Insights on the health benefits of the bioactive compounds of coffee silverskin extract

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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.

Abstract

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

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

1. Introduction

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

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

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

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

2. Material and Methods

2.1. Chemicals

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

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

2.2. Preparation of coffee silverskin extract

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

2.3. Evaluation of the bioaccessibility of coffee silverskin extract

2.3.1. *In vitro* oral gastrointestinal digestion

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

incubated at 37 °C for 5 min with constant stirring at 4 g. For the gastric digestion step, 10 ml of Milli-Q water was added and the pH of the samples was adjusted to 2.0 with 1 M HCl. Pepsin (110 µl, 5 mg/ml in 0.1 M HCl) was added to each sample and incubated in a final volume of 22.88 ml at 37 °C for 90 min under anaerobic conditions using an anaerobic chamber model Bactron II (Biogen, Weston, MA, USA) with constant stirring at 4 g. After this time, the pH of the samples was adjusted to 7 with 1M NaHCO₃ for the intestinal digestion step. One ml of pancreatin solution (287.59 mg/ml in 0.1 M NaHCO₃) was added, the final volume brought to 31.24 ml with Milli-Q water, and the mixture was incubated at 37 °C for 150 min under anaerobic conditions. Digested samples were centrifuged at 1677 g at 4 °C for 40 min. Enzymatic activity was stopped with liquid N₂ and supernatants were freeze-dried and stored under dark, dry conditions at 4 °C until analysis. The digestion of CSE was also carried out under the same conditions without adding the enzymes to gain insight into the effect of the pH in CGA and caffeine metabolism. Digestion experiments were carried out in triplicate.

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

Total phenolic content (TPC) was determined using the Folin-Ciocalteu's colorimetric assay (Singleton, Orthofer, & Lamuela-Raventós, 1999) adapted to a microplate reader. Briefly, CSE prepared at 2 mg/ml was used. Ten µl of the sample was combined with 200 µl of Folin reagent (0.017% (v/v)) and 50 µl of NaHCO₃ (30 mg/ml). The 96-well plate was incubated in darkness at room temperature for 2 h and was read at 725 nm using a BioTek PowerWave™ XS (Winoski, VT, USA) microplate reader. Calibration curves were constructed using a standard solution of CGA (0.1-0.8 mg/ml). Samples were analyzed in triplicate and results expressed as mg of CGA equivalents per g of CSE (mg CGA /g CSE).

The contents of CGA and caffeine in the CSE and their digested products was analyzed by capillary zone electrophoresis (CZE) as described by del) del Castillo, Ames, and Gordon

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

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

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

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

An aliquot of non-digested CSE (10 mg/ml) was subjected to fractionation using an Amicon[®] Ultra 0.5 ml centrifugal filter unit fitted with an Ultracel[®]-10K regenerated cellulose membrane (10 kDa cut-off) (Merck Millipore, Darmstadt, Germany). The antioxidant capacity of low (< 10 kDa) and high ($\geq$ 10 kDa) molecular weight fractions was also analysed using the ABTS⁺⁺ assay.

The ORAC_{FL} assay was performed following the procedure described by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002). Briefly, 25 μ l of sample of the appropriate dilution or standard were added to a 96-well microplate followed by the addition of 150 μ l of fluorescein work solution (8.5×10^{-5} mM) prepared in 75 mM phosphate buffer (pH 7.4). The Biotek Synergy[™] HT microplate reader (Biotek Instruments, Highland Park, Winooski, VT, USA) was programmed to incubate the plate at 37 °C and add 30 μ l of AAPH solution (153 mM in phosphate buffer) as a peroxy radical generator. Fluorescence was read with excitation at 485 nm and emission at 528 nm every two minutes for 90 min. A blank consisting of fluorescein, AAPH and phosphate buffer was also included. Calibration curves of Trolox (6.25-50 μ M) and CGA (3.12-25 μ M) were constructed. Standard calibration curves were composed by plotting the net area under the curve (AUC) as a function of Trolox or CGA concentration. ORAC_{FL} values were expressed as μ moles TE/g of CSE and as μ moles CGA/g of CSE.

2.4. Animals and the experimental design (ARRIVE guidelines)

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

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

2.4.1. Evaluation of the bioavailability of coffee silverskin extract

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

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

CGA, caffeine and related compounds, HA and paraxanthine were determined by UPLC-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 $\times$ 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 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$, 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 $^{\circ}\text{C}$, 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 ($[\text{M-H}]^{-}$) and
252 product ions for CGA and HA were m/z 353.2 $\rightarrow$ 191.1 and m/z 178.3 $\rightarrow$ 134.3, respectively,
253 whereas parent ($[\text{M-H}]^{+}$) and product ions for caffeine and paraxanthine were m/z 195.1 $\rightarrow$
254 138.2 and m/z 181.1 $\rightarrow$ 124.2, respectively.

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

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

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

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

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

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

2.5. Data analysis

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

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

3. Results

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

TPC and CGA and caffeine levels of 46.65 mg/g, 13.33 mg/g and 44.64 mg/g were detected in CSE, respectively. Overall antioxidant capacity values of 397 and 358 $\mu\text{mol CGA/g}$ (corresponding to 427 and 816 of $\mu\text{mol TE/g}$) were obtained for scavenging and hydrogen donating capacities in CSE, respectively (Table 1). The antioxidant capacity of low molecular weight compounds ($<10\text{ kDa}$) was 220 $\mu\text{mol CGA/g}$, while the antioxidant capacity of the fraction containing high molecular weight compounds ($\geq 10\text{ kDa}$) was 110 $\mu\text{mol CGA/g}$.

In vitro digestion of CSE decreased concentrations of caffeine (25%), TPC (40%) and CGA (82%). The overall antioxidant capacity of CSE as measured by ABTS and ORAC decreased by 15% and 50%, respectively (Table 1).

To evaluate the effect of changes in pH during digestion on the degradation of bioactive compounds, a digestion in the absence of digestive enzymes was performed. Changes in pH decreased TPC and CGA content by 38% and 83%, respectively, while caffeine content only decreased by 15%.

CSE can provide bioaccessible amounts of bioactive compounds such as TPC (79.26 $\mu\text{mol/g}$), CGA (6.86 $\mu\text{mol/g}$) and caffeine (172.37 $\mu\text{mol/g}$). Digests presented antioxidant capacities of 337 $\mu\text{mol/g}$ and 179 $\mu\text{mol/g}$ by ABTS and ORAC assays, respectively.

3.2. Metabolism of the bioactive compounds of coffee silverskin extract

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

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

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

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

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

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

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

reduce the rate of protein oxidation induced by the toxic agent. GSH content in the pancreas of T2DM rats decreased significantly ($p < 0.05$) (Figure 2B), and pre-treatment with CSE and CGA significantly reduced ($p < 0.05$) GSH depletion in the pancreas of diabetic rats. Untreated rats and those treated with CSE and CGA showed similar pancreatic GSH values ($p > 0.05$). GPX and GR values of all animals were of the same order of magnitude ($p > 0.05$) (Figure 2C). The physiological concentrations of the bioactive compounds forming CSE were able to protect pancreatic cells against oxidative stress produced by the diabetogenic agent STZ.

4. Discussion

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

decreased ($p < 0.05$) by the digestion processes (data not shown). Since this decrease was observed in the absence of digestive enzymes, it may be associated with changes in pH taking place during *in vitro* digestion. Several studies have shown that the bioaccessibility of TPC in different food matrixes was lower than that found for isolated polyphenols. Podio et al. (2015) observed a 5-fold lower TPC content in digested coffees than in native instant coffees. Campos-Vega et al. (2015) reported a considerable reduction of TPC (91%) in spent coffee grounds. Akillioglu and Karakaya (2010) showed that the bioaccessibility of TPC ranged from 19% to 39% in bean varieties. Phenolic compounds are less bioaccessible partly due to the presence of dietary fiber in the plant matrix (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). CSE contains high amounts of dietary fiber (362 mg/g) which affect the release of TPC in the digestion process. Hydroxycinnamic acid derivatives constitute the main phenolic component of CS (Bresciani, Calani, Bruni, Brighenti, & Del Rio, 2014). Vallejo, Gil-Izquierdo, Pérez Vicente, and García-Viguera (2004) observed an 87% decrease in CGA after intestinal digestion. Previous studies have suggested that a pH value of 7.5 and bile salts could contribute to lower CGA. Bermúdez-Soto, Tomás-Barberán, and García-Conesa (2007) reported a minor decrease in CGA (5%) in chokeberry extract due to the pH of intestinal digestion (pH 7.5). However, a bioaccessibility study of CGAs in spent coffee grounds showed a total recovery of this compound after digestion (Monente et al., 2015). These differences in CGA release suggest that bioaccessibility is also affected by the plant matrix.

In agreement with the data on TPC and CGAs, overall antioxidant capacity also decreased after *in vitro* gastrointestinal digestion. The main antioxidant compounds reported in CSE are CGAs, melanoidins and antioxidant fiber (Fernandez-Gomez, Martinez-Saez, Iriando, & Mesa, in press). According to our data, low molecular weight compounds (CGAs, other phenols and caffeine among others) seem to make a greater contribution to the *in vitro* overall antioxidant 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 $\text{CH}=\text{CH}-\text{COOH}$ group, which ensures greater H-donating ability and radical
428 stabilization. Caffeine effectively reacts with the hydroxyl radical ($\text{OH}\cdot$) 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, Srail, 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

plasma or urine of rats and humans fed pure CGA or CGA-containing foods (Booth, Emerson, Jones, & Deeds, 1957; Choudhury, Srail, Debnam, & Rice Evans, 1999; Azuma et al., 2000; Nardini, Cirillo, Natella, & Scaccini, 2002; Stalmach, Williamson, & Crozier, 2014).

CSE is also a good source of caffeine (1,3,7-trimethylxanthine). Methylxanthines are extensively absorbed in the gastrointestinal tract and metabolized in the liver to yield methylxanthine derivatives and methyluric acids as the main metabolites, which are finally excreted in urine (Figure 3) (Martínez-López et al., 2014). Paraxanthine (1,7 dimethylxanthine) is the main metabolite of caffeine biotransformation found in plasma and urine after caffeine intake (Arnaud, 2011). The pharmacokinetics of caffeine and paraxanthine excretion were evaluated after the consumption of 5.026 μmol pure caffeine and CSE containing 2.211 μmol caffeine. Caffeine was present in the urine of both groups of rats, which is in agreement with other studies that described incomplete biotransformation in humans (Bonati et al., 1982; Rodopoulos, Wisén, & Norman, 1995). According to CSE composition, the lower consumption of caffeine was in line with the lower excretion observed for this compound and its metabolite paraxanthine. These results are in agreement with previous findings of dose-dependent metabolism and the excretion of caffeine in humans (Martínez-López et al., 2014). Therefore, our data showed that the caffeine present in CSE is bioavailable, partially metabolized, and rapidly excreted.

The *in vivo* effect of CGA, caffeine and CSE on the prevention of oxidative damage in the pancreas of STZ-NA-induced T2DM rats was also evaluated. The cytotoxic action of STZ is associated with the generation of ROS and consequent β -cell destruction and suppression of insulin secretion (Szkudelski, 2001). Antioxidants are able to prevent pancreatic islets damage induced by STZ (Fernandez-Alvarez et al., 2004). Consequently, natural antioxidants may be considered promising candidates for the prevention or co-treatment of diabetes. In the present study, the administration of STZ to the animals produced a decrease in GSH and an increase in

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

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

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503 **Conflict of interest**

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

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Figure captions

Figure 1. Kinetics of the urinary excretion of hippuric acid (A), caffeine (B) and paraxanthine (C) after consumption of CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight), CGA (1.5 mg/kg body weight) and caffeine (5 mg/kg body weight). Results represent the concentration ($\mu\text{mol}/\text{mmol creatinine}$) as mean ($n=7$) $\pm$ SEM. CGA, chlorogenic acid; CSE, coffee silverskin extract.

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 $\pm$ SEM ($n=8$). Different letters denote statistically significant differences referred above in brackets. CGA, chlorogenic acid; CSE, coffee silverskin extract; NA, nicotinamide; STZ, streptozotocin.

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

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

749		
Sample	ABTS	ORAC _{FL} 750
CSE non-digested	397 ± 17 ^a	358 ± 25 ^a
CSE digested	337 ± 26 ^b	179 ± 13 ^b
752		

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.

754

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/ μ mol)	447.9 ± 53.0^a	1346.9 ± 274.9^b	$782.6 \pm 104.9^{a,b}$
	$T_{\max}$ (h)	0.8 ± 0.4^a	1.6 ± 0.3^a	1.4 ± 0.4^a
	AUC (mmol/ μ mol·h)	3727.0 ± 584.5^a	13842.7 ± 2182.8^b	7369.1 ± 1171.8^a
	$C_{\max}$ (mmol/ μ mol)	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/ μ mol·h)	14.8 ± 5.6^a		136.66 ± 17.7^b
	$C_{\max}$ (mmol/ μ mol)	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/ μ mol·h)	125.3 ± 34.7^a		265.56 ± 55.8^a

772 Values represent mean $\pm$ 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;