View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Digital.CSIC

1 # Insights on the health benefits of the bioactive compounds of coffee silverskin
2 extract

3 **Beatriz Fernandez-Gomez[a], Adriana Lezama[a], Miryam Amigo-Benavent[be], Mónica**
4 **Ullate[a], Miguel Herrero[a], María Ángeles Martín[b,d], Maria Dolores Mesa[c*], Maria Dolores**
5 **del Castillo[a*].**

6 [a]Department of Food Analysis and Bioactivity, Institute of Food Science Research (CIAL, CSIC-
7 UAM), 28049 Madrid, Spain.
8 [b]Department of Nutrition and Metabolism, Institute of Food Science, Technology and Nutrition
9 (ICTAN-CSIC), 28040 Madrid, Spain.
10 [c]Institute of Nutrition and Food Technology "José Mataix", Biomedical Research Center
11 (CIBM), University of Granada, 18100 Granada, Spain. Instituto de Investigación Biosanitaria
12 (IBS) Granada, Spain.
13 [d]Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders
14 (CIBERDEM), 28029 Madrid, Spain.
15 [e]Centre for Interventions in Inflammation, Infection and Immunity (4i), University of Limerick,
16 Limerick, Ireland.
17

18 [*]Corresponding authors:
19 Maria Dolores del Castillo, Department of Food Analysis and Bioactivity, Institute of Food
20 Science Research (CIAL, CSIC-UAM), C/ Nicolás Cabrera 9, Campus de la Universidad
21 Autónoma de Madrid, 28049, Madrid, Spain.
22 Phone: +34 910017953 - E-mail address: mdolores.delcastillo@csic.es

23 Maria Dolores Mesa, Institute of Nutrition and Food Technology "José Mataix", Biomedical
24 Research Center (CIBM), University of Granada, Avd del Conocimiento s/n 18100 Granada,
25 Spain
26 E.mail address: mdmesa@ugr.es

27 **Abbreviations:** CGA, chlorogenic acid; CSE, coffee silverskin extract, HA, hippuric acid;
28 GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; STZ,
29 streptozotocin, NA, nicotinamide; TE, trolox equivalent; ROS, reactive oxygen species, TPC,
30 total phenolic compounds.

31 **Abstract**

32 The bioaccessibility of chlorogenic acid (CGA) and caffeine in coffee silverskin extracts (CSE)
33 and the contribution of these substances to the prophylactic effect of CSE on the pathogenesis
34 of diabetes has not been reported. This study aimed to evaluate the bioaccessibility,
35 bioavailability and bioactivity of CGA and caffeine alone and in CSE in the pancreas of rats
36 treated with streptozotocin-nicotinamide (type 2 diabetes model). Metabolism of CGA and
37 caffeine started in the gastrointestinal tract due to changes of pH taking place during digestion.
38 Their metabolites protected pancreatic cells against the risk of diabetes. This is the first *in vivo*
39 study to demonstrate a specific chemo-protective effect of CSE in pancreatic tissue, and this
40 effect may be associated with its antioxidant capacity. Daily administration of CSE, CGA or
41 caffeine 35 d previous to the induction of diabetes significantly reduced ($p < 0.05$) pancreatic
42 oxidative stress and protein damage.

43
44
45
46
47
48
49
50
51
52
53
54
55

56 **Keywords:** coffee silverskin, chlorogenic acid, caffeine, bioaccessibility, metabolism,
57 pancreas oxidative stress, Type 2 diabetes.

58 Chlorogenic acid (PubChem CID: 1794427)

59 Hippuric acid (PubChem CID: 464)

60 Caffeine (PubChem CID: 2519)

61 Paraxanthine (PubChem CID: 4687)

62

63 ## 1. Introduction

64 According to the International Diabetes Federation (IDF), type 2 diabetes mellitus (T2DM) is
65 one of the most frequent diseases in the world, with 387 million cases in 2014 (IDF, Diabetes
66 Atlas Group, 2015). T2DM is a very complex and multifactorial metabolic disease
67 characterized by insulin resistance and β cell failure, leading to high blood glucose levels.
68 Oxidative stress plays an important role in hyperglycaemia-induced pancreas injury as well as
69 in the early events leading to the development of T2DM. Advanced glycation end-products
70 (AGEs) increase reactive oxygen species (ROS) formation and impair antioxidant systems.
71 Furthermore, the formation of some AGEs is induced *per se* under oxidative conditions
72 (Nowotny, Jung, Höhn, Weber, & Grune, 2015). There is also evidence that antiglycative
73 agents in foods and medicine may reduce the risk of diabetes and treat the pathology (Uribarri
74 et al., 2015).

75 Coffee silverskin (CS), the tegument of green coffee beans (outer layer), is the only by-product
76 of the roasting process. Previous studies have proposed the use of coffee silverskin extracts
77 (CSE) as a natural source of bioactive compounds, such as chlorogenic acid (CGA), caffeine,
78 melanoidins and dietary fibre among others, with putative health benefits (del Castillo,
79 Fernandez-Gomez, Martinez-Saez, Iriondo, & Mesa, in press). Indeed, glucoregulatory
80 properties have recently been ascribed to CSE by-products of roasting coffee (del Castillo,
81 Fernandez-Gomez, Ullate, & Mesa, 2014). The antidiabetic effect of CSE has been initially
82 associated with its capacity to inhibit the enzymatic activity of α-glucosidase and lipase taking
83 into account *in vitro* results (del Castillo, Fernandez-Gomez, Ullate, & Mesa,). 2014). CSE is
84 also able to inhibit the formation of AGEs. The anti-AGEs capacity of CSE may be ascribed to
85 CGA and other bioactive compounds composing the extract (Mesías et al., 2014). Fernandez-
86 Gomez et al. (2015) reported a novel antiglycoxidative mechanism of action of CGA.
87 Moreover, CSE may protect pancreatic tissue *in vitro* against oxidative stress induced by the

88 commonly-used diabetogenic agent streptozotocin (STZ) (Fernandez-Gomez et al., in press).
89 The antidiabetic mechanism of action of CSE *in vivo* should be elucidated and the present study
90 provides some insights on that matter.

91 Healthy effects associated with foodlargely depend on the bioaccesibility and bioavailability
92 of their bioactive components in the organism. CSE, like other food matrices, is a complex
93 mixture of bioactive compounds. Nowadays, the bioaccessibility of CGA and caffeine
94 composing CSE, and therefore, their true *in-vivo* potential are unknown. Moreover, the
95 contribution of CGA and caffeine to the prophylactic effect of CSE on the pathogenesis of
96 diabetes has not been reported. This study aimed to evaluate the bioaccessibility, bioavailability
97 and bioactivity of CGA and caffeine alone and in CSE in the pancreas of rats treated with STZ-
98 nicotinamide (NA), using phytochemomics (del Castillo, Martinez-Saez, Amigo-Benavent &
99 Silvan, 2013b). No data on that matter have been previously published. Although CSE and
100 coffee brews present some similarities in chemical composition they are significant different.
101 As a consequence, the effect in health of both matrices may be very different. On the other
102 hand, the valorisation of the coffee by-product into an antidiabetic product is of great interest
103 and it has not been previously reported by others. The present study provides relevant data to
104 support the interest of CSE as a functional product for diabetes and the potential of the coffee
105 sector in bioeconomy.

106 ## 2. Material and Methods

107 ### 2.1. Chemicals

108 Pancreatin (P-1625), α-amilase from human saliva type IX-A (A0521), 2.2′-azino-bis (3-
109 ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), CGA, hippuric acid (HA) ,
110 caffeine and paraxanthine solution, formic acid, *o*-phthaldehyde, 2,2′-Azobis (2-
111 amidinopropane) dihydrochloride (AAPH), glutathione reductase (GR), reduced and oxidized

112 glutathione (GSH and GSSG, respectively), NADH, NADPH, tert-butylhydroperoxide (t-
113 BOOH), 1,4-dithiothreitol (DTT) buffer, STZ and NA were purchased from Sigma Chemical
114 (Sigma-Aldrich, St Louis, MO, USA). The other chemicals and equipment used were: Pepsin
115 (Merck 1.07190) and Amicon® Ultra-0.5 ml centrifugal filter unit fitted with an Ultracel®-10
116 K regenerated cellulose membrane (30 kDa cut-off) (Merck, Darmstadt, Germany), Bradford
117 reagent for the protein assay (Bio-Rad, München, Germany), methanol (MeOH) HPLC-grade
118 (Lab-Scan, Gliwice, Sowinskiego, Poland). Distilled water was deionized using a Milli-Q
119 system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

120 **2.2. Preparation of coffee silverskin extract**

121 Arabica CSE was prepared by aqueous extraction following the procedure patented by del
122 Castillo et al. (2013a). Briefly, 50 ml of boiling water was added to 2.5 g of CS. The mixture
123 was stirred at 4 *g* for 10 min, filtered through no. 4 Whatman paper and freeze-dried. The
124 powdered extracts were stored in a dark and dry place until analysis. The sample (37 mg/ml
125 CSE solution) was filtered through a 0.45 µm pore diameter nylon membrane syringe filter
126 (Análisis Vínicos, Ciudad Real, Spain) and diluted 100-fold with Milli-Q water and 10 µl
127 aliquots analysed in triplicate by UPLC-MS/MS. CSE contained 19.87 ± 2.41 mg caffeine/g
128 dry matter and 6.88 ± 1.77 mg CGA/g dry matter.

129 **2.3. Evaluation of the bioaccessibility of coffee silverskin extract**

130 **2.3.1. *In vitro* oral gastrointestinal digestion**

131 The amount of CSE components potentially available for further uptake was determined
132 following the procedure of Hollebeeck, Borlon, Schneider, Larondelle, and Rogez (2013) with
133 slight modifications. To mimic *in vitro* oral digestion, 1.17 g of CSE was suspended in 9 ml of
134 Milli-Q water and the pH was adjusted to 6.9 with 1M HCl and brought to a volume of 9.98
135 ml. α-Amilase (0.45 ml of 0.562 mg/ml in phosphate buffer) was added to each sample and

136 incubated at 37 ºC for 5 min with constant stirring at 4 *g*. For the gastric digestion step, 10 ml
137 of Milli-Q water was added and the pH of the samples was adjusted to 2.0 with 1 M HCl.
138 Pepsin (110 µl, 5 mg/ml in 0.1 M HCl) was added to each sample and incubated in a final
139 volume of 22.88 ml at 37 ºC for 90 min under anaerobic conditions using an anaerobic chamber
140 model Bactron II (Biogen, Weston, MA, USA) with constant stirring at 4 *g*. After this time, the
141 pH of the samples was adjusted to 7 with 1M $NaHCO_3$ for the intestinal digestion step. One ml
142 of pancreatin solution (287.59 mg/ml in 0.1 M $NaHCO_3$) was added, the final volume brought
143 to 31.24 ml with Milli-Q water, and the mixture was incubated at 37 ºC for 150 min under
144 anaerobic conditions. Digested samples were centrifuged at 1677 *g* at 4 ºC for 40 min.
145 Enzymatic activity was stopped with liquid N_2 and supernatants were freeze-dried and stored
146 under dark, dry conditions at 4 ºC until analysis. The digestion of CSE was also carried out
147 under the same conditions without adding the enzymes to gain insight into the effect of the pH
148 in CGA and caffeine metabolism. Digestion experiments were carried out in triplicate.

149 **2.3.2. Chemical composition of digested and non-digested coffee silverskin extract**

150 Total phenolic content (TPC) was determined using the Folin-Ciocalteu's colorimetric assay
151 (Singleton, Orthofer, & Lamuela-Raventós, 1999) adapted to a microplate reader. Briefly, CSE
152 prepared at 2 mg/ml was used. Ten µl of the sample was combined with 200 µl of Folin reagent
153 (0.017% (v/v)) and 50 µl of $NaHCO_3$ (30 mg/ml). The 96-well plate was incubated in darkness
154 at room temperature for 2 h and was read at 725 nm using a BioTek PowerWave™ XS
155 (Winoski, VT, USA) microplate reader. Calibration curves were constructed using a standard
156 solution of CGA (0.1-0.8 mg/ml). Samples were analyzed in triplicate and results expressed as
157 mg of CGA equivalents per g of CSE (mg CGA /g CSE).
158 The contents of CGA and caffeine in the CSE and their digested products was analyzed by
159 capillary zone electrophoresis (CZE) as described by del) del Castillo, Ames, and Gordon

160 (2002). Previously, the digested CSE (10 mg of extract/ml) was filtered through 0.2 µm nylon
161 filters (Symta, Madrid, Spain). Determinations were carried out in an Agilent G1600 A (Santa
162 Clara, CA, USA) capillary electrophoresis instrument equipped with ChemStation software
163 and a diode array detector (DAD). CZE was performed in an uncoated fused 48.5 cm long silica
164 capillary (40 cm to the detector) with an internal diameter of 50 µm and a x3 bubble cell. The
165 other analysis conditions were as follows: 50 mM sodium borate buffer (pH 9.5), 20 kV
166 voltage, 25 ºC temperature and the injection was at 50 mbar for 5 s. Electropherograms (e-
167 grams) were monitored at 200 and 280 nm for CGA and caffeine, respectively; and spectra
168 collected from 190 to 600 nm. The capillary was conditioned after running each sample by
169 flushing with 0.1 M NaOH and buffer for 3 min. CGA (0.15-9 mM) and caffeine (0.15-10 mM)
170 calibration curves were used as standards for identification and quantification. Samples were
171 analyzed in triplicate and results were expressed as mg of CGA or caffeine/g CSE.

172 **2.3.3. Antioxidant capacity of digested and non-digested coffee silverskin extract**

173 Antioxidant capacity was determined by the ABTS and $ORAC_{FL}$ assays, respectively. The
174 ABTS assay was carried out according to Oki, Nagai, and Yoshinaga (2006). $ABTS^{•+}$ was
175 produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration in
176 10 ml of water). The mixture was incubated in the dark at room temperature for 16 h before
177 use. The aqueous $ABTS^{•+}$ solution was diluted 1:75 (v/v) with 5 mM phosphate buffer (pH 7.4)
178 obtaining an absorbance value of 0.7 ± 0.02 at 734 nm. Thirty microlitres of sample (0.2 mg of
179 non-digested CSE /ml or 0.2 mg of digested CSE/ml) or standard and 270 µl of $ABTS^{•+}$
180 working solution were placed in each well. Absorbance readings were recorded in a microplate
181 reader at 734 nm every minute. A standard calibration curve was constructed using Trolox
182 (0.01-0.25 mM), and results were expressed as µmoles of Trolox equivalents (TE)/g of CSE.
183 Another calibration curve using CGA, the major phenolic compound in coffee, was constructed

184 (0.05-0.25 mM), and results were expressed as µmoles of CGA equivalents (CGA)/g of CSE.
185 Samples were analyzed in triplicate.

186 An aliquot of non-digested CSE (10 mg/ml) was subjected to fractionation using an Amicon®
187 Ultra 0.5 ml centrifugal filter unit fitted with an Ultracel®-10K regenerated cellulose membrane
188 (10 kDa cut-off) (Merck Millipore, Darmstadt, Germany). The antioxidant capacity of low (<
189 10 kDa) and high (≥ 10 kDa) molecular weight fractions was also analysed using the ABTS$^{\bullet+}$
190 assay.
191 The ORAC$_{FL}$ assay was performed following the procedure described by Huang, Ou,
192 Hampsch-Woodill, Flanagan, and Prior (2002). Briefly, 25 µl of sample of the appropriate
193 dilution or standard were added to a 96-well microplate followed by the addition of 150 µl of
194 fluorescein work solution (8.5 x 10^{-5} mM) prepared in 75 mM phosphate buffer (pH 7.4). The
195 Biotek Synergy™ HT microplate reader (Biotek Instruments, Highland Park, Winooski, VT,
196 USA) was programmed to incubate the plate at 37 ºC and add 30 µl of AAPH solution (153
197 mM in phosphate buffer) as a peroxyl radical generator. Fluorescence was read with excitation
198 at 485 nm and emission at 528 nm every two minutes for 90 min. A blank consisting of
199 fluorescein, AAPH and phosphate buffer was also included. Calibration curves of Trolox (6.25-
200 50 µM) and CGA (3.12-25 µM) were constructed. Standard calibration curves were composed
201 by plotting the net area under the curve (AUC) as a function of Trolox or CGA concentration.
202 ORAC$_{FL}$ values were expressed as µmoles TE/g of CSE and as µmoles CGA/g of CSE.

203 **2.4. Animals and the experimental design (ARRIVE guidelines)**

204 The experimental protocols were approved by the Ethical Committee for the Use of Laboratory
205 Animals of the UGR, Universidad de Granada, Campus de la Cartuja, GR, Spain (CEEA: 2010-
206 287).

207 CSE doses administrated to the animals *in vivo* provided 0.150 and 0.434 mg/d of CGA and
208 caffeine, respectively. CGA and caffeine dose selection was based on moderate coffee
209 consumption (3 cups a day) in adults (del Castillo, Ames & Gordon, 2002; Heckman, Weil &
210 Gonzalez de Mejia, 2010), and the CSE dose was limited by its caffeine content (max. 300
211 mg/day).

212 **2.4.1. Evaluation of the bioavailability of coffee silverskin extract**

213 Twelve 6-week-old male Wistar rats (ENVIGO, Alconbury, United Kingdom) were housed
214 singly in metabolic cages with free access to standard food (2014S Teklad, ENVIGO,
215 Alconbury, United Kingdom) and water *ad libidum*. Food and water intake were measured by
216 subtracting the remaining amount of food and water in the containers from the total amount
217 given the day before, during the bioavailability study. Animals were divided into four groups
218 (n = 4): rats treated with CSE, rats treated with CGA, rats treated with caffeine and untreated
219 rats (control). At 8:00 in the morning, the CSE group received one single dose of CSE
220 (containing 2.2 mg caffeine/kg body weight; 0.8 mg CGA/kg body weight), the CGA group
221 received pure CGA (1.5 mg CGA/kg body weight) and the caffeine group received pure
222 caffeine (5 mg/kg body weight). Urine samples were then serially collected from treated rats
223 every hour for 6 hours, then every 2 h up to 10 h and finally after 24 h. Urine samples were
224 collected from untreated rats every 24 hours as a control. Samples were stored at -80 °C until
225 analysis. After 3 days of clearance, the bioavailability experiments were repeated with the same
226 animals.

227 Urinary creatinine was measured with the creatinine quantitative test kit (SPINREACT,
228 Gerona, Spain) based on the Jaffe reaction, as previously described by Murray (1984).

229 CGA, caffeine and related compounds, HA and paraxanthine were determined by UPLC-
230 MS/MS. Urine samples were defrosted, centrifuged at 10481 *g* for 10 min at 4 °C and

231 supernatants were filtered using a 0.45 μm pore-size nylon membrane syringe filter (Análisis
232 Vínicos, Ciudad Real, Spain). Aliquots (10 μl) were analysed in triplicate using an Accela
233 liquid chromatograph (Thermo Scientific, San Jose, CA, USA) equipped with a DAD and an
234 autosampler. The chromatograph was coupled to a TSQ TSQ Quantum (Thermo Scientific)
235 triple quadrupole analyzer via an electrospray ionization (ESI) interface. Xcalibur software
236 (Thermo Scientific) was used for data storage and evaluation. Analytical conditions consisted
237 of a ZORBAX SB-C18 (50 mm × 2.1 mm and 1.8 μm of particle diameter) column (Thermo
238 Scientific) using 1% (v/v) formic acid in methanol and 1% (v/v) formic acid in Milli-Q water
239 as A and B mobile phases, respectively. Elution was carried out according to the following
240 gradient: 0 min, 95% B; 0.35 min, 95% B; 7 min, 80% B; 9.5 min, 5% B; 10 min; 95% B; 15
241 min, 95% B. Optimum flow rate was 0.3 ml/min, whereas the injection volume was 10 μl. The
242 DAD recorded the spectra from 200 to 450 nm. Column and autosampler compartments were
243 kept at 30 °C and 4 °C, respectively. The mass spectrometer was operated in the positive ESI
244 mode to quantify caffeine and paraxanthine and in the negative ESI mode to quantify CGA and
245 HA. Spray voltage and capillary temperature were set at 3500 V and 250 °C, respectively.
246 Nitrogen was used as a sheath and auxiliary gas at pressures of 40 and 20 arbitrary units,
247 respectively. Ion sweep gas pressure was 2 units and collision gas (Ar) pressure was 190 mPa.
248 Scan width and scan time were fixed at 0.020 (m/z) and 0.100 s, respectively, and the system
249 was operated in selected reaction monitoring (SRM). SRM parameters were optimized by
250 direct infusion of standards. Two transition ions were monitored for identification but only the
251 most intense one for each precursor ion was used for quantification. Parent ($[M\text{-}H]^-$) and
252 product ions for CGA and HA were m/z 353.2 → 191.1 and m/z 178.3 → 134.3, respectively,
253 whereas parent ($[M\text{-}H]^+$) and product ions for caffeine and paraxanthine were m/z 195.1 →
254 138.2 and m/z 181.1 → 124.2, respectively.

255 **2.4.2. Evaluation of the bioactivity of coffee silverskin extract in the pancreas of**
256 **streptozotocin-nicotinamide diabetic rats**

257 Thirty-two 6-week-old male Wistar rats (ENVIGO, Alconbury, United Kingdom) were divided
258 into four groups (n = 8) paired by weight (average weight per group was 194 ± 2 g). Rats were
259 maintained at 23 ± 1 ˚C and 55 ± 5 % relative humidity on a 12:12-hour light-dark cycle with
260 free access to standard food (2014S Teklad, ENVIGO, Alconbury, United Kingdom) and water
261 *ad libidum*. Food and water intake were measured by subtracting the remaining amount of food
262 and water in the containers from the total amount given the day before, during the experimental
263 time. The rats in groups 1, 2 and 3 were supplemented by gastric gavage with CSE (2.2 mg
264 caffeine/kg body weight, 0.8 mg CGA/ kg body weight), pure CGA (1.5 mg CGA/kg body
265 weight) and pure caffeine (5 mg caffeine /kg body weight) dissolved in 1 ml of sterile water,
266 respectively, every day for a total of 42 days. The fourth group (the STZ group) was treated
267 similarly with sterile water. At day 35, all rats were injected with 200 mg/kg body weight of
268 NA dissolved in saline buffer, and 15 min later T2DM was induced by the intraperitoneal
269 injection of 60 mg/kg body weight of STZ dissolved in cold 0.1 M citrate buffer (pH 4.5)
270 immediately before use, according to Masiello et al. (1998). The order in which the animals
271 were injected was randomized among the groups. Blood samples were obtained from the tail
272 vein and glucose levels (mg/dl) were determined after T2DM induction every day for six days
273 using a glucometer (FreeStyle Lite®, Abbott Laboratories). Rats were considered diabetic when
274 blood glucose levels were above 200 mg/dl. An additional healthy control group (n = 8) was
275 also included in the experiment.

276 At day 42, overnight-fasting blood glucose was measured using a glucometer. The fasting rats
277 were then anaesthetised with Ketamine-Xylazine (1 ml/kg body weight and 0.5 ml/kg body
278 weight, respectively) and sacrificed. The pancreas was removed promptly, weighed, divided
279 into three parts and stored at −80 °C until required.

280 Glutathione peroxidase (GPx) and GR activity were determined in pancreas homogenates as
281 described by Rodríguez-Ramiro, Martín, Ramos, Bravo, and Goya (2011). Thus, pancreatic
282 tissues were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM DTT buffer pH
283 7.4 and centrifuged at 3000 *g* for 15 min. Determination of GPx activity was based on the
284 oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of
285 NADPH catalysed by GR which reduced GSSG. GR activity was determined based on the
286 decrease in absorbance due to the oxidation of NADPH used in the reduction of GSSG. Total
287 pancreatic protein content was measured by the Bradford method (Bradford, 1976).

288 GSH concentration was evaluated using the previously described fluorometric assay (
289 Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011). This method takes advantage of
290 the reaction of GSH with *o*-phthaldehyde at pH 8.0. Pancreatic tissues were homogenized (1:20
291 w/v) in 50 mM phosphate buffer pH 7.0, and proteins were precipitated with 5% trichloroacetic
292 acid and then centrifuged for 30 min at 10.000 *g*. Fluorescence was measured at an emission
293 wavelength of 460 nm and an excitation wavelength of 340 nm. Results were interpolated in a
294 GSH standard curve (5-1000 ng) and expressed as nmol/mg protein.

295 Pancreatic protein oxidation was measured as carbonyl groups content according to Granado-
296 Serrano, Martín, Bravo, Goya, and Ramos (2009). Pancreatic tissues were homogenized (1:5
297 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM DTT buffer pH 7.4 and centrifuged at 3000 *g*
298 for 15 min. Absorbance was measured at 360 nm, and carbonyl content was expressed as
299 nmol/mg protein using an extinction coefficient of 22000 nM/cm. Total pancreatic protein
300 content was measured by the Bradford reagent.

301 **2.5. Data analysis**

302 SPSS program version 22.0 was used for statistical analyses. Comparisons of excretion
303 pharmacokinetic parameters between treatments were done by Student's T-test. Kolmogorov-

304 Smirnov test and Q-Q graphics were used to test normality for the distribution of values by
305 group of treatment. The normality assumption was met for all data by group of treatment. Prior
306 to statistical analysis, all data were tested for homogeneity of variances using the Levene's test
307 in order to determine the appropriate statistical technique to be used for comparing means
308 between groups of treatmentsTo compare TPC, CGA and caffeine content of non-digested
309 CSE, digested CSE and CSE digested control (absence of enzymes) multiple comparisons were
310 carried out using one-way ANOVA followed by Bonferroni test when variances were
311 homogeneous or by Games-Howell test when variances were not. Antioxidant capacity
312 between CSE non-digested and CSE digested were analyzed with a T-test for dependent
313 samples, using the adjustment for heterogeneous variances when necessary. Maximum
314 concentration (C_{max}), AUC and the time required to reach maximum concentration (T_{max}) of
315 metabolites in urine was evaluated using Microsoft Excel functions (Usansky, Desai, & Tang-
316 Liu, 2010). To compare pharmacokinetic parameters of hippuric acid a multiple comparisons,
317 one-way ANOVA technique with the application of the Snedecor's F-test was carried out
318 followed by a Sidak posthoc test when variances were homogeneous (AUC and T_{max}) or by the
319 T3 Dunnet posthoc test when variances were not homogeneous (C_{max}). To compare
320 pharmacokinetic parameters of caffeine and paraxanthine, a T-test for independent samples
321 were performed, using the adjustment for heterogeneity when necessary. Carbonyl content,
322 GSH, GR and GPx activity comparisons between treatments were analyzed by one-way
323 ANOVA followed by Bonferroni test when variances were homogeneous or by T3-Dunnet
324 posthoc test when variances were not. The level of significance was $p < 0.05$ except in the case
325 of carbonyl content ($p < 0.1$).

326 **3. Results**

327 **3.1. *In vitro* bioaccessibility of the bioactive compounds of coffee silverskin extract**

328 TPC and CGA and caffeine levels of 46.65 mg/g, 13.33 mg/g and 44.64 mg/g were detected in
329 CSE, respectively. Overall antioxidant capacity values of 397 and 358 µmol CGA/g
330 (corresponding to 427 and 816 of µmol TE/g) were obtained for scavenging and hydrogen
331 donating capacities in CSE, respectively (Table 1). The antioxidant capacity of low molecular
332 weight compounds (<10 kDa) was 220 µmol CGA/g, while the antioxidant capacity of the
333 fraction containing high molecular weight compounds (≥10 kDa) was 110 µmol CGA/g.
334 *In vitro* digestion of CSE decreased concentrations of caffeine (25%), TPC (40%) and CGA
335 (82%). The overall antioxidant capacity of CSE as measured by ABTS and ORAC decreased
336 by 15% and 50%, respectively (Table 1).
337 To evaluate the effect of changes in pH during digestion on the degradation of bioactive
338 compounds, a digestion in the absence of digestive enzymes was performed. Changes in pH
339 decreased TPC and CGA content by 38% and 83%, respectively, while caffeine content only
340 decreased by 15%.
341 CSE can provide bioaccessible amounts of bioactive compounds such as TPC (79.26 µmol/g),
342 CGA (6.86 µmol/g) and caffeine (172.37 µmol/g). Digests presented antioxidant capacities of
343 337 µmol/g and 179 µmol/g by ABTS and ORAC assays, respectively.

344 **3.2. Metabolism of the bioactive compounds of coffee silverskin extract**

345 Figure 1 shows the kinetics of the urinary excretion of CGA, caffeine and their metabolites.
346 Urinary pharmacokinetic parameters of these compounds after CSE, caffeine and CGA
347 consumption are presented in Table 2. Intact CGA was not found in the urine of rats fed with
348 CSE (containing 0.150 mg of CGA/day), CGA (0.293 mg of CGA/day) or caffeine. The
349 baseline value of HA in urine excretion was set at 431.7 µmol/mmol creatinine and was
350 obtained by measuring HA in the 24 h urine of the animals before administering the products.
351 HA excretion was the greatest after the ingestion of CGA, reaching a peak in the 0-2 h interval,
352 and AUC was significantly higher than that found after the intake of CSE and caffeine (p <

353 0.001 and $p = 0.017$, respectively) (Table 2). The maximum concentration of HA in urine
354 (1346.9 mmol/μmol creatinine) was found 1.6 h after CGA consumption, which was higher
355 than the maximum HA concentrations found in urine after CSE intake ($p < 0.008$) (Figure 1A
356 and Table 2).

357 Excretion of caffeine and its metabolite paraxanthine were not detected in the urine of CGA
358 treated rats. Non-metabolized caffeine in urine excretion after the consumption of pure
359 caffeine was higher than that found after treatment with CSE ($p < 0.001$ for AUC and $p = 0.001$
360 for C_{max}) (Figure 1B and Table 2). Urinary excretion of caffeine was almost completed before
361 12 h in rats treated with caffeine or with the CSE (Figure 1B).

362 Urinary paraxanthine excretion after consumption of pure caffeine tended to be higher than
363 that found after the intake of CSE ($p = 0.056$ for AUC and $p = 0.050$ for C_{max}) (Figure 1C and
364 Table 2).In this case, paraxanthine needed 24 h to be completely excreted in urine after the
365 intake of both CSE and caffeine (Figure 1C).

366 Both studied compounds were metabolized. Free CGA was not detected in urine and caffeine
367 was metabolized to paraxanthine.

368 **3.3. Bioactivity of coffee silverskin extract in the pancreas of streptozotocin-**
369 **nicotinamide diabetic rats**

370 The effect of the CSE, CGA and caffeine treatments on oxidative stress biomarkers in the
371 pancreas of diabetic rats is shown in Figure 2. Rats were considered diabetic when blood
372 glucose levels were above 200 mg/dl. The STZ-NA treatment caused significant oxidation (p
373 < 0.1) of pancreatic proteins by increasing their carbonyl groups (Figure 2A). On the contrary,
374 animals pre-treated with CGA or caffeine for 35 d significantly prevented ($p < 0.1$) oxidative
375 protein damage induced by STZ. Protein carbonyl content decreased by 24% and 22% in the
376 pancreas of diabetic rats treated with CGA and caffeine, respectively. However, CSE did not

377 reduce the rate of protein oxidation induced by the toxic agent. GSH content in the pancreas of
378 T2DM rats decreased significantly ($p < 0.05$) (Figure 2B), and pre-treatment with CSE and
379 CGA significantly reduced ($p < 0.05$) GSH depletion in the pancreas of diabetic rats. Untreated
380 rats and those treated with CSE and CGA showed similar pancreatic GSH values ($p > 0.05$).
381 GPX and GR values of all animals were of the same order of magnitude ($p > 0.05$) (Figure 2C).

382 The physiological concentrations of the bioactive compounds forming CSE were able to protect
383 pancreatic cells against oxidative stress produced by the diabetogenic agent STZ.

384 **4. Discussion**

385 This is the first study assessing the role of the gastrointestinal digestion on the bioaccessibility
386 of CSE bioactive compounds and its remnant overall antioxidant capacity.
387 TPC values found in CSE are in agreement with those described by other authors (Martinez-
388 Saez et al., 2014; Mesías et al., 2014). Slightly higher CGA and caffeine concentrations were
389 found in CSE than in CS raw material (4.31 mg CGA/g and 10 mg caffeine/g) using a similar
390 analytical method (Bresciani, Calani, Bruni, Brighenti, & Del Rio, 2014). Results suggest that
391 aqueous extraction increases the bioaccessibility of the bioactive compounds present in the
392 plant matrix. Values of overall antioxidant capacity also agree with those reported by Mesías
393 et al. (2014). The highlighted chemical composition of CSE suggests that it could be a good
394 source of bioactive compounds with putative healthy benefits (del Castillo, Fernandez-Gomez,
395 Martinez-Saez, Iriondo, & Mesa, in press).
396 Our results indicate that *in vitro* digestion affected the composition of CSE reducing the
397 bioaccessibility of TPC, CGA and caffeine. However, digests presented antioxidant capacity
398 suggesting that antioxidants remained bioaccessible after the digestion process. The release of
399 compounds from the plant matrix depends on the chemical form and the properties of nutrients
400 and phytochemicals (Holst & Williamson, 2008). TPC and CGA contents were significantly

401 decreased ($p < 0.05$) by the digestion processes (data not shown). Since this decrease was
402 observed in the absence of digestive enzymes, it may be associated with changes in pH taking
403 place during *in vitro* digestion. Several studies have shown that the bioaccessibility of TPC in
404 different food matrixes was lower than that found for isolated polyphenols. Podio et al. (2015)
405 observed a 5-fold lower TPC content in digested coffees than in native instant coffees. Campos-
406 Vega et al. (2015) reported a considerable reduction of TPC (91%) in spent coffee grounds.
407 Akillioglu and Karakaya (2010) showed that the bioaccessibility of TPC ranged from 19% to
408 39% in bean varieties. Phenolic compounds are less bioaccessible partly due to the presence of
409 dietary fiber in the plant matrix (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011).
410 CSE contains high amounts of dietary fiber (362 mg/g) which affect the release of TPC in the
411 digestion process. Hydroxycinnamic acid derivatives constitute the main phenolic component
412 of CS (Bresciani, Calani, Bruni, Brighenti, & Del Rio, 2014).Vallejo, Gil-Izauierdo, Pérez
413 Vicente, and García-Viguera (2004) observed an 87% decrease in CGA after intestinal
414 digestion. Previous studies have suggested that a pH value of 7.5 and bile salts could contribute
415 to lower CGA. Bermúdez-Soto, Tomás-Barberán, and García-Conesa (2007) reported a minor
416 decrease in CGA (5%) in chokeberry extract due to the pH of intestinal digestion (pH 7.5).
417 However, a bioaccessibility study of CGAs in spent coffee grounds showed a total recovery of
418 this compound after digestion (Monente et al., 2015). These differences in CGA release suggest
419 that bioaccessibility is also affected by the plant matrix.
420 In agreement with the data on TPC and CGAs, overall antioxidant capacity also decreased after
421 *in vitro* gastrointestinal digestion. The main antioxidant compounds reported in CSE are CGAs,
422 melanoidins and antioxidant fiber (Fernandez-Gomez, Martinez-Saez, Iriondo, & Mesa, in
423 press). According to our data, low molecular weight compounds (CGAs, other phenols and
424 caffeine among others) seem to make a greater contribution to the *in vitro* overall antioxidant
425 capacity of CSE than the high molecular weight fraction (melanoidins, proteins and antioxidant

426 fiber). Rice-Evans, Miller and Paganga (1996) found that CGA antioxidant activity is related
427 to the CH=CH-COOH group, which ensures greater H-donating ability and radical
428 stabilization. Caffeine effectively reacts with the hydroxyl radical (OH·) and caffeine-derived
429 oxygen-centered radicals are formed in the reaction between caffeine and OH (Shi, Dalal, &
430 Jain, 1991). In this sense, Pellegrini et al. (2003) found a decrease of ~25–30% in the
431 antioxidant capacity of espresso coffee when the caffeine was removed.
432 The greatest part of the CGA ingested by rats is hydrolyzed to caffeic acid and quinic acid, and
433 further metabolized by gut microbiota into various aromatic acid metabolites including *m*-
434 coumaric acid and derivatives of phenylpropionic and benzoic acids (Figure 3) (Gonthier,
435 Verny, Besson, Rémésy, & Scalbert, 2003). Previous studies found that HA, a benzoic acid,
436 was the major CGA-derived metabolite observed in urine and plasma after the ingestion of
437 pure CGA or CGA from a food matrix (Gonthier, Verny, Besson, Rémésy, & Scalbert, 2003;
438 Mulder, Rietveld, & van Amelsvoort, 2005). We found amounts of HA in urine of 1346.9
439 mmol/μmol creatinine after the intake of a single dose of 0.825 μmol CGA. Urine HA
440 concentration after the intake of CSE containing 0.424 μmol CGA (447.9 mmol/μmol
441 creatinine) was of the same order of magnitude as basal values (431.7 mmol/μmol creatinine).
442 These results are in agreement with the low bioaccessibility observed for the CGA present in
443 CSE. The metabolic fate of CGAs ingested as a pure compound or present in coffee has been
444 previously investigated in rats (Choudhury, Srai, Debnam, & Rice-Evans, 1999; Gonthier,
445 Verny, Besson, Rémésy, & Scalbert, 2003) and humans (Monteiro, Farah, Perrone, Trugo, &
446 Donangelo, 2007; Stalmach, Williamson, & Crozier, 2014). Farah, Monteiro, Donangelo, and
447 Lafay (2008) reported high bioavailability of CGAs present in a green coffee extract in humans.
448 In this study, we did not detect intact CGA in urine after oral dosing of CGA and CSE. Results
449 suggest that CGA was absorbed and metabolized into different compounds to those tested in
450 the present study. In accordance with our findings, several authors failed to detect CGA in the

451 plasma or urine of rats and humans fed pure CGA or CGA-containing foods (Booth, Emerson,
452 Jones, & Deeds, 1957; Choudhury, Srai, Debnam, & Rice Evans, 1999; Azuma et al., 2000;
453 Nardini, Cirillo, Natella, & Scaccini, 2002; Stalmach, Williamson, & Crozier, 2014).
454 CSE is also a good source of caffeine (1,3,7-trimethylxanthine). Methylxanthines are
455 extensively absorbed in the gastrointestinal tract and metabolized in the liver to yield
456 methylxanthine derivatives and methyluric acids as the main metabolites, which are finally
457 excreted in urine (Figure 3) (Martínez-López et al., 2014). Paraxanthine (1,7 dimethylxanthine)
458 is the main metabolite of caffeine biotransformation found in plasma and urine after caffeine
459 intake (Arnaud, 2011). The pharmacokinetics of caffeine and paraxanthine excretion were
460 evaluated after the consumption of 5.026 µmol pure caffeine and CSE containing 2.211 µmol
461 caffeine. Caffeine was present in the urine of both groups of rats, which is in agreement with
462 other studies that described incomplete biotransformation in humans (Bonati et al., 1982;
463 Rodopoulos, Wisén, & Norman, 1995). According to CSE composition, the lower consumption
464 of caffeine was in line with the lower excretion observed for this compound and its metabolite
465 paraxanthine. These results are in agreement with previous findings of dose-dependent
466 metabolism and the excretion of caffeine in humans (Martínez-López et al., 2014). Therefore,
467 our data showed that the caffeine present in CSE is bioavailable, partially metabolized, and
468 rapidly excreted.
469 The *in vivo* effect of CGA, caffeine and CSE on the prevention of oxidative damage in the
470 pancreas of STZ-NA-induced T2DM rats was also evaluated. The cytotoxic action of STZ is
471 associated with the generation of ROS and consequent β-cell destruction and suppression of
472 insulin secretion (Szkudelski, 2001). Antioxidants are able to prevent pancreatic islets damage
473 induced by STZ (Fernandez-Alvarez et al., 2004). Consequently, natural antioxidants may be
474 considered promising candidates for the prevention or co-treatment of diabetes. In the present
475 study, the administration of STZ to the animals produced a decrease in GSH and an increase in

476 GR activity ($p = 0.173$), while GPx activity remained unaltered in pancreas antioxidant defense.
477 This indicates that the depletion of GSH may induce GR activity but this induction is not
478 enough to regenerate the basal GSH concentration. Protein oxidation was significant ($p < 0.1$)
479 in the pancreas of STZ induced T2DM rats. Interestingly, the daily administration of CSE,
480 CGA or caffeine 35 d previous to the induction of diabetes significantly prevented ($p < 0.05$)
481 pancreatic oxidative stress. Treatments with CGA and caffeine significantly inhibited cellular
482 protein damage. *In vitro* studies have shown that CGA (Ahn et al., 2014; Deng et al., 2013;
483 Nam et al., 2015) and caffeine (Chen, Yu, Shen, Xia, & Xu, 2015) protect pancreatic β-cells
484 from the oxidative stress damage caused by free radicals. Furthermore, *in vivo* studies have
485 demonstrated that CGA (Karthikesan, Pari, & Menon, 2010) and caffeine (Abunasef, Amin, &
486 Abdel-hamid, 2014; Kagami, Morita, Onda, Hirano, & Oka, 2008) could also prevent STZ-
487 induced oxidative stress and protect β-cells *in vivo*. The present study is the first to demonstrate
488 a specific chemo-protective effect of CSE on pancreas tissue, possibly associated to its
489 antioxidant capacity.

490 In conclusion, the present study provides, for the first time, information on the bioaccessibility,
491 metabolism and *in vivo* bioactivity of bioactive compounds present in CSE. The
492 bioaccessibility of CGA and caffeine was affected by changes in pH during digestion. CGA
493 (0.91 µmol) and caffeine (5.53 µmol) were metabolized and protected pancreatic cells against
494 the oxidative stress induced by the diabetogenic agent.
495
496
497

498 **Acknowledgments**

499 This work was supported by the grants SUSCOFFEE (AGL2014-57239-R) and SAMID
500 (RD12/0026). B. Fernandez-Gomez thanks PhD. program of MINECO for supporting her
501 research career (BES-2011-046827). The authors thank Llenalia García Fernández for
502 statistical analysis.

503 **Conflict of interest**

504 The authors declare that there are no conflicts of interest.

505 **References**

506 Abunasef, S. K., Amin, H. A., & Abdel-hamid, G. A. (2014). A histological and
507 immunohistochemical study of beta cells in streptozotocin diabetic rats treated with
508 caffeine, *52*, 42–50.

509 Ahn, H. R., Lee, H. J., Kim, K. A., Kim, C. Y., Nho, C. W., Jang, H., & Jung, S. H. (2014).
510 Hydroxycinnamic acids in *Crepidiastrum denticulatum* protect oxidative stress-induced
511 retinal damage. *Journal of Agricultural and Food Chemistry*, *62*, 1310–23.
512 http://doi.org/10.1021/jf4046232

513 Akillioglu, H. G., & Karakaya, S. (2010). Changes in total phenols, total flavonoids, and
514 antioxidant activities of common beans and pinto beans after soaking, cooking, and in
515 vitro digestion process. *Food Science and Biotechnology*, *19*, 633-639.
516 http://doi.org/10.1007/s10068-010-0089-8

517 Arnaud, M. J. (2011). Pharmacokinetics and metabolism of natural methylxanthines in animal
518 and man. *Handbook of Experimental Pharmacology*, *200*, 33–91.
519 http://doi.org/10.1007/978-3-642-13443-2_3

520 Azuma, K., Ippoushi, K., Nakayama, M., Ito, H., Higashio, H., & Terao, J. (2000). Absorption
521 of chlorogenic acid and caffeic acid in rats after oral administration. *Journal of*
522 *Agricultural and Food Chemistry*, *48*, 5496-5500. http://doi.org/10.1021/jf000483q

523 Bermúdez-Soto, M., Tomás-Barberán, F., & García-Conesa, M. (2007). Stability of
524 polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and
525 pancreatic digestion. *Food Chemistry*, *102*, 865–874.
526 http://doi.org/10.1016/j.foodchem.2006.06.025

527 Bonati, M., Latini, R., Galletti, F., Young, J. F., Tognoni, G., & Garattini, S. (1982). Caffeine
528 disposition after oral doses. *Clinical Pharmacology and Therapeutics*, *32*, 98–106.

529 Booth, A. N., Emerson, O. H., Jones, F. T., & Deeds, F. (1957). Urinary metabolites of caffeic
530 acid and chlorogenic acids. *The Journal of Biological Chemistry*, *229*, 51-59.

531 Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities
532 of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–
533 54.

534 Bresciani, L., Calani, L., Bruni, R., Brighenti, F., & Del Rio, D. (2014). Phenolic composition,
535 caffeine content and antioxidant capacity of coffee silverskin. *Food Research*
536 *International*, *61*, 196–201. http://doi.org/10.1016/j.foodres.2013.10.047

537 Campos-Vega, R., Vázquez-Sánchez, K., López-Barrera, D., Loarca-Piña, G., Mendoza-Díaz,
538 S., & Oomah, B.D. (2015). Simulated gastrointestinal digestion and in vitro colonic
539 fermentation of spent coffee (*Coffea arabica* L.): Bioaccessibility and intestinal
540 permeability. *Food Research International*, *77*, 156-161. http://doi.org/156-161.
541 10.1016/j.foodres.2015.07.024

542 Chen, L., Yu, M., Shen, T., Xia, J., & Xu, B. L. (2015). Impact of caffeine on β cell proliferation
543 and apoptosis under the influence of palmitic acid. *Genetics and Molecular Research*, *14*,
544 5724–5730. http://doi.org/10.4238/2015.May.29.4

545 Choudhury, R., Srai, S. K., Debnam, E., & Rice-Evans, C. A. (1999). Urinary excretion of
546 hydroxycinnamates and flavonoids after oral and intravenous administration. *Free*
547 *Radical Biology and Medicine*, *27*, 278–286. http://doi.org/10.1016/S0891-

548 5849(99)00054-4

549 del Castillo, M. D., Ames, J. M., & Gordon, M. H. (2002). Effect of roasting on the antioxidant
550 activity of coffee brews. *Journal of Agricultural and Food Chemistry*, *50*, 3698–3703.

551 del Castillo, M. D., Ibañez, E., Amigo-Benavent, M., Herrero, M., Plaza, M., & Ullate, M.
552 (2013a). Application of products of coffee silverskin in anti-ageing cosmetics and
553 functional food. WO 2013/004873.

554 del Castillo, M. D., Martinez-Saez, N., Amigo-Benavent, M., & Silvan, J. M. (2013b).
555 Phytochemomics and other omics for permitting health claims made on foods. *Food*
556 *Research International*, *54*, 1237-1249.

557 del Castillo, M. D., Fernandez-Gomez, B., Ullate, M., & Mesa, M. D. (2014). Uso de productos
558 de la cascarilla de café para la prevención y tratamiento de las patologías que conforman
559 el síndrome metabólico y de sus factores de riesgo. P201431848.

560 del Castillo, M. D., Fernandez-Gomez, B., Martinez-Saez, N., Iriondo, A. & Mesa, M. D. (in
561 press). Coffee By-Products. In A. Farah (Ed.), *Coffee: Chemestry, Quality and Health*
562 *Implications*. RSC Publishing Inc.

563 Deng, J., Qi, X. L., Guan, Z. Z., Yan, X. M., Huang, Y., & Wang, Y.L. (2013). Pretreatment
564 of SH-SY5Y cells with dicaffeoylquinic acids attenuates the reduced expression of
565 nicotinic receptors, elevated level of oxidative stress and enhanced apoptosis caused by
566 β-amyloid peptide. *The Journal of Pharmacy and Pharmacology*, *65*, 1736–1744.
567 http://doi.org/10.1111/jphp.12096

568 Farah, A., Monteiro, M., Donangelo, C, M., & Lafay, S. (2008). Chlorogenic acids from green
569 coffee extract are highly bioavailable in humans. *The Journal of Nutrition*, *138***,** 2309-
570 2315. http://doi.org/10.3945/jn.108.095554

571 Fernandez-Alvarez, J., Barberà, A., Nadal, B., Barceló-Batllori, S., Piquer, S., Claret, M., &
572 Gomis, R. (2004). Stable and functional regeneration of pancreatic beta-cell population in
573 nSTZ-rats treated with tungstate. *Diabetologia*, *47*, 470–477.
574 http://doi.org/10.1007/s00125-004-1332-8

575 Fernandez-Gomez, B., Ullate, M., Picariello, G., Ferranti, P., Mesa, M. D. & del Castillo, M.
576 D. (2015). New knowledge on the antiglycoxidative mechanism of chlorogenic acid. *Food*
577 *& Function*, *6*, 2081-2090.

578 Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M.D., del Castillo, M.D., & Martín, M. A.
579 (in press). Coffee silverskin extract improves glucose-stimulated insulin secretion and
580 protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food*
581 *Research International.* doi:10.1016/j.foodres.2016.03.006

582 Gonthier, M. P., Verny, M.A., Besson, C., Rémésy, C., & Scalbert, A. (2003). Chlorogenic
583 acid bioavailability largely depends on its metabolism by the gut microflora in rats. *The*
584 *Journal of Nutrition*, *133*, 1853–1859.

585 Granado-Serrano, A. B., Martín, M. A., Bravo, L., Goya, L., & Ramos, S. (2009). A diet rich
586 in cocoa attenuates N-nitrosodiethylamine-induced liver injury in rats. *Food and*
587 *Chemical Toxicology, 47*, 2499–2506. http://doi.org/10.1016/j.fct.2009.07.007

588 Heckman, M. A, Weil, J., & Gonzalez de Mejia, E. (2010). Caffeine (1, 3, 7 trimethylxanthine)
589 in foods: a comprehensive review on consumption, functionality, safety, and regulatory
590 matters. *Journal of Food Science*, *75*, R77–87. http://doi.org/10.1111/j.1750-

591 3841.2010.01561.x

592 Hollebeeck, S., Borlon, F., Schneider, Y. J., Larondelle, Y., & Rogez, H. (2013). Development
593 of a standardised human *in vitro* digestion protocol based on macronutrient digestion
594 using response surface methodology. *Food Chemistry*, *138*, 1936–1944.
595 http://doi.org/10.1016/j.foodchem.2012.11.041

596 Holst, B., & Williamson, G. (2008). Nutrients and phytochemicals: from bioavailability to
597 bioefficacy beyond antioxidants. *Current Opinion in Biotechnology*, *19*, 73–82.
598 http://doi.org/10.1016/j.copbio.2008.03.003

599 Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-
600 throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel
601 liquid handling system coupled with a microplate fluorescence reader in 96-Well format.
602 *Journal of Agricultural and Food Chemistry*, *50*, 4437–4444.
603 http://doi.org/10.1021/jf0201529

604 IDF Diabetes Atlas Group. (2015) Update of mortality attributable to diabetes for the IDF
605 Diabetes Atlas: Estimates for the year 2013. Diabetes Research and Clinical Practice.
606 http://dx.doi.org/10.1016/j.diabres.2015.05.03

607 Kagami, K., Morita, H., Onda, K., Hirano, T., & Oka, K. (2008). Protective effect of caffeine
608 on streptozotocin-induced beta-cell damage in rats. *The Journal of Pharmacy and*
609 *Pharmacology*, *60*, 1161–1165. http://doi.org/10.1211/jpp.60.9.0007

610 Karthikesan, K., Pari, L., & Menon, V. P. (2010). Protective effect of tetrahydrocurcumin and
611 chlorogenic acid against streptozotocin-nicotinamide generated oxidative stress induced
612 diabetes. *Journal of Functional Foods*, *2*, 134–142.
613 http://doi.org/10.1016/j.jff.2010.04.001

614 Martínez-López, S., Sarriá, B., Gómez-Juaristi, M., Goya, L., Mateos, R., & Bravo-Clemente,
615 L. (2014). Theobromine, caffeine, and theophylline metabolites in human plasma and
616 urine after consumption of soluble cocoa products with different methylxanthine contents.
617 *Food Research International*, *63*, 446–455. http://doi.org/10.1016/j.foodres.2014.03.009

618 Martinez-Saez, N., Ullate, M., Martin-Cabrejas, M. A., Martorell, P., Genovés, S., Ramon, D.,
619 & del Castillo, M. D. (2014). A novel antioxidant beverage for body weight control based
620 on coffee silverskin. *Food Chemistry*, *150*, 227–234.
621 http://doi.org/10.1016/j.foodchem.2013.10.100

622 Masiello, P., Broca, C., Gross, R., Roye, M., Manteghetti, M., Hillaire-Buys, D., & Ribes, G.
623 (1998). Experimental NIDDM: development of a new model in adult rats administered
624 streptozotocin and nicotinamide. *Diabetes*, *47*, 224–229.

625 Mesías, M., Navarro, M., Martinez-Saez, N., Ullate, M., del Castillo, M. D., & Morales, F. J.
626 (2014). Antiglycative and carbonyl trapping properties of the water soluble fraction of
627 coffee silverskin. *Food Research International*, *62*, 1120–1126.
628 http://doi.org/10.1016/j.foodres.2014.05.058

629 Monente, C., Ludwig, I. A., Stalmach, A., de Peña, M. P., Cid, C., & Crozier, A. (2015). In
630 vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in
631 Caco-2 cells of chlorogenic acids from spent coffee grounds. *International Journal of*
632 *Food Sciences and Nutrition*, *66*, 657–664.
633 http://doi.org/10.3109/09637486.2015.1064874

634 Monteiro, M., Farah, A., Perrone, D., Trugo, L. C., & Donangelo, C. (2007). Chlorogenic acid

635 compounds from coffee are differentially absorbed and metabolized in humans. *The*
636 *Journal of Nutrition*, *137*, 2196–2201. http://doi.org/137/10/2196.

637 Mulder, T. P., Rietveld, A. G., & van Amelsvoort, J. M. (2005). Consumption of both black
638 tea and green tea results in an increase in the excretion of hippuric acid into urine. *The*
639 *American Journal of Clinical Nutrition*, *81*, 256S-260S.

640 Murray R L. (1984). Creatinine, In Clinical Chemistry; Theory, Analysis and Correlation,. In
641 L. A. Kaplan, and A. J. Pesce (Eds.), (pp. 1257–1253). St Louis: The C.V. Mosby
642 Company.

643 Nam, Y. H., Hong, B. N., Rodriguez, I., Ji, M. G., Kim, K., Kim, U.J., & Kang, T. H. (2015).
644 Synergistic potentials of coffee on injured pancreatic islets and insulin action via KATP
645 channel blocking in zebrafish. *Journal of Agricultural and Food Chemistry*, *63*, 5612–
646 5621. http://doi.org/10.1021/acs.jafc.5b00027

647 Nardini, M., Cirillo, E., Natella, F., & Scaccini, C. (2002). Absorption of phenolic acids in
648 humans after coffee consumption. *Journal of Agricultural and Food Chemistry*, *50*, 5735-
649 5741. http://doi.org/10.1021/jf0257547

650 Nowotny, K., Jung, T., Höhn, A., Weber, D., & Grune, T. (2015). Advanced glycation end
651 products and oxidative stress in type 2 diabetes mellitus. *Biomolecules*, *5*, 194–222.
652 http://doi.org/10.3390/biom5010194

653 Oki, T., Nagai, S., & Yoshinaga, M. (2006). Contribution of .BETA.-carotene to radical
654 scavenging capacity varies among orange-fleshed sweet potato cultivars. *Food Science*
655 *and Technology Research*, *12*, 156-160.

656 Palafox-Carlos, H., Ayala-Zavala, J. F., & González-Aguilar, G. A. (2011). The role of dietary
657 fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *Journal*
658 *of Food Science*, *76*, R6-R15. http://doi.org/10.1111/j.1750-3841.2010.01957.x

659 Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M., & Brighenti,
660 F. (2003). Total antioxidant capacity of plant foods, beverages and oils consumed in Italy
661 assessed by three different *in vitro* assays. *The Journal of Nutrition*, *133*, 2812–2819.

662 Podio, N. S., López-Froilán, R., Ramirez-Moreno, E., Bertrand, L., Baroni, M. V., Pérez-
663 Rodríguez, M. L., Sánchez-Mata, M. C., & Wunderlin, D. A. (2015). Matching *in vitro*
664 bioaccessibility of polyphenols and antioxidant capacity of soluble coffee by boosted
665 regression trees. *Journal of Aricultural and Food Chemistry*, *63*, 9572-9582. http://doi:
666 10.1021/acs.jafc.5b04406

667 Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity
668 relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*,
669 933–956. http://doi.org/10.1016/0891-5849(95)02227-9

670 Rodopoulos, N., Wisén, O., & Norman, A. (1995). Caffeine metabolism in patients with
671 chronic liver disease. *Scandinavian Journal of Clinical and Laboratory Investigation*, *55*,
672 229–42.

673 Rodríguez-Ramiro, I., Martín, M. A., Ramos, S., Bravo, L., & Goya, L. (2011). Comparative
674 effects of dietary flavanols on antioxidant defences and their response to oxidant-induced
675 stress on Caco2 cells. *European Journal of Nutrition*, *50*, 313–22.
676 http://doi.org/10.1007/s00394-010-0139-2

677 Shi, X., Dalal, N. S., & Jain, A. C. (1991). Antioxidant behaviour of caffeine: efficient

scavenging of hydroxyl radicals. *Food and Chemical Toxicology, 29*, 1–6.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In I. L. Packer (Ed.), *Methods in Enzymology* (pp. 152-178). Philadephia. Elsevier. http://doi.org/10.1016/S0076-6879(99)99017-1

Stalmach, A., Williamson, G., & Crozier, A. (2014). Impact of dose on the bioavailability of coffee chlorogenic acids in humans. *Food & Function*, *5*, 1727–1737. http://doi.org/10.1039/c4fo00316k

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological Research / Academia Scientiarum Bohemoslovaca*, *50*, 537–546.

Uribarri, J., del Castillo, M. D., de la Maza, M. P., Filip, R., Gugliucci, A., Luevano-Contreras, C., & Garay-Sevilla, M. E. (2015). Dietary advanced glycation end products and their role in health and disease. *Advances in Nutrition*, *6*, 461–473. http://doi.org/10.3945/an.115.008433

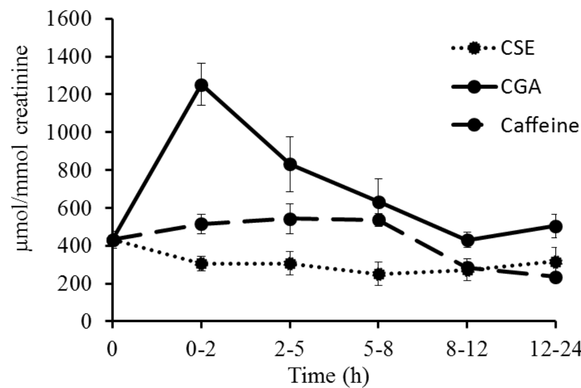
Usansky, J. L., Desai, A., & Tang-Liu, D. (2010). PK functions for Microsoft Excel. *Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA*.

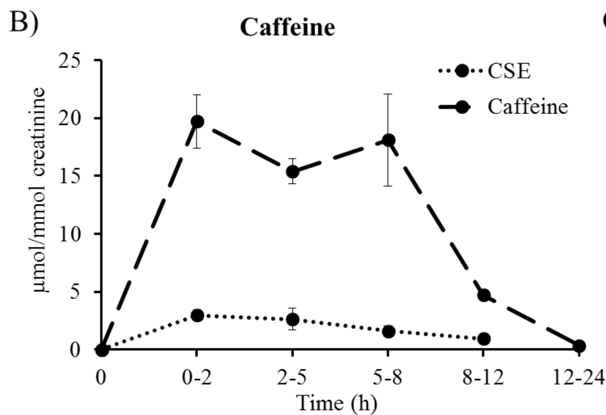
Vallejo, F., Gil-Izquierdo, A., Pérez-Vicente, A., & García-Viguera, C. (2004). *In vitro* gastrointestinal digestion study of broccoli inflorescence phenolic compounds, glucosinolates, and vitamin C. *Journal of Agricultural and Food Chemistry*, *52*, 135–138. http://doi.org/10.1021/jf0305128

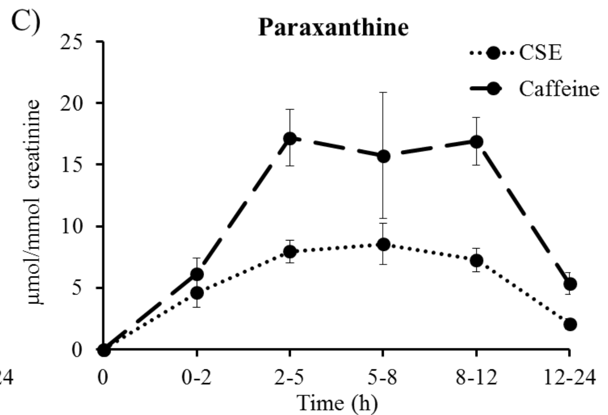
701 **Figure captions**

702

703 **Figure 1**. Kinetics of the urinary excretion of hippuric acid (A), caffeine (B) and paraxanthine
704 (C) after consumption of CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight),
705 CGA (1.5 mg/kg body weight) and caffeine (5 mg/kg body weight). Results represent the
706 concentration (µmol/mmol creatinine) as mean (n=7) ± SEM. CGA, chlorogenic acid; CSE,
707 coffee silverskin extract.

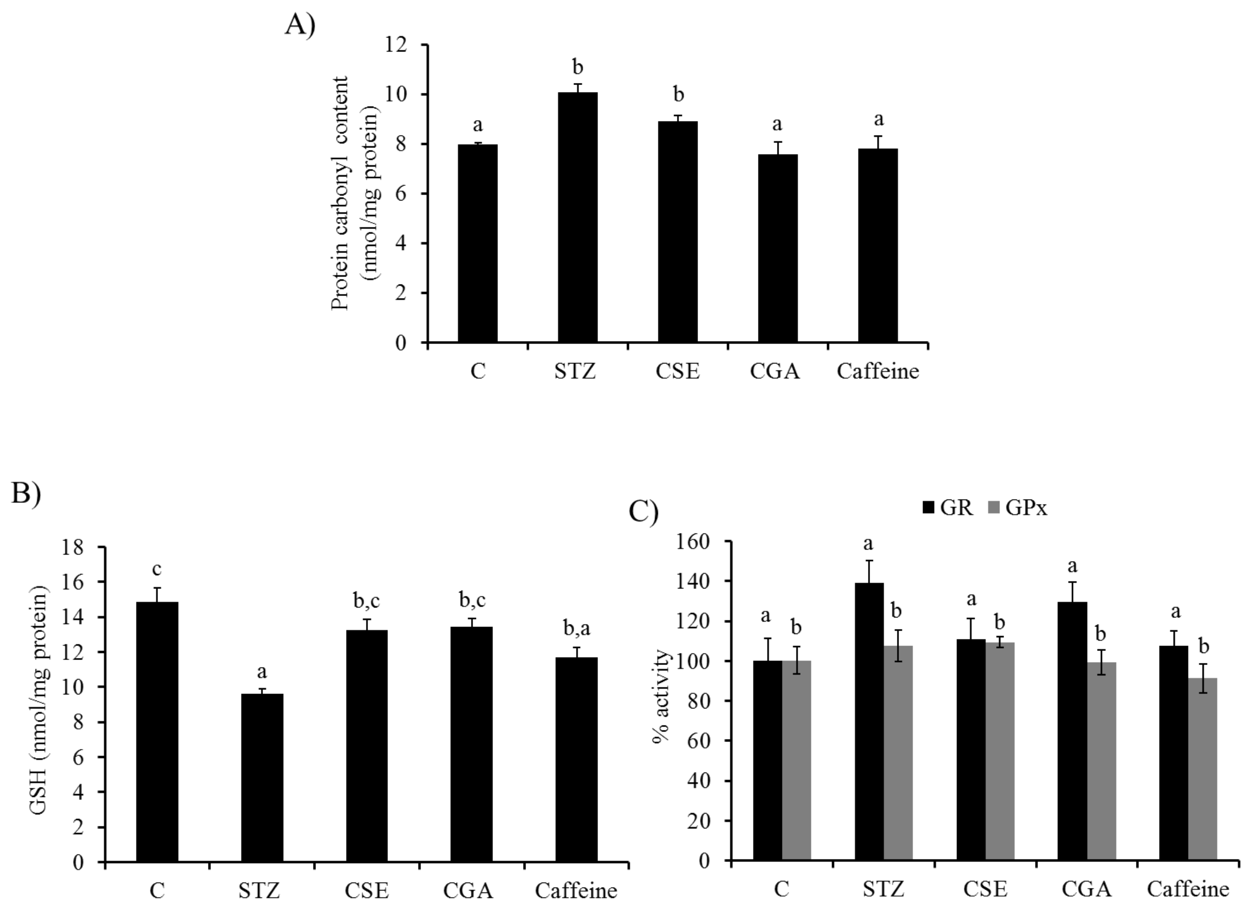




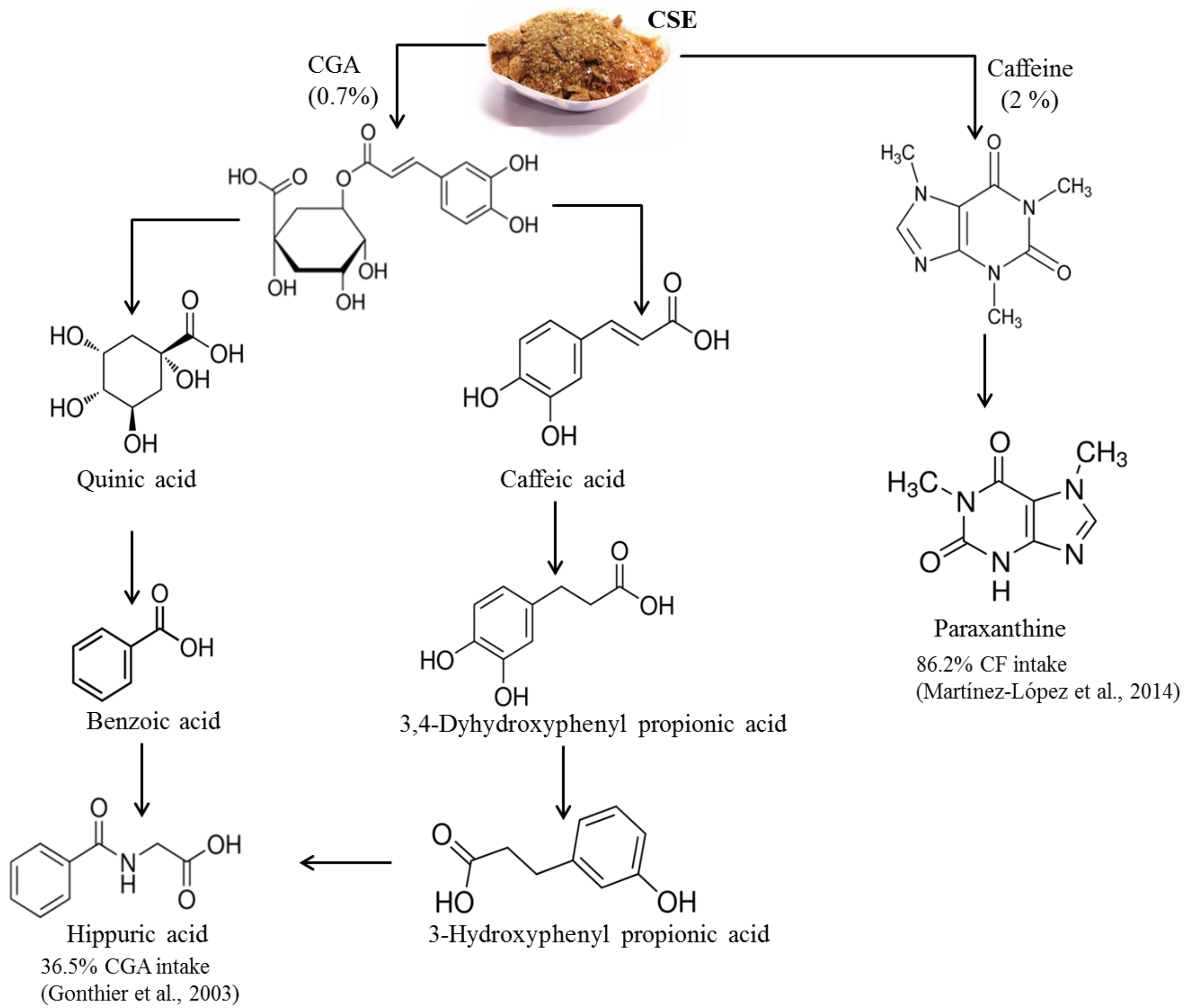


708
709
710
711
712
713
714
715
716
717

Figure 2. Effect of CSE, CGA and caffeine on oxidative status in pancreatic tissues of STZ-NA induced diabetic rats. C, untreated healthy control rats; STZ, rats treated with STZ (60 mg/kg body weight) and NA (200 mg/kg body weight); CSE, rats treated with STZ-NA and CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight); CGA, rats treated with STZ-NA and 1.5 mg CGA/kg body weight; Caffeine, rats treated with STZ-NA and 5 mg caffeine/kg body weight; (A) GSH levels ($p < 0.05$, (B) GR and GPx activities ($p < 0.05$) and (C) Carbonyl groups production ($p < 0.1$) were evaluated. Data represent means ± SEM (n=8). Different letters denote statistically significant differences referred above in brackets. CGA, chlorogenic acid; CSE, coffee silverskin extract; NA, nicotinamide; STZ, streptozotocin.



733 **Figure 3**. Simplified scheme of CGA and caffeine metabolism studied in the present study.



734
735
736
737
738
739
740
741
742
743
744
745
746

Table 1. Antioxidant capacity (µmol CGA/g) of non-digested and digested CSE.

Sample	ABTS	$ORAC_{FL}$
CSE non-digested	397 ± 17[a]	358 ± 25[a]
CSE digested	337 ± 26[b]	179 ± 13[b]

Results are expressed as mean ± SD for n = 3. Different letters in the same column indicate significant differences ($p < 0.05$). CGA, chlorogenic acid; CSE, coffee silverskin extract.

768

769 **Table 2**. Pharmacokinetic parameters (C_{max}, AUC and T_{max}) of metabolites detected in urine
770 after treatments consumption with CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/kg body
771 weight), CGA (1.5 mg CGA/kg body weight); and caffeine (5 mg caffeine/kg body weight).

Metabolite	Parameters	CSE	CGA	Caffeine
	C_{max} (mmol/µmol)	447.9 ± 53.0 [a]	1346.9 ± 274.9 [b]	782.6 ± 104.9 [a,b]
	T_{max} (h)	0.8 ± 0.4 [a]	1.6± 0.3 [a]	1.4± 0.4 [a]
	AUC (mmol/µmol·h)	3727.0 ± 584.5 [a]	13842.7 ± 2182.8 [b]	7369.1 ± 1171.8 [a]
	C_{max} (mmol/µmol)	3.3 ± 1.1 [a]	nd	28.88 ± 4.6 [b]
	T_{max} (h)	1.8 ± 0.4 [a]		2.50 ± 0.5 [a]
	AUC (mmol/µmol·h)	14.8 ± 5.6 [a]		136.66 ± 17.7 [b]
	C_{max} (mmol/µmol)	10.7 ± 2.7 [a]	nd	22.4 ± 4.2 [a]
	T_{max} (h)	5.5± 1.6 [a]		4.62 ± 0.6 [a]
	AUC (mmol/µmol·h)	125.3 ± 34.7 [a]		265.56 ± 55.8 [a]

772 Values represent mean ± SEM, n=7. Different letters in the same row indicate significant
773 differences between treatments ($p < 0.05$). AUC, area under the curve; C_{max}, maximum
774 concentration reached; CGA, chlorogenic acid; CSE, coffee silverskin extract; n.d., not
775 detected, T_{max}, time to reach;

776