

Proceedings

# Bioaccessibility of Phenolic Compounds from Cocoa Shell Subjected to In Vitro Digestion and Its Antioxidant Activity in Intestinal and Hepatic Cells †

Silvia Cañas <sup>1,2\*</sup>, Miguel Rebollo-Hernanz <sup>1,2\*</sup>, Yolanda Aguilera <sup>1,2</sup>, Vanesa Benítez <sup>1,2</sup>,  
Cheyenne Braojos <sup>1,2</sup>, Silvia M. Arribas <sup>3</sup> and María A. Martín-Cabrejas <sup>1,2</sup>

<sup>1</sup> Department of Agricultural Chemistry and Food Science, Universidad Autónoma de Madrid, 28049 Madrid, Spain; yolanda.aguilera@uam.es (Y.A.); cheyenne.braojos@uam.es (V.B.); vanesa.benitez@uam.es (C.B.); maria.martin@uam.es (M.A.M.-C.)

<sup>2</sup> Institute of Food Science Research, CIAL (UAM-CSIC), 28049 Madrid, Spain

<sup>3</sup> Department of Physiology, Universidad Autónoma de Madrid, 28029 Madrid, Spain; silvia.arribas@uam.es

\* Correspondence: silvia.cannas@uam.es (S.C.); miguel.rebollo@uam.es (M.R.-H.)

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**Abstract:** The cocoa shell is a by-product generated by the cocoa processing industry that could be used as a nutraceutical owing to the significant amounts of bioactive compounds it contains. This work aimed to study the bioaccessibility of phenolic compounds present in the flour (CSF) and an aqueous extract (CSE) from cocoa shells through an in vitro simulated digestion and to assess their antioxidant capacity in vitro and by using intestinal and hepatic cell culture models (IEC-6 and HepG2 cells). The bioaccessibility of phenolic compounds was determined using a simulated in vitro digestion model (INFOGEST). Total phenolic compounds (TPC) and antioxidant activity were measured using in vitro techniques. Reactive oxygen species (ROS) were evaluated in IEC-6 and HepG2 cells after *t*-BOOH stimulation. TPC present in CSE were more bioaccessible than phenolic compounds present in CSF. During digestion, the bioaccessibility of phenolic compounds from CSF fluctuated in the gastric (2.8 mg/g), intestinal phase (7.6 mg/g), and the colonic (5.7 mg/g) phases. Similarly, for the phenolics of CSE, the bioaccessibility increased from 50.6 mg/g (gastric) to 53.4 mg/g (intestinal) and decreased in the colonic phase (37.2 mg/g). The in vitro antioxidant capacity followed a similar behavior, increasing throughout the digestion in CSF (8.8 to 10.6-fold) and CSE (6.0 to 7.4-fold). Digested CSF and CSE were not cytotoxic for IEC-6 and HepG2 cells and protected their viability under oxidative stress conditions (93–100%). *t*-BOOH-induced ROS were prevented by CSF (72–88%) and CSE (81–94%) bioaccessible fractions in both intestinal and hepatic cells. In conclusion, cocoa shells are a source of potentially bioavailable antioxidant phenolic compounds that may protect cells from oxidative stress.

**Keywords:** cocoa by-products; cocoa shell; in vitro digestion; bioaccessibility; oxidative stress; antioxidant capacity

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## 1. Introduction

The cocoa processing industry generates large quantities of by-products, including cocoa shells, representing between 12 and 20% of the cocoa bean [1]. Global cocoa production is estimated at 4.8 million tons in 2019–2020, resulting in a substantial amount of cocoa shells [2]. Cocoa shells are

generally underused as fuel, fertilizer, or animal feed. The scarcity of natural resources and severe environmental problems has increased interest in utilizing agricultural by-products such as cocoa shells for use as new food sources [3]. Several studies have reported the attractive nutritional value of cocoa shells, owing to their high content of bioactive compounds such as dietary fiber, phenolic compounds, theobromine, and a lipid profile similar to cocoa butter [1]. Recently the cocoa shell has been validated as a novel antioxidant dietary fiber food ingredient, allowing its incorporation to develop foods with health-promoting properties [4]. In this regard, the determination of bioactive compounds is not enough to predict the possible beneficial effects *in vivo* of the cocoa shell, as the digestive process can transform the original compounds present in food into metabolites that finally reach the blood system. It is, therefore, essential to determine the bioaccessibility. This term refers to the fraction of a food constituent released from the food matrix in the gastrointestinal tract and is potentially available for absorption [5]. The bioaccessibility of the compounds can be evaluated using an *in vitro* digestion model. Although *in vitro* models do not entirely simulate human conditions, they provide a more straightforward and cheaper alternative to *in vivo* models [6]. In this sense, evaluating the impact of bioactive antioxidant compounds at the cellular level is essential since they can exert a protective effect against oxidative stress conditions. Oxidative stress is caused by the imbalance between reactive oxygen species (ROS) and antioxidants' availability. The imbalance in favor of ROS can cause molecular and cellular damage [7]. Cocoa shells as a source of phenolic compounds with antioxidant potential could modulate ROS levels [8,9]. Thus, the objective of this study was to evaluate the bioaccessibility of phenolic compounds in cocoa shell flour and in an extract obtained from cocoa shell flour through an *in vitro* digestion model, as well as the study of the antioxidant capacity *in vitro* and by using intestinal and hepatic cell culture models (IEC-6 and HepG2 cells).

## 2. Experiments

### 2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and 0.25% trypsin-EDTA were purchased from GE Healthcare Life Sciences. Fetal Bovine Serum (FBS) and penicillin/streptomycin (100 X) were obtained from Gibco Life Technologies. Chlorogenic acid, Folin-Ciocalteu reagent, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulfate, ( $\pm$ )-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), tert-butyl hydroperoxide (*t*-BOOH), dimethyl sulfoxide (DMSO), 2',7'-dichlorodihydro-fluorescein diacetate (DCFDA), quercetin, and insulin were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA). All other chemicals and reagents were obtained from Panreac unless otherwise specified. Cocoa shell was kindly supplied by Chocolates Santocildes (Castrocontrigo, León, Spain).

### 2.2. Cocoa Shell Flour and Aqueous Extracts Preparation

Cocoa shell was ground in a mill, obtaining cocoa shell flour. The cocoa shell extract was prepared according to the extraction conditions described by Aguilera et al. [10].

### 2.3. *In Vitro* Simulated Digestion

*In vitro* simulated gastrointestinal digestion was performed following the harmonized INFOGEST method [11] with slight modifications. *In vitro* simulated colonic digestion was carried out according to Papillo et al. [12].

#### 2.4. Extraction and Analysis of Total Phenolic Compounds and Antioxidant Capacity

##### 2.4.1. Extraction of Free and Bound Phenolic Compounds

Free and bound phenolic compounds were extracted according to the method described by Rebollo-Hernanz et al. [13]. For the extraction of free phenolic compounds, cocoa shell flour (1 g) was mixed with 50 mL of a solution of methanol: HCl (1‰)–water 80:20 (*v/v*). The mixture was ultrasonicated for 30 min, incubated under agitation (40 °C, 16 h), and finally centrifuged at 4000 g for 10 min, collecting the supernatants obtained. The extraction process was repeated twice without incubation. The extracts obtained were mixed and concentrated using a rotary evaporator. The residue obtained was reserved for the extraction of bound phenolic compounds. 5 mL of NaOH 4 mol L<sup>-1</sup> was added to the residue. Subsequently, the sample was stirred under nitrogen gas for 1 h, and then, the pH was adjusted to 2.0. The sample was centrifuged at 4000 g for 10 min, collecting the organic fraction. Finally, three additional extractions were performed with methanol: HCl (1‰)–water 80:20 (*v/v*). The supernatants collected from each extraction were mixed and concentrated using a rotary evaporator.

##### 2.4.2. Total Phenolic Compounds

Total phenolic compounds were analyzed by the Folin-Ciocalteu assay [13]. The experiment was carried out in a 96-well microplate. Briefly, 10 µL of the sample, 150 µL of Folin-Ciocalteu reagent (diluted 1:14, *v/v* in Milli-Q water), and 50 µL of Na<sub>2</sub>CO<sub>3</sub> 20% were added to each well. The plate was incubated in the dark at room temperature for 2 h. Absorbance was measured at 750 nm in a microplate reader. A standard gallic acid curve (0.01–0.2 mg mL<sup>-1</sup>) was performed, and the results were expressed as mg gallic acid equivalents per gram (mg GAE g<sup>-1</sup>).

##### 2.4.3. In Vitro Antioxidant Capacity

Antioxidant capacity was assessed by the ABTS<sup>•+</sup> assay [14]. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cations (ABTS<sup>•+</sup>) were obtained by reacting 7 mmol L<sup>-1</sup> ABTS solution with 2.45 mmol L<sup>-1</sup> potassium persulfate and stirring it in the dark at room temperature for 16 h before use. The ABTS<sup>•+</sup> solution obtained was diluted in 5 mmol L<sup>-1</sup> PBS, pH 7.4, by adjusting the solution to an absorbance of 0.70 at 734 nm. The assay was carried out in a 96-well microplate by adding 30 µL of the sample and 270 µL of the diluted solution ABTS<sup>•+</sup> to each well. After 10 min of incubation, the absorbance was read at 734 nm on a microplate reader. A calibration curve was made using Trolox as a standard solution (0–0.06 mg mL<sup>-1</sup>). The results were expressed as mg Trolox equivalent per gram (mg TE g<sup>-1</sup>).

#### 2.5. Cell Culture

HepG2 and IEC-6 cells were kindly provided by Dr. Blanca Hernandez-Ledesma and the Bioanalytical Techniques Unit (BAT), from the Food Science Research Institute (CIAL) (Madrid, Spain), respectively. HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. IEC-6 cells were cultured in DMEM, supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1 U mL<sup>-1</sup> insulin. Cells were grown in a humidified incubator containing 5% CO<sub>2</sub> and 95% air at 37 °C.

##### 2.5.1. Cell Viability

HepG2 and IEC-6 cells were seeded at 5 × 10<sup>5</sup> cells mL<sup>-1</sup> in 96-well plates. After 24 h, cells were treated with CSF and CSE diluted in DMEM culture medium (50–1000 µg mL<sup>-1</sup>) and incubated for 24 h at 37 °C in a humidified atmosphere. Cell viability was measured using the CellTiter 96<sup>®</sup> AQueous

(MTS) assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The non-citotoxic concentrations were used in further experiments.

### 2.5.2. Determination of Reactive Oxygen Species (ROS)

The production of Reactive Oxygen Species was quantified by the dichlorofluorescein assay [15]. The cells were treated with CSF and CSE extracts (50–500  $\mu\text{g mL}^{-1}$ ) for 24 h. After pre-treatment, DCFDA (25  $\mu\text{M}$ ) was added for 30 min. The cells were washed with PBS. Afterward, the cells were treated with CSF and CSE extracts for 1 h. Tert-butyl hydroperoxide was used as an oxidant. The fluorescent intensity of the cell suspensions was detected using a fluorescence spectrophotometer. Excitation and emission wavelengths were 485 and 530 nm, respectively.

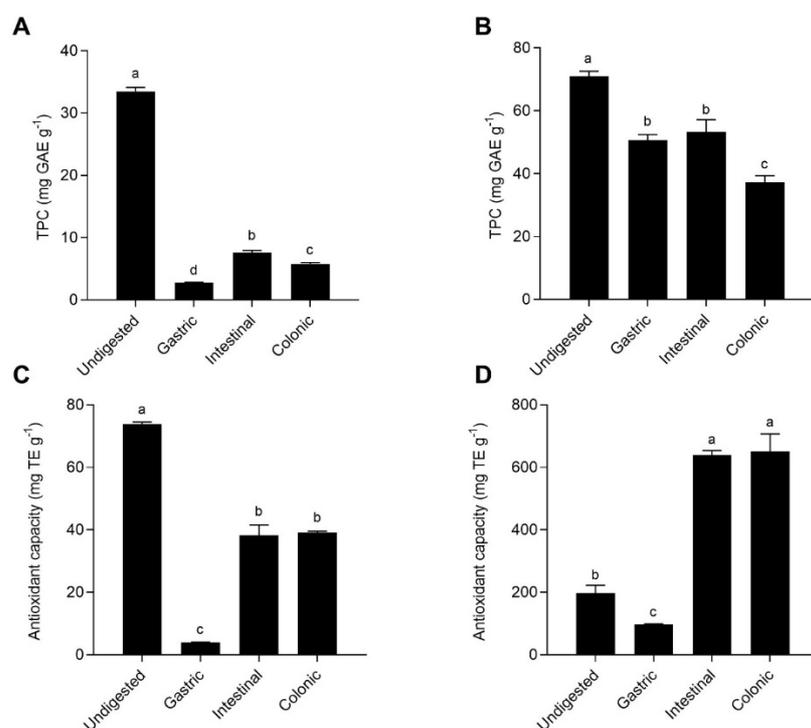
### 2.6. Statistical Analysis

Statistical analysis was performed using the statistical program SPSS 26.0. The results were expressed as mean  $\pm$  standard deviation (SD) ( $n = 3$ ). The data were analyzed by one-way analysis of variance (ANOVA) and *post hoc* Tukey's test. Differences were significant at  $p < 0.05$ .

## 3. Results

### 3.1. Bioaccessibility of Total Phenolic Compounds and Antioxidant Activity

The effect of *in vitro* digestion on the total phenolic compound content of cocoa shell flour is shown in Figure 1A. Digested cocoa shell flour exhibited a significantly lower TPC content ( $p < 0.05$ ) compared to undigested CSF. Significant differences ( $p < 0.05$ ) were found between all digestion stages. Bioaccessibility increased significantly ( $p < 0.05$ ) from the gastric to the intestinal phase and decreased in the colonic phase ( $p < 0.05$ ). Likewise, TPC's content in CSE (Figure 1B) was higher in the undigested extract. No significant differences ( $p < 0.05$ ) were observed between the gastric and intestinal phases' bioaccessibility. Bioaccessibility decreased from the intestinal to the colonic phase.



**Figure 1.** Total phenolic compounds in cocoa shell flour (A) and cocoa shell extract (B), and antioxidant capacity of cocoa shell flour (C) and cocoa shell extract (D) in the different stages of

simulated in vitro digestion. Data are presented as mean  $\pm$  SD ( $n = 3$ ). Different letters among columns indicate significant ( $p < 0.05$ ) differences between samples.

In vitro antioxidant activity showed similar behavior. The antioxidant activity of CSF (Figure 1C) increased through the digestive process, being lower ( $p < 0.05$ ) in the gastric phase than in the intestinal and colonic phases. The antioxidant activity of CSE (Figure 1D) increased significantly ( $p < 0.05$ ) from the gastric phase to the intestinal and colonic phases.

### 3.2. Evaluation of CSF and CSE Cytotoxicity

Cell viability assay was performed to determine non-toxic concentrations of CSF and CSE in HepG2 and IEC-6 cells. No significant decrease ( $p < 0.05$ ) in cell viability was observed compared to untreated control cells when cells were treated with CSF at the highest concentration ( $1000 \mu\text{g mL}^{-1}$ ). In cells treated with CSE (intestinal extract) at  $1000 \mu\text{g mL}^{-1}$ , a significant reduction ( $p < 0.05$ ) in cell viability was observed compared to untreated control cells. Thus, non-cytotoxic concentrations in intestinal and hepatic cells ( $50\text{--}500 \mu\text{g mL}^{-1}$ ) were selected to study their effect in preventing intracellular ROS formation.

### 3.3. Effect of CSF and CSE on ROS Production

CSF and CSE's effect on induced intracellular ROS was evaluated in intestinal (IEC-6) and hepatic (HepG2) cells. Oxidation was induced by *t*-BOOH, which significantly increased ( $p < 0.05$ ) intracellular ROS production. In contrast, quercetin significantly reduced induced ROS formation ( $p < 0.05$ ). HepG2 and IEC-6 cells treated with CSF and CSE ( $50\text{--}500 \mu\text{g mL}^{-1}$ ) evoked a significant reduction ( $p < 0.05$ ) in the cellular ROS generation.

## 4. Discussion

Cocoa shell is an agro-food by-product that is currently discarded and could be revalued due to its high concentration of bioactive compounds [16]. The present study investigated for the first time the bioaccessibility of the antioxidant compounds contained in cocoa shell flour and a cocoa shell extract by in vitro enzymatic digestion. The knowledge generated in this study is essential since phenolic compounds released from cocoa shells can exert antioxidant activity. TPC's low bioaccessibility in cocoa flour might be due to phenolic compounds in vegetable matrices are bound to non-digestible components resistant to digestion, mainly dietary fiber [17]. Several studies have shown that cocoa shell contains a high amount of dietary fiber [18]. Phenolic compounds bound to dietary fiber and are not released in the gastrointestinal tract can reach the colon intact and can be released by microflora bacteria, promoting an antioxidant environment [19]. Besides, bound phenolic compounds' bioaccessibility can be increased by using processing technologies, such as mechanical treatment, thermal treatment, or extrusion cooking [20]. The antioxidant capacity of CSF and CSE is attributed to the presence of phenolic compounds. The simulated digestive process released phenolic compounds with antioxidant capacity from the matrix. Antioxidant capacity could play an important role in protecting against oxidative damage [21]. To determine CSF and CSE's potential in preventing oxidative damage, intestinal and hepatic cells were used. Our results show that polyphenols from the digested samples managed to inhibit and reverse the production of ROS, protecting the cells from oxidation. Some studies have shown cocoa and cocoa shell phenolic extracts' ability to protect cells from oxidative stress [22,23]. However, this study describes for the first time the effect of digested cocoa shells on the formation of intracellular ROS. Our study confirms that cocoa shells represent a potential source of antioxidant compounds potentially bioavailable, which can protect cells from oxidative stress.

## 5. Conclusions

In conclusion, the cocoa shell is an agri-food by-product that can be revalued for use as a novel food ingredient since it is a source of potentially bioavailable antioxidant compounds that can protect cells from oxidative stress.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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