It comprises a plethora of nutrients and bioactive phytochemicals with diverse preventive and therapeutic effects for chronic diseases (del Castillo et al., 2017). Nowadays, coffee beans are almost exclusively used for the preparation of the beverage. While the coffee industry is very diverse, coffee processing can generally be described by ten steps: planting, harvesting and processing the cherries, drying, milling and trading, tasting, roasting, grinding and finally brewing. Even though most of the health-promoting phytochemicals and nutrients are found in the coffee cherry, 90% of the cherry is discarded during processing as agricultural waste or by-product (del Castillo, Fernandez-Gomez, Martinez-Saez, Iriondo-DeHond, & Mesa, 2018).

According to the European Commission, a “food waste” is defined as “food (including inedible parts) lost from the food supply chain, not including food diverted to material uses such as bio-based products, animal feed, or sent for redistribution” (European Commission, 2014). The coffee industry is responsible for the generation of large amounts of residues that represent a great pollution hazard if discharged into the environment (Chanakya & De Alwis, 2004). The future of coffee depends on sustainable practices, and a wide range of initiatives can be implemented in every part of the coffee supply chain: improving the conditions at origin, recycling packaging materials, reducing emissions, developing eco-friendly facilities or designing new coffee products. The conversion of coffee waste into food ingredients is a research priority in the Nutrition and Food Chemistry field.

Esquivel et al. (2012) defined coffee husk as the outer skin and pulp obtained from the wet processing of coffee berries (Esquivel & Jiménez, 2012). Coffee husk encloses the coffee beans and compromises nearly 45% of the berry. It has a high content in

85 carbohydrates (35-85%), soluble fibers (30.8 %), minerals (3-11%) and proteins (5-11%).
86 It is also rich in insoluble dietary fiber and can be a source of phytochemicals such as
87 tannins (5-9%) and cyanidins (20%) for the food and pharmaceutical industries (M.
88 Dolores del Castillo et al., 2018; Esquivel & Jiménez, 2012). Phytochemicals have
89 recently received considerable interest because of their safety and potential positive
90 physiological effects on the human body (Singh & Geetanjali, 2013). To date, coffee husk
91 has been used as a source of dietary fiber (www.pectcof.com) and also as a source of
92 anthocyanins (Murthy, Manjunatha, Sulochannama, & Madhava Naidu, 2012).
93 Considering its chemical composition and the proposed applications, coffee husk has a
94 great potential as a food ingredient and as a natural source of nutrients and bioactive
95 compounds.

96 Parchment is a strong fibrous endocarp that covers both hemispheres of the coffee seed
97 and separates them from each other (Belitz, Grosch, & Schieberle, 2009). It represents
98 5.8% of berry dry weight and is formed by (α-) cellulose (40–49%), hemicellulose (25–
99 32%), lignin (33–35%) and ash (0.5–1%) (del Castillo et al., 2018). In wet coffee
100 processing, the parchment is removed after drying and hulling, which allows it to be
101 collected and used separately from other by-products (Esquivel & Jiménez, 2012). To
102 date, no applications have been found for coffee parchment as a food ingredient.

103 Coffee silverskin (CS) represents 4.2 % (w/w) of the coffee cherry and constitutes a thin
104 tegument of the outer layer of the two beans forming the green coffee seed. It is obtained
105 as a by-product of the roasting process. CS has a high dietary fiber content (68-80%),
106 polysaccharides are also abundant components (60-70 %) and total sugar content varies
107 greatly (1.6−12%) (Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013). CS contains
108 protein, fat, and ash, at 16.2–19.0%, 1.56–3.28%, and 5-7%, respectively (del Castillo et
109 al., 2018). Borrelli and colleagues found that CS, obtained from several Italian roasting

110 plants, had a high amount of soluble dietary fiber (about 14% of the total fiber) and a very
111 high antioxidant activity (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002).
112 Previous studies have suggested the use of CS as a source of dietary fiber and prebiotics
113 (Ballesteros, Teixeira, & Mussatto, 2014; Borrelli, Esposito, Napolitano, Ritieni, &
114 Fogliano, 2004).

115 In 2016, the European Food Safety Authority (EFSA) reported that only 1.5% of 83,000
116 food samples analyzed exceeded the legal limit of pesticides, and the national competent
117 authorities had to take appropriate enforcement actions (European Food Safety Authority
118 (EFSA), 2016). To date, no studies have analyzed pesticide content in coffee by-products.
119 Moreover, coffee is susceptible to contamination by mycotoxins, toxic compounds that
120 result from fungal secondary metabolism under certain conditions, which cause different
121 toxicological effects in humans (Afsah-Hejri, Jinap, Hajeb, Radu, & Shakibazadeh, 2013;
122 Gamboa-Gaitán, 2012). The most studied mycotoxin in coffee is ochratoxin A (OTA),
123 the only mycotoxin subjected to European legislation (European Commission (EC)
124 1881/2006). Other contaminants may also be formed during food processing, e.g.
125 Maillard reaction products such as acrylamide. This low molecular weight compound is
126 highly soluble in water, and has a well-documented carcinogenic potential (European
127 Commission, 2017). It is formed in food processing as a degradation product of
128 asparagine and sugars, typically at 120 °C and low moisture (European Commission,
129 2017). Therefore, it could be found in roasted coffee beans and CS. Previous studies have
130 confirmed the presence of acrylamide in CS (333 μg/kg) (Garcia-Serna, Martinez-Saez,
131 Mesias, Morales, & Castillo, 2014) in the same amount as that reported in roasted coffee
132 and approximately 10 times lower than that reported in instant coffee (European
133 Commission, 2017).

134 The aim of this research was to perform a comparative study of the three coffee by-
135 products obtained before (husk and parchment) and after the roasting process (silverskin);
136 validate their potential and safety as sustainable food ingredients; explore novel
137 applications for them through environment-friendly procedures and implement novel
138 analytical tools such as vibrational spectroscopy.

139 ## 2. Materials and methods

140 *2.1. Reagents*

141 Chlorogenic acid, 2,21-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium
142 salt (ABTS), caffeine, Folin-Ciocalteu reagent, tert-butyl hydroperoxide (tBOOH),
143 dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazole-y)-2,5-diphenyltetrazolium
144 bromide (MTT) and 21,71-dichlorodihydro-fluorescein diacetate (DCFH-DA) were
145 purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA). Dulbecco's
146 Modified Eagle's Medium (DMEM) was purchased from Lonza (Basel, Switzerland).

147 *2.2. Raw materials*

148 Coffee husk and parchment from Arabica species were kindly provided by Delikia from
149 Finca Morenitas in Nicaragua. CS from Arabica and Robusta species from Colombia was
150 provided by Fortaleza S.A. (Spain). All coffee by-products were derived from wet-
151 processing.

152 *2.3. Raman and infrared spectroscopy*

153 Fourier-Transform (FT) Raman spectra were recorded with an RFS-100/S spectrometer
154 (Bruker, Billerica, USA). A single 4x4 mm^2 endocarp shell was positioned between a thin
155 quartz window (1 mm) and a silver-mirror. Spectra were recorded according to previous

156 measuring protocols for green coffee (Keidel, von Stetten, Rodrigues, Máguas, &
157 Hildebrandt, 2010).

158 Complementary endocarp FT-infrared (IR) spectra were recorded in a Tensor27 FT-
159 spectrometer (Bruker, Billerica, USA), equipped with a diamond attenuated total
160 reflection (ATR) cell (Durascope Resultec). The single endocarp shell was positioned and
161 pressed towards the ATR-diamond surface. Absorbance spectrum was calculated from
162 the recorded sample spectrum and a previously measured reference spectrum (empty
163 ATR-cell). Further details regarding spectrometers and data processing are given
164 elsewhere (Ulijasz et al., 2009).

165 For the band assignment of the endocarp IR and the Raman spectra, we refer to previous
166 experimental works in cellulose, lignin and further related compounds (Adebajo, Frost,
167 Kloprogge, & Kokot, 2006; Gierlinger, Keplinger, Harrington, & Schwanninger, 2013;
168 Nabais et al., 2008).

169 *2.4. Characterization of coffee by-product extracts*

170 *2.4.1. Preparation of aqueous extracts from coffee by-products*

171 Coffee by-product extracts were produced as described in the patent WO 2013/004873
172 (del Castillo et al., 2013). Briefly, 50 mg of coffee husk, parchment or silverskin were
173 added per milliliter of boiling water. This mixture was stirred at 250 rpm for 10 min;
174 filtered by a 250 μm filter and then through Whatman paper No. 4; and the filtrate and
175 the insoluble residue were freeze-dried. Extracts were prepared three times.

176 *2.4.2. Caffeine and 5-CQA*

177 Caffeine and 5-CQA content was determined by ultra-performance liquid
178 chromatography - tandem mass spectrometer (UPLC-MS/MS) (Thermo-Scientific, San

179 José, CA, USA) as described by Fernandez-Gomez, Lezama, et al., (2016). For
180 quantification, samples were diluted in milli-Q water and added with 50 µg/ml of
181 phluoroglucinol as internal standard for the most abundant chlorogenic acid (CGA)
182 described for coffee (5-caffeoylquinic acid, 5-CQA) and 50 µg/ml of salicylic acid as
183 internal standard for caffeine. To verify the reproducibility and possible variations, all
184 experiments were performed in triplicate. Compound concentrations were expressed in
185 mg caffeine or 5-CQA/ g of sample.

186 *2.4.3. Overall antioxidant capacity*

187 The trapping capacity of cationic free radicals was evaluated using the method of radical
188 $ABTS^+$ bleaching described by Re et al., (1999) and modified by Oki, Nagai, Yoshinaga,
189 Nishiba, & Suda (2006) for its use in microplate. Aqueous solutions of CGA (0.15–2.0
190 mmol/l) were used for calibration. Absorbance was measured in microplate using a UV-
191 Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). All
192 measurements were performed in triplicate, and results were expressed as mg CGA eq.
193 per gram of sample.

194 *2.4.4. Total phenolic compounds*

195 Folin–Ciocalteu adapted to a micromethod format was used for the analysis of total
196 phenolic compounds in samples (Contini, Baccelloni, Massantini, & Anelli, 2008). The
197 reaction was initiated by mixing 10 µl of sample with 150 µl of Folin–Ciocalteu solution.
198 After 3 minutes at room temperature, 50 µl of sodium bicarbonate solution were added.
199 The kinetics of the reaction at 37 ºC was followed for 120 minutes by measuring the
200 absorbance at 735 nm once every minute using a UV-Visible Spectrophotometer (BioTek
201 Instruments, Winooski, VT, USA). Sample blank and reagent blank were also analyzed
202 in each set of samples. A CGA calibration curve was used for quantification (0.1–0.8

203 mg/ml). Results were expressed as μmol CGA eq./g. All measurements were performed
204 in triplicate.

205 *2.4.5. Total flavonoid content*

206 Total flavonoid content was determined using a previously described colorimetric method
207 (Xu & Chang, 2007). Briefly, 100 μL of samples or rutin (RUT) standard solution were
208 mixed with 30 μL of a 5 % $NaNO_2$ solution. After 5 minutes of incubation, 30 μL of a
209 10% $AlCl_3$ solution were added and allowed to stand for another 6 minutes before adding
210 100 μL of 2M NaOH. Absorbance was measured immediately at 510 nm using a UV-
211 Visible Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Results were
212 calculated and expressed as milligrams of RUT equivalents per 100 mg of extract. The
213 determination was carried out in triplicate.

214 *2.4.6. Cell culture and treatments*

215 HepG2 human hepatocyte cell line was kindly provided by Dr. Paloma Morales (Facultad
216 de Veterinaria, Universidad Complutense de Madrid, Spain). Cells were cultured in
217 Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine
218 serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin in standard conditions (37
219 ºC, 5% CO2, in a humidified incubator, BINDER CB series 2010, Tuttlingen, Germany).

220 *2.4.7. Cell viability assays*

221 The effect of different concentrations of husk, parchment, and CS extracts on cell viability
222 was measured using the MTT assay (Bakondi et al., 2003). Powdered extracts were
223 prepared in PBS and sterile filtered when used in cell culture. Cells were cultured at a
224 density of $1.0x10^5$ cells per well of a 96-well plate. After 24 hours, cells were treated with
225 husk, parchment, and CS extracts diluted in DMEM culture medium (0.1, 1, 10 and 20
226 mg/ml) for 24 hours. DMSO (50%) was used as death control. Subsequently, cells were

227 incubated in MTT solution (0.5 mg/ml) for 1 h at 37 ºC. The supernatant was then
228 removed, 100 µL of DMSO were added, and the optical density at 570 nm was measured
229 using a microplate reader (BioTek Synergy HT Multi-Mode Microplate Reader,
230 Winooski, VT, USA). Experiments were carried out in triplicate.

231 *2.4.8. Intracellular ROS scavenging assay*

232 The analysis was performed by measuring the fluorescence intensity of the DCFH-DA
233 probe, which was proportional to the amount of ROS formed (Gomes, Fernandes, &
234 Lima, 2005). A 10 mM solution of DCFH-DA was prepared (5 mg in 1 ml DMSO), and
235 a 50 µL aliquot was separated. Then, 800 µL of DMSO were added to the 50 µL solution.
236 After 24 hours of extract incubation, cells were pre-loaded with 2.5 µL/well of this last
237 solution for 30 minutes at 37 ºC. After incubation, DCFH becomes dichlorofluorescein
238 (DCF) due to intracellular oxidants and will emit fluorescence. Next, the culture medium
239 was removed; cells were washed with PBS; and the three coffee by-product extracts (1
240 mg/ml) were added for 1 hour. tBOOH 1 mM was used as a positive control. Then,
241 fluorescence was measured at 485 nm/528 nm (BioTek Synergy HT Multi-Mode
242 Microplate Reader). Experiments were carried out in triplicate.

243 *2.5. Characterization of the insoluble fraction obtained from coffee by-products during*
244 *the extraction process*

245 *2.5.1. Dietary fiber*

246 Insoluble (IDF), soluble (SDF) and total (TDF) dietary fiber content was determined
247 using the Total Dietary Fiber Assay Kit (Megazyme International Ireland, Ireland)
248 following the manufacturer's instructions, and based on the enzymatic–gravimetric
249 method. Results are expressed as percentage (%).

250 *2.5.2. Total protein*

251 Protein content was determined by Kjeldahl mineralization followed by a colorimetric
252 analysis of nitrogen for quantification (AOAC-32.1.22, 920.87). NH_4Cl was used for a
253 standard calibration curve. A conversion factor (6.25) was used to calculate protein
254 content. Results were expressed as % dry matter (d.m.).

255 *2.5.3. Total lipid content*

256 Using a modified protocol described by Folch, Lees, & Sloane Stanley (1987), lipid
257 content was determined by a solid-liquid extraction with n-hexane. Fifty milliliters of n-
258 hexane were added to the insoluble fractions (1 g) and the mixture was homogenized
259 using an Ultra-Turrax for 3 minutes and subjected to an ultrasonic bath for 20 minutes to
260 improve lipid extraction. Then, samples were centrifuged at 1620 g for 20 minutes.
261 Supernatant's solvent was then gently removed under controlled vacuum. Total lipid
262 content was obtained by weighing difference. The assay was performed in duplicate and
263 the results expressed as % lipid content.

264 *2.5.4. Overall antioxidant capacity*

265 Direct $ABTS^+$ assay was carried out according to Açar, Gökmen, Pellegrini, & Fogliano
266 (2009). Ten mg of sample were mixed with 90 mg of cellulose and stirred, 10 mg of the
267 mixture were mixed together with 1.7 ml of $ABTS^+$ solution in a thermomixer (25 ºC, 2
268 minutes, 600 rpm). After centrifugation, absorbance of the supernatant was measured in
269 a microplate. A CGA calibration curve (0–250 µg/ml) was used. Measurements were
270 performed in triplicate and results expressed as mg CGA eq. per gram of sample.

271 *2.6. Safety assays*

272 *2.6.1. Pesticides*

273 The gas chromatography system (HPGC-7890A) was equipped with split/splitless
274 injector and a MS detector (model MS-5975-VL-MSD with Triple –Axis Detector). The
275 automatic split/splitless injector operated at 250ºC and the split (1:10) was used to detect
276 the analytes with helium as the carrier gas at an approximate flowrate of 1 ml/min. Gas
277 chromatographic analysis of samples (husk extract and insoluble fraction, raw parchment
278 and Arabica CS extract and insoluble fraction) was performed using a 30 m × 0.25 mm
279 ID capillary fused silica column coated with a 0.25 μm film of TRB-5 (Teknokroma,
280 Spain). Oven temperature-ramp followed a 12 °C/min increase-rate from 60ºC (2 min) to
281 140ºC and 6°C/min up to 320ºC. Desired temperature was maintained for 5 min.

282 The ion source temperature of the MS was 230ºC, mass range 40–500 amu, scan speed
283 1666 amu/s, interface temperature 230ºC. First, the SCAN mode and then the selected ion
284 monitoring (SIM) mode were used for the analyses. An advanced pressure control
285 supplied helium to the interface at constant pressure (95 kPa). In all cases, analyses were
286 made in triplicate. Pesticide identification was based on the retention time and the relative
287 abundance of the specific ions (used in the SIM mode), compared from the sample extract
288 (Supplementary Material, Table S1). The number of monitored ions, preferably and if
289 possible a minimum of three, was dependent on the pesticide. Analyzed pesticides were
290 HCH-α, HCH-β, HCH-γ, HCH-δ, heptachlor, Aldrin, hexachlorbenzene, endosulfan-α,
291 endosulfan-β, chlorpyrifos methyl, parathion methyl, parathion ethyl, dieldin, endrin, 4,4-
292 DDE, 2,4-DDD, 44DDT, 24DDE and mirex.

293 *2.6.2. Mycotoxins*

294 Samples (husk extract and insoluble fraction, raw parchment and Arabica CS extract and
295 insoluble fraction) were ground with an A-10 Basic laboratory mill (IKA, Staufen,
296 Germany) and stored at -20 ºC until use. Ground samples (1 g) where mixed with
297 acetonitrile:water (80:20, v/v, 2.5 ml) in a 15 ml centrifuge tube, vortexed (5 min),

298 sonicated (3 min), vortexed again (1 min) and then samples were centrifuged (3000 g, 10
299 min). Subsequently, the supernatant was separated. The residue was extracted again
300 following the same procedure and the supernatants were combined. One ml of the
301 supernatant was transferred to a new 15 ml centrifuge tube and diluted with water (7 ml)
302 to a total volume of 8 ml and centrifuged (3000 g, 10 min).

303 Mycotoxins were extracted using ISOLUTE® Myco 60 mg/3 ml (Biotage, Sweden) SPE
304 columns following the procedure recommended by the supplier. Briefly, SPE column was
305 conditioned with 2 ml acetonitrile, equilibrated with 2 ml of ammonium acetate (10 mM)
306 and 3 ml of the diluted sample extract were loaded. Then, the column was washed with
307 10 mM ammonium acetate (3 ml) and with 10 mM ammonium acetate:acetonitrile (90:10,
308 v/v, 3 ml) and dried for 30 seconds at maximum vacuum. Finally, the sample was eluted
309 with 0.1% formic acid in acetonitrile (2 ml) and then with 0.1% formic acid in methanol
310 (2 ml). The combined eluates were dried under vacuum (5 mbar at 35 °C) and
311 reconstituted in 250 µL of 0.1 % acetic acid in 20 % acetonitrile:methanol (1:1, v/v).

312 HPLC-QToF analysis: The analytical system used consisted of a 1260 Infinity HPLC
313 system coupled to a 6545 quadrupole-time of flight (Q-ToF) mass spectrometer detector
314 (Agilent, Waldbronn, Germany). Control software was Mass Hunter Workstation
315 (version B.06.11). The Q-ToF used a Dual Jet Stream Electrospray Ionization (Dual AJS-
316 ESI) source operated in the positive ionization mode, and the following parameters were
317 set: capillary voltage, 4000 V; fragmentor, 120 V; nozzle voltage 500 V; gas temperature,
318 130 ºC; drying gas,13 L/min; nebulizer, 30 psig; sheath gas temperature, 300 ºC; sheath
319 gas flow, 11 L/min; acquisition range, 80-1000 m/z. Samples were analyzed after
320 injection (30 µl) on a Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1 × 50
321 mm, 1.8 µm, Agilent, Santa Clara, CA) protected with a 5 mm guard column of the same
322 material thermostated at 30 ºC. The solvents system were 5 mM ammonium formate +

323 0.1% formic acid (solvent A) and 5 mM ammonium formate + 0.1% formic acid in
324 methanol (solvent B). The elution gradient was (time, % of solvent A): 0 min, 90%; 0.5
325 min, 90%; 10 min, 30%; 15 min, 2%; 18 min, 2%; 20 min, 90% and a post time of 5 min.
326 Compounds were identified and quantified using the algorithm "Find by Formula" that
327 evaluated the mass accuracy together with the isotopic relative abundance and isotopic
328 separation.

329 *2.6.3. Acrylamide*

330 The quantification of acrylamide in Arabica CS extract and insoluble fraction was carried
331 out in a laboratory that participates in appropriate proficiency testing schemes (which
332 comply with the 'International Harmonised Protocol for the Proficiency Testing of
333 (Chemical) Analytical Laboratories') developed under the auspices of
334 IUPAC/ISO/AOAC and using approved analytical methods for detection and
335 quantification (HPLC-MS/MS, ISO 18862:2016) according to Commission Regulation
336 (EU) 2017/2158. This analysis was performed by Coffee Consulting S.L.

337 *2.6.4. Acute Toxicity Assay*

338 In accordance to OECD (Organization for Economic Co-operation and Development)
339 Test Guidelines 425 (Up and Down Procedure), healthy young adult nulliparous and
340 nonpregnant female rats, weighing 180,52±6,42 g (8 weeks old) at the start of the
341 experiment, were procured from Charles River (Sant Cugat del Vallés, Spain) . The
342 present study was approved by the Institutional Animal Ethics Committee (Reg. No.
343 PROEX 011/17) of Community of Madrid, Spain.

344 Rats were housed in cages with free access to standard food (A04 Safe Diets, Augy,
345 France) and water *ad libidum*. According to OECD Test Guidelines 425 (OECD, 2008),
346 limit test of raw parchment (section 2.2) and husk and Arabica CS extracts (section 2.4.1)

347 was performed at 2000 mg/kg b.w. by gavage as a single dose to one rat. Husk and CS
348 extracts were dissolved in water and parchment was dissolved in corn oil. As a control,
349 one rat was dosed with water and another rat with corn oil to establish a comparative
350 negative control group. Rats were closely observed for the first 30 min, then for 4 h. After
351 survival of the treated rat, 4 additional female rats per group were administered with the
352 same dose under the same conditions.

353 Body weight changes, signs of toxicity, behavior and mortality were observed for 24
354 hours after administration and once daily for 14 days. Then, rats were sacrificed by
355 exposure to excess carbon dioxide in a gas chamber for necropsy examination. Internal
356 organs (heart, lungs, liver, kidneys, spleen, adrenals, sex organs and brain) were collected
357 for histopathological examination.

358 Tissue samples were routinely processed for histology and fixed in buffered formalin 10%
359 (Panreac©, Barcelona, Spain, stabilized with methanol at pH 7) for 24 hours at room
360 temperature. Samples were then embedded in synthetic paraffin (Casa Alvarez, Madrid,
361 Spain) with a melting point of 56 °C, using an automatic tissue processor (ASP300,
362 Leica®, Wetzlar, Germany), with a program of automatic transmissions alcohols
363 increased histological grading. Blocks were performed in a block forming unit (console
364 Leica© EG1140H and cold plate Leica© EG1130) and 4 micron thick sections were
365 obtained from rotation microtome Leica© brand, model RM 2155. Sections were
366 deparaffinized in xylene and hydrated in alcohol and water. Conventional staining
367 method, hematoxylin & eosin, was used by means of Leica© auto stainer SP4040. Then,
368 dehydrated first ascending alcohol series and xylene were used, and finally, mounted with
369 DPX (Nustain©, Nottingham, UK).

370 *2.7. Statistical analysis*

371 Data were expressed as the mean ± SD. Prior to statistical analysis, all data were tested
372 for variance homogeneity using the Levene's test. Logarithmic transformation of the
373 response variable was used to achieve homocedasticity when needed. One-way analysis
374 of variance (ANOVA) was performed. Statistical comparisons of the different treatments
375 were performed using Tukey's test. Values of $p < 0.05$ were considered statistically
376 significant. All statistical analyses were performed using SPSS Statistics 24.

377 **3. Results and discussion**

378 *3.1. Characterization of coffee husk and its potential applications*

379 Coffee husk extract (Figure 1a) showed the highest extraction yield (28 %), followed by
380 CS extract (14 %) and parchment extract (2.3 %). Data on the characterization of coffee
381 husk extract are summarized in Table 1. 5-CQA and caffeine were detected in all three
382 extracts. Thus, caffeine concentration was higher than that corresponding to the phenolic
383 compound whose concentration increased towards the fruit's core. Therefore, extract
384 from coffee husk, the outer part of the cherry, showed the lowest values of the two
385 bioactive compounds (1.7 mg 5-CQA/g and 13.9 mg caffeine /g). Husk extract also
386 presented the lowest ($p < 0.05$) amount of total phenolic compounds (TPC) and total
387 flavonoid content (TFC) under the tested conditions (15.6 mg CGA eq./g and 0.9 g RUT
388 eq./g, respectively). The lowest total antioxidant capacity (TAC) corresponded to husk
389 extract, in accordance with the obtained data of TPC, TFC and 5-CQA content. Some
390 authors have previously reported the amount of caffeine and CGA in raw coffee husk, 13
391 and 25 mg/g, respectively (Murthy & Naidu, 2012; Pandey et al., 2000). Husk extract
392 obtained under the conditions described in this work (WO 2013/004873) presented the
393 same levels of caffeine as in the starting material.

394 Table 2 shows the viability of HepG2 cells after 24 hours of incubation with husk extract
395 at different concentrations (0.1 to 20 mg/ml). At concentrations of 0.1 and 1 mg/ml, cell
396 viability was not significantly ($p > 0.05$) altered, while 20 mg/ml resulted cytotoxic. To
397 date, there are no published studies about the effect of water soluble bioactive compounds
398 from coffee husk on cell viability. Table 2 also shows the effect of husk extract on the
399 prevention of intracellular ROS formation. The addition of tBOOH 1 mM to the culture
400 medium significantly increased ($p < 0.05$) intracellular ROS production (≈44%)
401 compared to non-treated cells. Cells pre-treated for 24 hours with husk extract (1 mg/ml)
402 showed a significant reduction ($p < 0.05$) in ROS levels compared to cells treated with
403 tBOOH. ROS values were similar for non-treated cells (control) and those treated with
404 the extract in absence of tBOOH, suggesting no effect in the physiological production of
405 radicals. This is the first time that the effect of coffee husk on intracellular ROS formation
406 has been described. Understanding the cellular defense action mechanism requires further
407 research beyond the scope of the current study. Nevertheless, coffee husk extract should
408 be considered a potential agent for the prevention of cellular damage induced by oxidative
409 stress.

410 Soluble dietary fiber (SDF) was found in the insoluble fraction generated during the
411 extraction process of coffee husk in significantly higher concentrations ($p < 0.05$) than in
412 the rest of the studied samples (12.3 %, Table 3). Total dietary fiber (TDF) values of husk
413 insoluble residue are in the range described by Gouvea, Torres, Franca, Oliveira, &
414 Oliveira (2009), but in lower amounts compared to the results obtained by Navya &
415 Pushpa (2013). Coffee husk has been characterized as a source of soluble dietary fiber
416 aimed for use in food and pharmaceutical applications. Due to its properties as an
417 emulsifier and stabilizer, this extracted dietary fiber is a promising new ingredient for the
418 food and beverage industry (www.pectcof.com).

419 In addition, husk residue presented a protein content of 8.4 % (Table 3), which is within
420 the range reported by other authors (Brand et al., 2001; Gouvea et al., 2009). Lipid content
421 was also in the same range reported by Gouvea et al. (2009). Regarding the total
422 antioxidant capacity of this (water-insoluble) fraction, coffee husk showed the highest
423 values, with 48.6 mg CGA/g ($p < 0.05$). This is the first time the antioxidant character
424 of the dietary fiber from coffee husk is described. In addition to its antioxidant properties,
425 consumption of SDF has nutritional relevance due to its positive impact on health by
426 decreasing serum cholesterol, postprandial blood glucose and insulin contents in the
427 human body (Bruntha Devi, Vijayabharathi, Sathyabama, Gurusiddappa Malleshi, &
428 Brindha Priyadarisini, 2011). The ability of SDF to retard the absorption of glucose in the
429 small intestine is a desirable characteristic in the development of foods for diabetic
430 populations (Onyango, Noetzold, Bley, & Henle, 2004).

431 With regard to contaminants, no pesticides were detected either in husk extract or husk
432 insoluble fraction. OTA was detected in husk insoluble fraction (4.3 µg/kg) in amounts
433 below the maximum levels of 5 µg/kg established by the European Commission
434 (European Commission, 2005) (Table 4). In contrast, OTA levels in husk extract were
435 below the quantification limit (< 0.3 µg/kg). Furthermore, neither aflatoxin B1 nor
436 enniantin B were detected in husk extract or its solid residue. García-Moraleja *et al.*
437 (2015) reported OTA, Aflatoxin B1 and Enniantin B as the most frequent mycotoxins in
438 coffee beverages (Garcia-Moraleja, Font, Manes, & Ferrer, 2015).

439 The chemical composition and antioxidant capacity of coffee husk underline its potential
440 as a food ingredient with promising health-promoting properties. For instance, coffee
441 pulp has been proposed as a potential source of anthocyanins (pigments widely distributed
442 in colored fruits and flowers). Murthy et al. (2012) reported cyanadin-3-rutinoside as the
443 major anthocyanin in coffee pulp. Interest in anthocyanins has emerged due to their

444 potential health benefits as antioxidant, anticarcinogenic, anti-inflammatory and
445 hypoglycemic agents and as insulin sensitivity promoters (Lila, 2004). Coffee husk has
446 also been used in the production of fructooligosaccharides (FOSs), low caloric, non-
447 carcinogenicity fructose oligomers which promote decreasing levels of phospholipids,
448 triglycerides, and cholesterol. This helps gut absorption of calcium and magnesium
449 thereby stimulating probiotic growth in the human colon (Mussatto, Aguilar, Rodrigues,
450 & Teixeira, 2009).

451 Considering the extraction yield, the chemical composition of both fractions, the *in vitro*
452 antioxidant capacity and the effect on HepG2 intracellular ROS production, we propose
453 the fractioning of coffee husk into two novel ingredients. First, an enriched extract with
454 potential implementation as a food preservative, natural colorant or health-promoting
455 food ingredient, and another fraction used as a source of antioxidant dietary fiber.

456 *3.2. Characterization of coffee parchment and its potential applications*

457 As an extended approach to standard analytical characterization, we investigated
458 parchment using vibrational spectroscopy as a non-invasive technique. Vibrational
459 spectroscopy is a reliable tool to characterize structural composition in coffee (Keidel et
460 al., 2010) and a rapid analytical tool for extracts and future food and health applications.
461 In the Raman spectrum (Figure 2A), a strong, broad envelope containing C-H stretching
462 modes was observed, possibly of CH2-functional groups in the highest frequency region
463 (2700-3600 cm-1). Main peaks were at 2937 and 2896 cm-1. At lower frequencies a peak
464 at 2712 cm-1 was identified, which is characteristic of the H-stretching modes of a O-
465 CH2-O group or an CH3 umbrella overtone. Within the broad band envelope, C-H
466 stretching modes rising also aromatic systems have the strong contribution, since they are
467 highly Raman active. Conversely, fewer IR-bands (Figure 2B 2700-3600cm-1 region)
468 were found. At even higher frequencies, namely at 3322 cm-1, we found a weak Raman-

469 active yet strong and broad IR-active peak. This was assigned to alcohol O-H stretching.
470 Only two IR-active peaks were identified at lower frequencies at 2854 and 2924 cm-1,
471 both related to CH2-stretching coordinates (Supplementary Material, Table S2).

472 At 1602 and 1658 cm-1, two C=C stretching modes with strong Raman activity and
473 negligible IR intensity were identified. In fact, this region in the IR spectrum was rather
474 broad. As the broad peak at 1632 cm-1 might contain small contributions of amid-I
475 modes, a low, yet detectable protein concentration in the parchment (*vide infra*) is
476 assumed. The band at 1602 cm-1 was assigned to an aromatic ring def., whereas the peak
477 at higher frequencies contained contributions rising from a C=C double bond, possibly in
478 the vicinity of a benzyl (or phenyl) group. At 1732 cm-1 a strong IR-active and a weak
479 Raman band associated with carbonyl C=O stretching coordinates were detected.

480 The further assignment mainly refers to the analysis of the parchment Raman spectrum.
481 At lower frequencies, namely between 1000 and 1500 cm-1, (in-plane) bending and
482 deformation modes gave rise to the main contribution in this region. These allowed a
483 further structural assignment of endocarp composition. The band at 1461 cm-1 was
484 assigned to the CH3 (or CH2) flowering mode of methyl (methylene) coordinates,
485 whereas the peak at 1378 cm-1 contained further coordinates of the CH2, HCC, HCO and
486 COH bending modes. Unlike double bond stretching modes, the potential energy
487 distribution of these vibrations is less localized and includes relatively large contributions
488 of distinct coordinates. Deformation of the O-H groups in aliphatic and aryl alcohols
489 contribute to the bands at 1333 and 1278 cm-a, respectively. A band containing glycoside
490 symmetric ring breathing was located at 1095 cm-1, whereas the peak at 1038 cm-1
491 mainly contained contributions of C-C and C-O stretching coordinates. Finally, the peak
492 at 899 cm-1 was assigned to HCC and HCO bending modes. The adjacent peak at 926
493 cm-1 probably contained further angle bending modes (CHO, CCH or similar), however

494 an unambiguous assignment was not possible. While the Raman spectrum contained very
495 strong bending modes, only a broad and prominent envelope at 1011 cm-1 with band
496 shoulders at 1048 and 1100 cm-1 was found. Here the highest peak and further low-
497 frequency peaks could be assigned to C-O stretching modes of aryl alcohols, whereas the
498 high frequency shoulders contained C-O stretching contributions of aliphatic alcohols,
499 esters or ethers.

500 In agreement with physicochemical studies on coffee parchment composition, the bands
501 at 1658 and 1602 cm-1 have been assigned to lignin. Conversely, cellulose did not display
502 any bands in this region. The corresponding marker bands were observed at 899, 1095
503 and 1121 cm-1. In the high frequency region (2700-3500 cm-1), the strongest peak at
504 2937 cm-1 corresponds to lignin, whereas the adjacent peaks at 2896 and 2712 cm-1
505 correspond to the C-H stretching modes of cellulose. The O-H stretching peak has
506 contributions from both molecules. The strong C=O stretching mode observed at 1732
507 cm-1 could not be assigned to either of the previously mentioned compounds. It could
508 possibly reflect a third compound group, such as hemicellulose. The observed IR and
509 Raman activity of the C=O group (vide supra), can only be understood if the C=O group
510 in discussion is conjugated, as for example in ferulated xylan. The presence of ashes, coal
511 and dark materials give rise to strong thermal emission in the Raman spectrum. If present,
512 the contribution is low or negligible.

513 Two components could be unambiguously confirmed in coffee parchment, namely lignin
514 and cellulose. Bekalo and Reinhardt (2010) also reported the presence of these
515 compounds in coffee parchment. Strong indications for a third species were presented.
516 This combined spectroscopic approach provides complementary information. However,
517 further studies of different parchment varieties, vibrational spectra of isolated compounds
518 as well as extended chemometric analysis are required. It is interesting to note that the

519 obtained results provide the first vibrational spectroscopy (IR and Raman)
520 characterization of coffee by-products.

521 Parchment showed the lowest extraction yield (2.3 %). This extract (Figure 1b) presented
522 low levels of 5-CQA (6.1 mg/g); however, it had the highest caffeine content (58.2 mg/g)
523 ($p < 0.05$). No bibliographical references have been found regarding caffeine and CGA
524 content in aqueous extracts of coffee parchment. As shown in Table 1, parchment extract
525 had the greatest antioxidant capacity ($p < 0.05$) of all the samples analyzed, followed by
526 CS extracts. Parchment and Robusta CS extract showed the highest TPC and TFC ($p <
527 0.05$). Table 2 includes data on the effect of parchment extract on cell viability and
528 intracellular ROS formation. At concentrations of 0.1 and 1 mg/ml cell viability was not
529 significantly ($p > 0.05$) altered, while 10 and 20 mg/ml resulted cytotoxic. Pre-treatment
530 of cells with parchment extract for 24 hours (1 mg/ml) significantly reduced ($p < 0.05$)
531 ROS levels compared to non-treated cells and cells treated with tBOOH. Parchment
532 extract had a greater antioxidant effect on HepG2 cells compared to husk extract. Results
533 are in line with those corresponding to TAC and TPC. Parchment extract presented the
534 highest values for these parameters among the studied samples. To the best of our
535 knowledge, no studies have been published on the effect of coffee parchment on cell
536 viability and intracellular ROS formation.

537 The insoluble fraction of coffee parchment obtained after extraction presented the highest
538 amount of TDF ($p < 0.05$) (92.6 %) mainly composed of IDF (92.1 %) (Table 3). Protein
539 content in the parchment insoluble fraction was 3.1%, which was the lowest value
540 compared to the rest of the samples. Our results showed that parchment insoluble fraction
541 also displayed the lowest ($p < 0.05$) lipid content (0.3 %). To date, no other data on protein
542 and lipid content of coffee parchment have been published. Furthermore, it also had the
543 lowest antioxidant capacity (4.1 mg CGA/g).

544 Considering the food safety of raw parchment, none of the studied pesticides were
545 detected in this sample. Aflatoxin B1 and enniantin B were not present in parchment
546 (Table 4). However, OTA was detected at 2.7 μg/kg, which is under the limit established
547 by the European Commission (5 μg/kg). Therefore, thermal stabilization of the raw
548 material should be thermally stabilized to avoid microbial contamination and the
549 production of mycotoxins during its storage, to increase the shelf-life of the sample and
550 to ensure its safe use as a food ingredient for human beings.

551 Until now, applications for coffee parchment as a food ingredient remain largely
552 unexplored. Despite the promising chemical composition of aqueous parchment extract
553 and its high *in vitro* antioxidant capacity, we suggest that coffee parchment should be
554 used as it is after a simple thermal stabilization, since extraction yields are rather poor.
555 New applications of coffee parchment in the food industry as a natural source of
556 antioxidant dietary fiber should be considered to revalue this by-product.

557 *3.3. Characterization of coffee silverskin and its potential applications*

558 The extraction yield of water-soluble compounds from CS (Figure 1c) was similar for
559 Arabica and Robusta species. As CS is in direct contact with the coffee seeds, CS extract
560 presented the highest content of 5-CQA (9.4 mg/g and 21.3 mg/g, for Arabica and
561 Robusta species, respectively). The concentration ratio of caffeine/5-CQA was 2.5 in both
562 the Arabica and Robusta CS extract. However, values of both compounds were lower in
563 the Arabica CS extract (Table 1). The obtained caffeine content was higher than that
564 reported by other authors (Bresciani, Calani, Bruni, Brighenti, & Del Rio, 2014;
565 Napolitano, Fogliano, Tafuri, & Ritieni, 2007). In agreement with our results, Mesías et
566 al. (2014) found that 5-CQA and caffeine content were significantly higher in Robusta
567 than in Arabica CS extract.

568 EFSA reported a safety level for daily caffeine consumption of 400 mg for the general
569 population and 200 mg for lactating women. There is currently not enough information
570 available to determine a safe level of caffeine intake for children (European Food Safety
571 Authority (EFSA), 2015). Caffeine also presents beneficial health effects due to its
572 stimulating properties (Nehlig, 2016). Arabica and Robusta CS extracts could be a natural
573 source of sustainable caffeine. So far, published results suggest that caffeine content
574 should not be considered as a safety concern in the application of coffee by-products as
575 food ingredients (Garcia-Serna et al., 2014; Martinez-Saez et al., 2014). However, more
576 conclusive studies are required.

577 Robusta CS extract presented significantly higher amounts of TPC and TFC than Arabica
578 CS extract ($p < 0.05$), and TPC and TFC concentration was significantly higher in these
579 two species than in husk extract ($p < 0.05$) (Table 1). In this case, the TPC value obtained
580 for CS extracts was in the same order of magnitude as the values obtained by Mesías et
581 al. (2014). The antioxidant capacity values of the different CS extracts did not differ
582 significantly ($p > 0.05$). There is usually a greater proportion of antioxidants in Robusta
583 CS than in Arabica CS (Mesías et al., 2014; Napolitano et al., 2007). Other authors have
584 also confirmed the antioxidant character and high phenolic and flavonoid content in this
585 sample (Borrelli et al., 2004; Rodrigues, Palmeira-de-Oliveira, et al., 2015a). Although
586 TAC was significantly greater in parchment extract ($p < 0.05$), parchment extraction yield
587 is low. Therefore, CS could be a more suitable source of bioactive compounds with a
588 higher antioxidant capacity. The application of CS as a natural source of antioxidants has
589 been previously described (del Castillo et al., 2013; Garcia-Serna, Martinez-Saez, Mesias,
590 Morales, & Castillo, 2014; Iriondo-DeHond et al., 2016; Mesías et al., 2014; Rodrigues,
591 Palmeira-de-Oliveira, et al., 2015).

592 Since Arabica coffee represents around 70 % of global production (Bunn, Läderach,
593 Ovalle Rivera, & Kirschke, 2015), this species was used in the *in vitro* studies. Arabica
594 CS extract was not cytotoxic in HepG2 cells at none of the tested concentrations. For
595 doses of 10 and 20 mg/ml of Arabica CS extract, cell viability increased significantly (p
596 < 0.05) (Table 2). The non-cytotoxic character of CS has also been studied in other cell
597 lines (Iriondo-DeHond et al., 2016; Iriondo-DeHond, Haza, Ávalos, del Castillo, &
598 Morales, 2017; Rodrigues, Gaspar, et al., 2015). Arabica CS extract was more effective
599 in the reduction of induced ROS than husk extract, and as effective as parchment extract.
600 This is in agreement with their corresponding antioxidant capacity studied *in vitro.* The
601 protective effect of CS in the reduction of induced ROS formation has been reported for
602 other cell lines (Fernandez-Gomez, Ramos, et al., 2016; Iriondo-DeHond et al., 2016).

603 The TDF content in the remaining CS solid insoluble fraction after the aqueous extraction
604 process was similar for Arabica and Robusta varieties (Table 3). Values obtained for CS
605 dietary fiber are similar to those previously described (Ballesteros et al., 2014; Borrelli et
606 al., 2004; Garcia-Serna et al., 2014). Most of the dietary fiber found in the sample was
607 insoluble, and values of SDF were lower than those recently described (Esquivel &
608 Jiménez, 2012; Napolitano et al., 2007). In agreement with previous results (Ballesteros
609 et al., 2014; Borrelli et al., 2004; Pourfarzad et al., 2013), the highest protein content
610 corresponded to Robusta CS (19 %). Lipid content of the insoluble residues of CS was
611 2.6 % and 3.6 % for Arabica and Robusta, respectively, in the same range as that
612 previously described by Ballesteros et al. (2014) and Borrelli et al. (2004).

613 Food safety of Arabica CS was validated by the absence of pesticides, aflatoxin B1 and
614 enniantin B (Table 4). OTA was present in the insoluble fraction of this sample (2.9
615 µg/kg) in a concentration below the limit established by the European Commission (5
616 µg/kg) and was not detected in CS extract. Other authors have described the presence of

617 OTA in CS (Toschi, Cardenia, Bonaga, Mandrioli, & Rodriguez-estrada, 2014). The
618 analysis of this contaminant is mandatory to ensure the safety of CS extract as a food
619 ingredient. Since CS is the only by-product generated during the roasting process of
620 coffee beans, acrylamide content was also studied in this sample. Levels of 489 µg/kg of
621 this water-soluble compound were found in CS extract. This value is between the values
622 established by the European Commission for roasted coffee (400 µg/kg) and instant
623 coffee (850 µg/kg) (European Commission, 2017). In contrast, CS insoluble fraction
624 presented levels < 50 µg/kg. Mitigation of acrylamide in CS can be achieved following
625 the instructions recommended for roasted and instant coffees by the European
626 Commission.

627 Arabica CS, CS extract and the insoluble fraction recovered from the water extraction
628 process have been implemented to improve the formulation of biscuits. In this study, CS
629 was used as a natural colorant and as a source of fiber (Garcia-Serna et al., 2014). CS has
630 also been used to improve the quality, shelf-life, sensory qualities and appearance of
631 Barbari bread (Pourfarzad et al., 2013). Other authors proposed the use of CS as a source
632 of prebiotics (Borrelli et al., 2004; Jiménez-Zamora, Pastoriza, & Rufián-Henares, 2015;
633 Mussatto et al., 2013). In addition, CS has been used in combination with roasted coffee
634 and cocoa powder to obtain innovative coffee blends that were rich in bioactive
635 compounds, such as CGAs, trigonelline, theobromine, and caffeine (Ribeiro, Leitão,
636 Ramalho, & Lidon, 2014). Other novel antioxidant beverages based on raw CS and CS
637 extract from Arabica and Robusta species have been developed to study their inhibitory
638 effect on fat accumulation using *Caenorhabditis elegans* as an *in vivo* model (Martinez-
639 Saez et al., 2014). According to our results, improved fractioning of CS will yield a
640 superior aqueous extract rich in bioactive compounds with the potential to reduce diabetes

641 and obesity, among other conditions. Similarly, the potential of coffee husk extract to
642 reduce the risk of chronic diseases should also be studied.

643 Considering the extraction yield and chemical characterization of each by-product
644 fraction, we suggest single-fraction separation and development of two new
645 multifunctional ingredients. As proposed for coffee husk, CS is a suitable source for an
646 enriched extract which could be implemented as a food ingredient with antioxidant and
647 antidiabetic properties (del Castillo et al., 2016). Furthermore, the insoluble fraction can
648 be used as a source of antioxidant dietary fiber.

649 *3.4. Food toxicity of novel ingredients*

650 Single oral administration of the three validated coffee by-products (raw parchment and
651 husk and Arabica CS extracts) at a dose of 2000 mg/kg b.w. showed no visible signs of
652 toxicity, abnormal behavior or mortality. Relative organ weights are shown in Table S3
653 (Supplementary Material). No significant differences ($p > 0.05$) were found among
654 groups. Intake of an acute dose of ingredients (2000 mg/kg b.w) did not cause significant
655 changes in histological parameters of vital organs. Livers of the control and treated rats
656 showing normal hepatic lobules, polyhedral hepatocytes with central vesicular nuclei and
657 eosinophilic granular cytoplasm are shown in Figure 3a and 3b, respectively. Similarly,
658 the control and treated kidney sections showed a normal histological picture composed
659 of renal corpuscles, which appear as rounded structures, and glomeruli are surrounded by
660 narrow Bowman's spaces and cortical tubules with a small number of distal convoluted
661 tubules and collecting tubules (Figure 3c and 3d).

662 To the best of our knowledge, very few studies regarding the presence of contaminants
663 in coffee by-products have been previously published. No lethal effects were observed in
664 rats treated with 2000 mg/kg b.w. by oral administration. Results are supported by data

665 on contaminants. As previously discussed, pesticides, aflatoxin B1 and enniantin B were
666 not found in the coffee by-products; OTA was detected in raw parchment and in the
667 insoluble fraction of husk and CS in concentrations lower than 5 µg/kg; acrylamide was
668 only detected in CS and its derivatives in lower or similar concentrations to those found
669 in roasted coffee (400 µg/kg) (European Commission, 2017). Caffeine concentrations can
670 be considered safe and were lower than concentrations reported for coffee beverages
671 (European Food Safety Authority (EFSA), 2015).

672 **4. Conclusions**

673 This study provided data for the validation of CS and coffee husk as two novel safe food
674 ingredients: an enriched extract with multifunctional properties and an insoluble fraction
675 composed of dietary fiber of different nature (soluble and insoluble). The fractioning
676 procedure is a sustainable, low-cost process which could be easily performed by the
677 coffee industry without the production of new waste. We also propose that coffee
678 parchment be used as a single food ingredient composed of antioxidant insoluble dietary
679 fiber after a thermal stabilization process. Thus, coffee by-products can be converted into
680 promising safe health-promoting food ingredients, providing sustainable economic and
681 environmental benefits. Analysis of contaminants is essential to achieve this goal.

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