JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Effect of Fat Content on the Digestibility and Bioaccessibility of Cocoa Polyphenol by an in Vitro Digestion Model

Nàdia Ortega,[†,‡] Jordi Reguant,[‡] Maria-Paz Romero,[†] Alba Macià,[†] and Maria-Jose Motilva*,[†]

[†]Food Technology Department, Escuela Técnica Superior de Ingeniería Agraria Universidad de Lleida, Av/Alcalde Rovira Roure 191, 25198 Lleida, Spain, and [‡]R+D+i Department, La Morella Nuts, S.A., Camí Ample s/n, 43392 Castellvell del Camp, Spain

This work describes the applicability of an in vitro digestion model for the evaluation of the digestibility and bioaccessibility of cocoa polyphenols (procyanidins, phenolic acids, and flavones) and for the study of the food matrix effect in relation with the fat content. For this purpose, two cocoa samples, cocoa liquor (≈50% fat content) and cocoa powder (≈15% fat content), were used. The results showed an important increase of the concentration of procyanidin (monomers and dimers), probably due to the hydrolysis of procyanidins with a high degree of polymerization (pentamers to nonamers) submitted to the digestion procedure. In relation to flavones, the concentration of aglycone forms remained almost constant after the digestion steps; in contrast, the concentration of the glycoside forms an increase in the digestion mixtures mainly after the duodenal step, probably as a result of the partial digestion of the dietary fiber present in the cocoa. The higher fat content in the cocoa liquor seemed to have a protective effect, probably related with a better micellarization that favors the stability of polyphenols during digestion.

INTRODUCTION

It is well-known that cocoa contains a wide range of polyphenols, especially procyanidins, with a high degree of polymerization. Procyanidins are a class of polyphenolic polymer composed of flavan-3-ol (catechin and epicatechin as monomers). Moreover, small amounts of flavonoids and phenolic acids have been found in various cocoa-derived products (1). Numerous studies have reported a relation between the consumption of cocoa derivatives, especially dark chocolate, with beneficial health effects on cardiovascular diseases related with the antioxidant activity of procyanidins (2, 3). Related with the health-protective effect of cocoa polyphenols, increasing interest has been devoted to evaluate their modifications, in composition and quantity, during the multiple steps from cocoa bean to chocolate that involve seed fermentation, roasting, nib-grinding, alkalizing, and conching (4).

These health benefits have led many studies to focus on the bioavailability of cocoa polyphenols. Several in vivo studies have detected procyanidin monomers and dimers in human plasma (5, 6) and the small intestine of rat (7) after the ingestion of cocoa. The bioavailability of procyanidins oligomers from cocoa samples (3) and the effects of the chiral nature of monomers, catechin and epicatechin, have also been studied (8). Schramm et al. (9) concluded that the food matrix could have an important effect on polyphenol absorption and bioavailability.

A factor common to all of the in vivo studies is the intersubject variability, which influences the interpretation of the obtained data to determine the bioavailability of the cocoa polyphenols. In addition to this individual variability, the composition of the food matrices could also determine the bioavailability of polyphenols.

Several in vitro digestion models have been applied to different food matrices to measure the release of polyphenols during digestion (10, 14). These studies have shown different trends in the polyphenol profile after gastrointestinal digestion in the function of which phenolic groups are studied. Besides, some in vitro studies have evaluated the stability and the transformation of the cocoa phenols during digestion by using pure synthesized compounds or ones extracted from cocoa food sources (8, 15, 16). However, the digestibility of these compounds might be different considering the whole cocoa samples and their derived products. The food components including proteins, carbohydrates, fiber, and fat and the interaction between these and the polyphenols have rarely been taken into account when determining polyphenol digestion. The presence of protein in the food matrix has been ascribed to a complex form with procyanidins with a high degree of polymerization and could induce low bioaccessibility of these phenolic compounds. However, a negative effect of cocoa polyphenols bioavailability after the intake of the milk-powder cocoa beverage has not been shown (17).

Taking into account that all of the in vivo trials, with either animals or human volunteers, are expensive long-term studies and they have also shown high variability between subjects (3), this work proposed an in vitro digestion model to be used as a

*To whom correspondence should be addressed. Tel: +34 973 702817. Fax: +34 973 702596. E-mail: motilva@tecal.udl.es.

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simple, fast way to measure how the food matrix influences the digestibility and bioaccessibility of polyphenols in new functional food products. So, defining the conditions that influence bioaccessibility of polyphenols can provide potential information for maximizing the use of the polyphenols as healthy nutrients. In this study, the digestibility and bioaccessibility of the phenolic fraction (procyanidins, phenolic acids, and flavones) of cocoa during the gastric and duodenal phases (small intestinal) are studied, and the effect of the fat content of the food matrix is evaluated. For this purpose, two different cocoa sources were used, cocoa liquor with a 50% fat content and cocoa powder with a 15% fat content.

MATERIALS AND METHODS

Chemicals and Reagents. (+)-Catechin, (−)-epicatechin, caffeic acid, syringic acid, protocatechuic acid, epigallocatechin, hidroxybenzoic acid, naringenin, myricetin, α-amylase, pepsin, pancreatine, and bile salts were obtained from Sigma Aldrich (St. Louis, MO). Kaempferol, dimer B$_2$, vanillic acid, and quercetin-rhamnoside were acquired from Fluka Co. (Buchs, Switzerland). Ferulic acid, coumaric acid, cinnamic acid, chlorogenic acid, quercetin, kaempferol-rutinoside, apigenin, apigeninglycoside, luteolin, kaempferol-glycoside, and luteolin-glycoside were purchased from Extrasynthese (Genay, France).

Samples. Two cocoa derivatives (liquor and powder) obtained by standard manufacturing processes were submitted to in vitro digestion. The criterion for selecting the test food was mainly based on the high and low fat contents; the cocoa liquor had a fat content of around 50%, while the cocoa powder had a fat content around 15%. Another reason that these cocoa derivatives were selected was based on the minor differences in the phenolic compositions related with the minor physical treatments to transform the cocoa liquor into powder. Cocoa liquor is produced by taking cocoa beans that have been fermented, dried, roasted, and separated from their shells and grinding their center, the cotyledon. The liquor contains both cocoa solids and cocoa butter in roughly equal proportions. The cocoa liquor is pressed, and the remaining cakes of cocoa solids are pulverized into "cocoa powder". Before the digestion, cocoa liquor and cocoa powder were ground in a laboratory mill for 30 s to obtain a homogeneous material.

Simulated Gastrointestinal Digestion. The digestion model was a slightly modified version of the technique developed by Gil-Izquierdo et al. (*10*). The model describes a three-step procedure to mimic the digestive process in the mouth, stomach (gastric digestion), and small intestine (duodenal digestion) (**Figure 1A**). The digestion starts by adding 10 mg of α-amylase (100−300 ud/g) in phosphate buffer solution (pH 6.9 with 0.04% NaCl and 0.004% CaCl$_2$) to 1 g of the cocoa sample, which is incubated for 5 min. The gastric digestion includes the pH adjusted to 2 by adding HCl concentrate and 15 mg of porcine−pepsin solution (24.750 ud/g pepsin) in 1 mL of 0.01 N HCl. This mixture is shaken in an incubator for 2 h at 37 °C. Finally, for the duodenal digestion, the pH is adjusted to 6.5 by adding NaHCO$_3$. Then, 5 mL of duodenal juice, including 2.5 mL of bile salts and 2.5 mL of pancreatin (8 g/L), is added and shaken for another 2 h at 37 °C. The bile salt concentration was previously adjusted to the fat content of the two cocoa samples (80 g/L for cocoa liquor and 50 g/L for cocoa powder). All of the digestive mixtures are incubated in the dark in an orbital incubator (Orbital Incubator Cooler, Gallenkamp) at 200 rpm.

At the end of the gastric and duodenal steps, the digestion mixtures are centrifuged for 15 min at 12000*g* at 4 °C, yielding the chyme (supernatant or soluble fraction) and the pellet (cloud or nonsoluble fraction) (**Figure 1A**). Both of these chyme and pellet fractions are freeze-dried (Lyobeta, Telstar, Barcelona, Spain) and stored for chromatographic analysis of the phenol contents (procyanidins, phenolic acids, and flavones).

Design of Dialysis Duodenal Digestion. For duodenal digestion phase with dialysis, two models were considered, static (DD-SD) and dynamic (continuous-flow) (DD-DD), respectively (**Figure 1A**). The dialysis tub used for both methods was a cellulose dialysis tub, with a molecular mass cutoff at 12400 Da. The dialysis membrane was previously conditioned by washing the tub in running water for 3−4 h. The tub was then treated with a 0.3% (w/v) solution of sodium sulfide at 80 °C for

1 min followed by acidification with a 0.2% (v/v) sulfuric acid solution. Finally, the tub was rinsed with hot water to remove the acids. All of these pretreatments were designed to remove the glycerol included as a humectant and to ensure the optimum dialysis of all of the compounds.

Static Dialysis System (DD-SD). The procedure was based on Gil-Izquierdo's et al. (*10*) modified procedure. The duodenal mixture was placed inside the conditioned dialysis tub (400 mm long × 40 mm diameter), and the dialysis tub was completely covered with a phosphate buffer solution (pH 7.4). The system was placed in a beaker and agitated for 2 h at 37 °C in an orbital agitator.

Continuous-Flow Dialysis System (DD-DD). The proposed system was designed with an adapted Liebig−West condenser and some end-fitting fluid connectors (**Figure 1B**). The first chamber contained the dialysis tub, through which the duodenal mixture flowed during the duodenal digestion step by using a peristaltic pump with a flow rate of 1 mL/min and a phosphate buffer solution (pH 7.4), which covered the dialysis tub. A temperate water solution was pumped from a bath through the water jacket to keep the system's temperature constant, under 37 °C.

In both dialysis systems, two fractions were collected at the end of the duodenal digestion step and analyzed separately. These were the outside dialysis solution (OUT), which was considered the dialyzable fraction, and the inside dialysis tub content (IN), referring to the nondialyzable fraction (**Figure 1A**). The dialyzable fraction (OUT) was considered to be the fraction that could be available for absorption into the systematic circulation by passive diffusion. Meanwhile, the nondialyzable fraction (IN) was attributed to the digested fraction that would reach colon fermentation intact. Both fractions were freeze-dried and stored for chromatographic analysis of the phenol content (procyanidins, flavones, and phenolic acids).

Chromatographic Analysis of Phenols. The two tested samples, cocoa liquor and powder, were characterized as in our previous work (*1*). The digested freeze-dried fractions obtained (chyme, pellet, IN, and OUT) were dissolved in acetone/Milli-Q water/acetic acid (70:29.5:0.5 v/v/v) at a ratio of 1:5, after which the tubes were vortexed for 3 min and centrifuged at 12500 rpm. The supernatant containing the solubilized phenols was filtered through 0.22 μm nylon filters prior to chromatographic analysis by ultraperformance liquid chromatography mass spectroscopy (UPLC-MS/MS).

UPLC-MS/MS. The chromatographic analyses were performed using a Waters Acquity Ultra-Performance Liquid Chromatography system (Waters, Milford, MA), equipped with a binary pump system (Waters). The UPLC system was coupled to a TQD mass spectrometer (Waters) using an electrospray ionization (ESI) source Z-spray. The MS was operated in negative mode to analyze the phenolic compounds. The data were acquired in selected reaction monitoring (SRM). The dwell time established for each transition was 30 ms. Data acquisition was carried out by MassLynx v 4.1 software.

The UPLC analyses were performed using an Acquity High Streng Silica (HSS) T3 column (100 mm × 2.1 mm i.d.; 1.8 μm particle size) (Waters) with a binary mobile phase; solvent A was water/acetic acid (0.2%), and solvent B was acetonitrile. Two chromatography methods were applied to characterize the phenolic profile in the cocoa samples. The first one was to analyze procyanidins, and a second one was to analyze phenolic acids and flavones. The analysis of procyanidins in cocoa samples and the analysis of phenolic acids and flavones are those reported in a previous work (*1*). In both analytical methods, the flow rate was 0.4 mL/min, and the sample volume injected was 2.5 μL.

The quantification of the procyanidins is expressed as μg of catechin, and the phenolic acids and the aglycon flavones are expressed as μg of the respective standard. All of the flavone glycosides are quantified as μg of the corresponding aglycon.

Digestibility and Bioaccesibility Indexes. To evaluate the food matrix effect on the polyphenol digestion, two different indexes were studied for each phenol group: the percentage of digestibility and the percentage of bioaccessibility. The digestibility allows the amount of phenol present in the complete digesta (chyme and pellet) after gastric or duodenal digestion of 1 g of test food (liquor or powder cocoa) to be measured

$$\text{digestibility index (\%)} = (PC_{DF}/PC_{TF}) \times 100 \qquad (1)$$
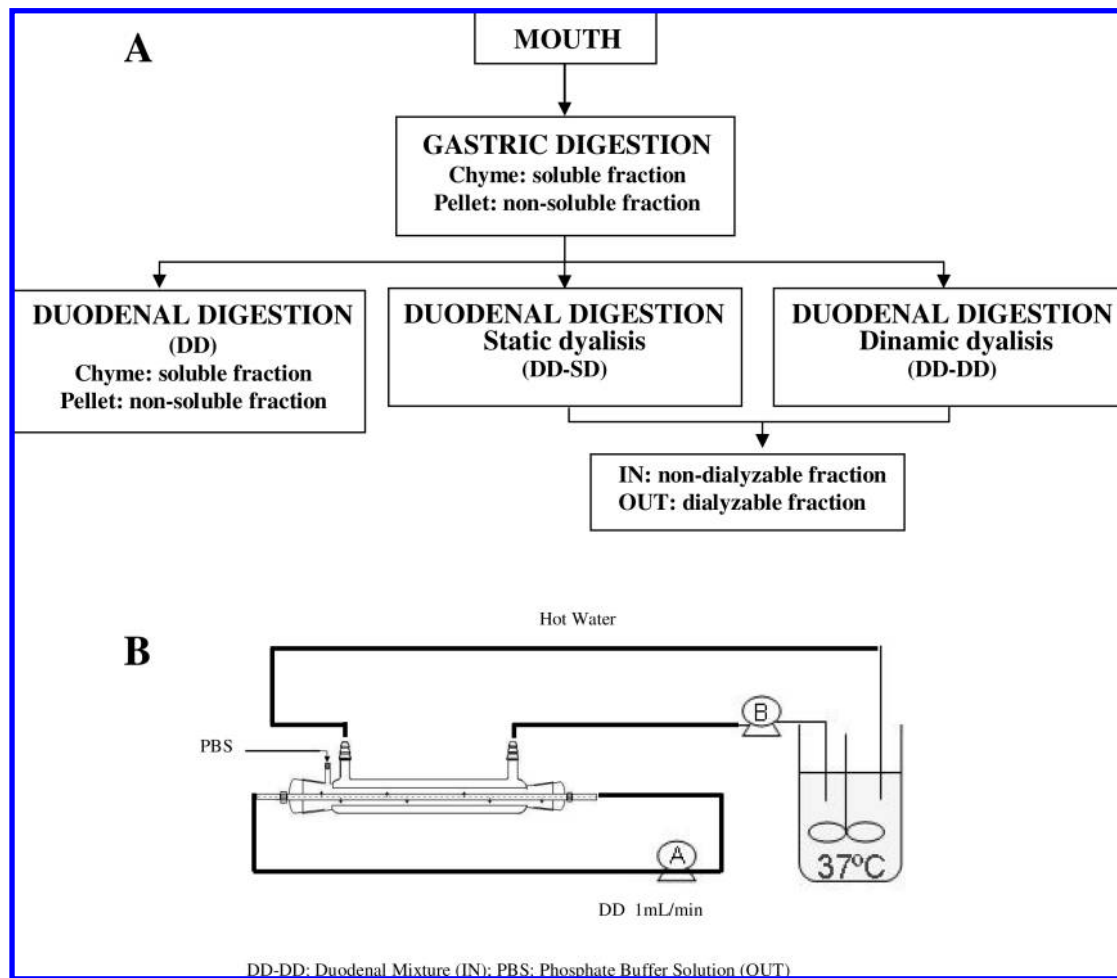
Figure 1. (**A**) Steps of the proposed in vitro digestion model: gastric digestion, duodenal digestion without dialysis (DD), duodenal digestion with static dialysis (DD-SD), and duodenal digestion with dynamic dialysis (DD-DD). (**B**) Schema of the duodenal digestion with dynamic dialysis.

where PC_{DF} = the total phenol content (μg) in the digested (chyme + pellet) and PC_{TF} = the total phenol content (μg) quantified in 1 g of test food (cocoa liquor or powder).

For each phenol group, the bioaccessibility defines the percentage of phenol that is solubilized in the chyme (supernatant) after each step of digestion. Thus, this index allows the proportion of the phenol group that could become available for absorption into the systematic circulation to be estimated:

$$\text{bioaccessibility index } (\%) = (PC_S / PC_{DF}) \times 100 \qquad (2)$$

where PC_S = the total phenol content (μg) in the chyme fraction and PC_{DF} = the total phenol content (μg) in the digested matrix (chyme + pellet). Both indexes are calculated for each digestion step, gastric and duodenal, and for both foods tested, cocoa liquor and powder.

Statistical Analysis. All data are expressed as the average of the five replicates. To simplify the results shown in the tables, the standard deviation is omitted because all of these values are lower than 10%. The data are analyzed by a one-way analysis of variance test to assess differences between cocoa sources with different fat contents. A level of $p < 0.05$ is considered a significant difference. All of the statistical analysis was carried out using Statgraphics Plus 5.1.

RESULTS AND DISCUSSION

In Vitro Simulated Digestion Method. This study is designed first of all to assess the digestibility and the stability of the phenolic fraction after the simulation of an in vitro digestion procedure applied to two different cocoa sources. The selection of these two cocoa sources was based on their different fat contents: 50% in the liquor and 15% in the powder. After the gastric and

duodenal digestion steps, the phenol content was measured in the soluble and nonsoluble (undigested) fractions, respectively called chyme and pellet (**Figure 1A**). **Tables 1−3** show the phenolic profile, based on procyanidins, phenolic acids, and flavones, respectively, after the application of the in vitro digestion model to the cocoa samples. For both the gastric and the duodenal steps, the total content (μg) of each phenol was quantified in the chyme and pellet fractions. Analyzing the procyanidin profile (**Table 1**) shows that the oligomers with a high degree of polymerization (pentamers to nonamers) present in the cocoa liquor are completely hydrolyzed during gastric digestion, leading to an important increase in the concentration of monomers and mainly of dimers. This is probably due to the acidic conditions during this step. In comparison with the liquor, the oligomer content of the cocoa powder is lower, and the increase in the concentration of dimers is not as evident after gastric digestion. In addition to their increase, monomer and dimer molecules appeared stable under acidic conditions in both cocoa samples tested. These results partially agree with some previous studies, in which monomers to pentamers from isolated procyanidins were also stable under acidic conditions (*15*).

The procyanidin content of the digested cocoa liquor after duodenal digestion was 1.8 times higher than after gastric digestion (**Table 1**). However, the effect of duodenal digestion was not as evident in the digested cocoa powder. The greater stability of procyanidins in digested cocoa liquor could be related to the protective effect of the fat-forming micellar structures within the digested matrix that probably protect the procyanidin molecules

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Table 1. Procyanidin Contents in the Different Fractions of the Gastric and Duodenal Steps of the Simulated Gastrointestinal Digestion of Cocoa Liquor and Powder[a]

| procyanidins (μg) | 1 g of food | gastrointestinal digestion model | | | | | |
| | | gastric | | | duodenal | | |
		chyme[b]	pellet[c]	total gastric	chyme[b]	pellet[c]	total duodenal
			cocoa liquor				
catechin	52.3	137	43.0	180	176	83.7	260
epicatechin	219	331	168	499	760	374	1134
dimer	284	639	306	945	888	650	1538
trimer	118	125	44.4	170	130	102	232
tetramer	25.6	18.2	2.8	21.0	10.0	17.6	27.6
pentamer	3.58	ND	ND		ND	ND	
hexamer	4.21	ND	ND		ND	ND	
heptamer	3.9	ND	ND		ND	ND	
octamer	2.85	ND	ND		ND	ND	
nonamer	2.49	ND	ND		ND	ND	
total	**716**	**1250**	**564**	**1814**	**1964**	**1226**	**3191**
			cocoa powder				
catechin	119	140	120	260	166	58.2	224
epicatechin	106	103	76	179	123	80.8	204
dimer	117	156	140	296	233	182	415
trimer	27.0	5.71	12.4	18.1	5.37	21.3	26.6
tetramer	6.73	0.85	1.65	2.49	ND	ND	
pentamer	ND	ND	ND		ND	ND	
hexamer	ND	ND	ND		ND	ND	
heptamer	ND	ND	ND		ND	ND	
octamer	ND	ND	ND		ND	ND	
nonamer	ND	ND	ND		ND	ND	
total	**376**	**405**	**350**	**755**	**528**	**342**	**870**

[a] The results are expressed as μg of phenol in relation to the phenol content of 1 g of tested food, cocoa liquor, and powder, respectively. ND, not detected. [b] Soluble fraction. [c] Nonsoluble fraction.

during enzymatic digestion. The surface-active agents, including the bile salts, present in the duodenal digestion media, form micellar and vesicular structures within the digesta (*18*), and these colloidal particles could provide further protection to the digested phenols. In fact, changes in the emulsion interface properties (droplet size and surface area) in the duodenal fluid was observed in the presence of green and black tea (*19*). The results of this study suggest that some epicatechins may be incorporated into the lipid phase of the emulsion. Considering the results of our study, a greater fat content in the digesta seems to favor the formation of the lipid emulsion droplets and the incorporation of the cocoa phenols into the lipid phase, achieving a protective effect during duodenal digestion.

In the case of the phenolic acids fraction (**Table 2**), it is interesting to note the increased content of several acids in the digesta after the gastric and duodenal steps. The increase in the hydroxybenzoic, vanillic, chlorogenic, and protocatechic acids in the two cocoa samples after both the gastric and the duodenal digestion steps should be noted. In contrast, a total loss of ferulic acid can be observed in both foods tested. Apparently, the fat content of the food tested does not affect the digestibility and stability of the phenolic acids fraction during the gastric and duodenal steps, unlike the protective effect of the fat observed in the procyanidin group.

Finally, observing the content of flavones (**Table 3**) in the digesta of both cocoa samples after the gastric step, an increase can be seen in the concentration of the aglycone forms. These results show a trend in both cocoa samples toward conversion

from glycosides to aglycone forms under acid conditions. After duodenal digestion of the cocoa liquor, significant increases were observed in the flavone content and the aglycone and the glucoside forms. This fact was not observed after duodenal digestion of the cocoa powder, so the flavone content in the digesta remained almost constant in relation to the gastric step. From the results obtained after the duodenal step, it can be concluded that the higher fat content in cocoa liquor (50% approximately) could protect the flavone glycoside forms during the duodenal digestion, probably also as a consequence of a better micellarization similar to that observed in the procyanidin group.

The increase in the total content of the phenolic acids and flavones observed in the different digested fractions (chyme and pellet) during the gastric and duodenal steps of the two cocoa matrices could be the result of the partial digestion of the dietary fiber in the tested food. Various studies have previously reported the bonding of phenolic compounds to fiber in wine (*20*) and in cocoa powder (*21*). Taking this into account and comparing the phenolic content in the tested foods and after the digestion procedure, the solubility of polyphenols seems to be higher after the simulated digestion of the tested food, where the phenols are extracted with the solvent mixture (acetone/Milli-Q water/acetic acid, 70:29.5:0.5 v/v/v) at room temperature. These softer conditions during phenol extraction could mean insufficient cellular disruption in the cocoa samples, which only allows the solubilization of the free phenolic compounds from the cocoa matrices. Subsequently, a part of these phenolic groups, phenolic acids and flavones associated with the fiber fraction in the cocoa samples before digestion, could be digested and released into the digesta, chyme, and pellet, by effect of the temperature (37 °C), time (2 h) under acidic conditions, and the enzymatic treatments during the gastric and duodenal digestion steps.

The stability of phenols under acidic conditions has been previously observed in different food matrices, such as orange juice, frozen strawberries (*10, 11, 14*), red cabbage (*12*), and chokoberries (*13*). Studies have been carried out on the stability of certain procyanidins as pure compounds under the same conditions (*8, 16*). However, all of these studies report polyphenol losses after duodenal digestion. All of the mentioned food matrices contain lower levels of fat in comparison with cocoa samples or as pure compounds with either low fat or without fat. In this study, by contrast, after the duodenal digestion of the tested food matrices, the phenolic fractions seemed stable; an increase was even observed in the phenol concentration in the chyme and pellet fractions. As mentioned above, this could be related to the interaction of the fat fraction with the polyphenols, which create a stable emulsion that favors the stability of the polyphenols.

Evaluation of the Food Matrix Effect on the Digestibility and Bioaccessibility of Phenolic Compounds. There were differences in the initial phenol content concentration in the two cocoa sources subjected to the in vitro digestion model (**Tables 1–3**). These differences make it difficult to compare the two cocoa sources to assess the matrix effect on the polyphenol digestibility and stability under gastric and duodenal conditions. To normalize the results, two different indexes for each phenolic group, digestibility and bioaccessibility, were calculated as described in the Materials and Methods.

During gastric digestion, it is interesting to note the digestibility (**Figure 2**) of the phenolic acids, especially hydroxybenzoic, syringic, and chlorogenic acids, mainly in cocoa liquor (**Table 2**). The high digestibility index of these phenolic acids could be due to a combination of factors, that is, the ring scission of the other phenolic compounds (procyanidins or flavones) in the food matrix, which leads to a major concentration of these simple phenolic molecules, and/or different rates of solubilization from

Table 2. Phenolic Acid Contents in the Various Fractions of the Gastric and Duodenal Steps of the Simulated Gastrointestinal Digestion of Cocoa Liquor and Powder[a]

phenolic acids (μg)	1 g of food	gastric			duodenal		
		chyme[b]	pellet[c]	total gastric	chyme[b]	pellet[c]	total duodenal
cocoa liquor							
hydroxybenzoic acid	1.53	7.67	3.83	11.5	15.0	5.53	20.5
vanillic acid	0.67	17.0	7.31	24.3	19.3	10.0	29.2
caffeic acid	2.79	3.43	4.24	7.67	4.50	ND	4.50
syringic acid	0.31	5.30	2.60	7.90	5.21	ND	5.21
ferulic acid	0.06	ND	ND		ND	ND	
vanillin	0.11	1.33	2.27	3.60	2.88	4.88	7.76
coumaric acid	0.70	0.86	ND	0.86	1.14	1.67	2.82
cinnamic acid	0.65	1.55	ND	1.55	3.06	ND	3.06
chlorogenic acid	1.35	54.5	15.9	70.4	53.8	18.4	72.2
protocatechuic acid	18.3	63.8	24.7	88.5	93.5	43.1	137
total	**26.5**	**156**	**60,9**	**216**	**198**	**83.6**	**282**
cocoa powder							
hydroxybenzoic acid	7.39	28.1	11.7	39.8	41.9	6.33	48.3
vanillic acid	2.16	72.4	11.9	84.3	35.2	7.95	43.2
caffeic acid	10.2	5.48	3.57	9.05	7.23	ND	7.23
syringic acid	1.75	6.05	3.28	9.33	7.43	ND	7.43
ferulic acid	0.11	ND	ND		ND	ND	
vanillin	0.25	1.49	2.18	3.67	5.56	5.10	10.7
coumaric acid	3.49	1.30	1.20	2.50	1.72	0.37	2.09
cinnamic acid	4.11	2.15	2.49	4.63	7.19	0.68	7.87
chlorogenic acid	2.58	10.8	6.66	17.4	22.3	ND	22.3
protocatechuic acid	150	240	78.9	319	403	36.8	439
total	**182**	**368**	**122**	**490**	**531**	**57**	**588**

[a] The results are expressed as μg of phenol in relation to the phenol content of 1 g of tested food, cocoa liquor, and powder, respectively. ND, not detected. [b] Soluble fraction. [c] Nonsoluble fraction.

Table 3. Flavone Contents in the Various Fractions of the Gastric and Duodenal Steps of the Simulated Gastrointestinal Digestion of Cocoa Liquor and Powder[a]

flavones (μg)	1 g of food	gastric			duodenal		
		chyme[b]	pellet[c]	total gastric	chyme[b]	pellet[c]	total duodenal
cocoa liquor							
epigallocatechin	4.21	4.24	2.07	6.31	10.3	5.25	15.6
quercetin	9.14	ND	14.1	14.1	7.32	13.6	20.9
quercetin-glycoside	84.7	16.5	57.9	74.4	79.6	66.4	146
quercetin-rutinoside	0.95	ND	ND		ND	ND	
quercetin-arabinoside	103	14.2	76.1	90.3	77.4	89.2	167
dihydroquercetin	4.28	ND	5.10	5.10	7.47	6.70	14.2
dihydroxykaempferol	27.7	0.83	2.34	3.17	26	8.07	34.1
kaempferol-rutinoside	21.1	5.4	13.6	19.0	11.6	9.87	21.5
kaempferol-glycoside	74.2	29.4	100	129	64.0	118	182
naringenin	1.03	0.15	1.25	1.40	0.72	0.99	1.71
naringenin-glycoside	2.47	0.42	0.72	1.14	2.49	1.59	4,08
myricetin-glycoside	5.29	ND	22.5	22.5	ND	ND	
luteolin	4.07	0.38	3.30	3.68	1.47	3.08	4.55
luteolin-glycoside	8.66	0.67	2.08	2.75	1.28	3.05	4.33
apigenin	0.15	ND	0.57	0.57	0.35	0.52	0.87
apigenin-glycoside	0.04	ND	ND		ND	ND	
total aglycone forms	**18.6**	**4.78**	**21.3**	**26.0**	**20.2**	**23.4**	**43.6**
total glycoside forms	**258**	**67.4**	**281**	**321**	**270**	**303**	**559**
cocoa powder							
epigallocatechin	7.54	4.24	4.08	8.32	5.57	1.52	7.09
quercetin	7.07	ND	7.57	7.57	4.87	5.82	10.7
quercetin-glycoside	72,7	16.5	40.8	57.3	29.6	32.5	62.1
quercetin-rutinoside	2.85	ND	ND		ND	ND	
quercetin-arabinoside	81.9	14.2	55.2	69.4	28.9	43	71.9
dihydroquercetin	7.42	ND	ND		ND	ND	
dihydroxykaempferol	21.1	3.7	11.4	15.1	8.07	11.5	19.6
kaempferol-rutinoside	71.3	5.4	26.9	32.3	4.97	19.9	24.9
kaempferol-glycoside	15.7	29.4	222	251	68.2	195	263
naringenin	0.62	0.15	0.78	0.93	ND	0.71	0.71
naringenin-glycoside	2.49	0.42	0.60	1.02	0.93	0.65	1.58
myricetin-glycoside	19.8	ND	ND		ND	ND	
luteolin	3.58	0.38	5.52	5.90	0.99	2.03	3.02
luteolin-glycoside	19.3	0.67	4.63	5.30	1.58	4.42	6.12
apigenin	0.12	ND	0.43	0.43	0.20	0.38	0.58
apigenin-glycoside	0.09	ND	ND		ND	ND	
total aglycone forms	**18.9**	**4.78**	**18.4**	**23.2**	**11.6**	**10.1**	**21.7**
total glycoside forms	**299**	**70.3**	**361**	**431**	**142**	**296**	**438**

[a] The results are expressed as μg of phenol in relation to the phenol content of 1 g of tested food, cocoa liquor, and powder, respectively. ND, not detected. [b] Soluble fraction. [c] Nonsoluble fraction.

cocoa fiber. In contrast to the phenolic acids, no differences were observed in the digestibility of glycoside flavones between the cocoa matrices after the gastric digestion step. However, with regard to the flavone group, the aglycone forms did show a significantly higher digestibility in both cocoa samples. This could be due to the greater stability of the aglycone form under acidic conditions and/or to the induction of the hydrolysis of glycosides, resulting in the respective aglycones.

The higher phenol digestibility in cocoa liquor may be due to fat digestion under the acidic conditions and the action of the bile salts during the gastric and duodenal steps, respectively, which may favor disruption of the food matrix. Furthermore, the major fat content of cocoa liquor can mean higher micellarization efficiency, leading to a major surface interaction with the solubilized phenols or greater solubility of phenols in micelles, leading to protection during duodenal digestion. This possible protective role of the fat is especially evident in the digestibility percentage of procyanidins (mainly monomers and dimers) and of some phenolic acids (**Figure 2**). Moreover, it is interesting to note that the percentage of the flavones' digestibility is lower than for the other phenolic groups, mainly after the gastric step; this may be due to the lack of stability in acidic conditions. In contrast, the digestibility of these two groups, aglycones and glycosides, increases after the duodenal step. In brief, the fat content in the digestion media can improve the digestibility of the phenols in a combination of factors, that is, a better solubilization from the food matrix and greater protection during digestion due to the major micellarization of the solubilized phenols.

The index of bioaccessibility is aimed at evaluating the quantity of phenols digested (pellet + chyme) that are solubilized into the water phase (chyme) and become available for potential absorption in the systematic circulation in biological systems. Unlike the digestibility index, the bioaccessibility of the phenols was not affected by the fat content in the digestion media. In contrast, the main differences in this index were observed between phenolic groups, probably related to their hydrophilic nature. As **Figure 3** shows, the highest solubilization into the water phase corresponds to phenolic acids, followed by procyanidins. Flavones showed the lowest water phase solubilization, and it could be related with the presence of a glycosidic residue in their molecular structure. The bioaccessibility indexes of flavone glycosides showed significant differences ($p < 0.05$) between cocoa matrices in both digestion steps (**Figure 3**). As can be seen in the quantification of flavones
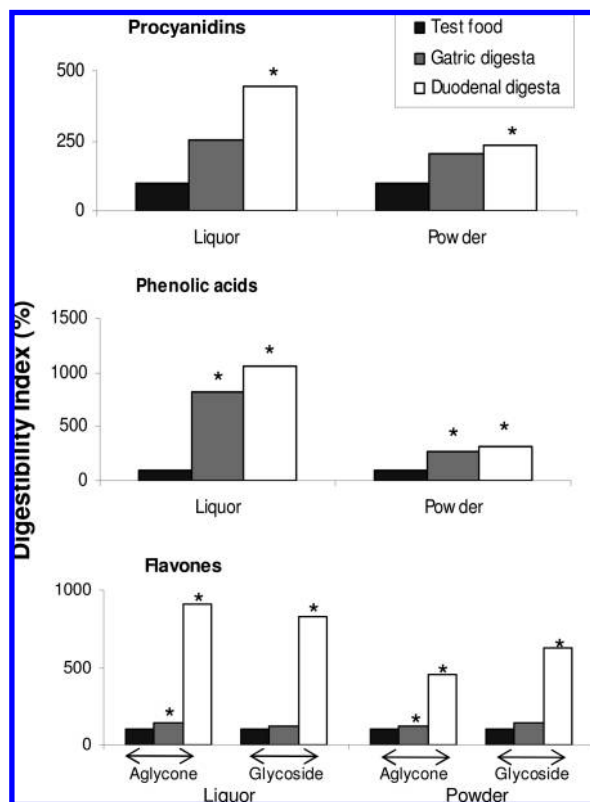
Figure 2. Digestibility index of procyanidins, phenolic acids, and flavones (aglycone and glycoside forms) after the gastric and duodenal digestion steps of cocoa liquor and powder. The index has been calculated according to eq 1 of the Materials and Methods. *Denotes significant differences ($p <$ 0.05) between cocoa matrices, liquor, and powder in each digestion step.
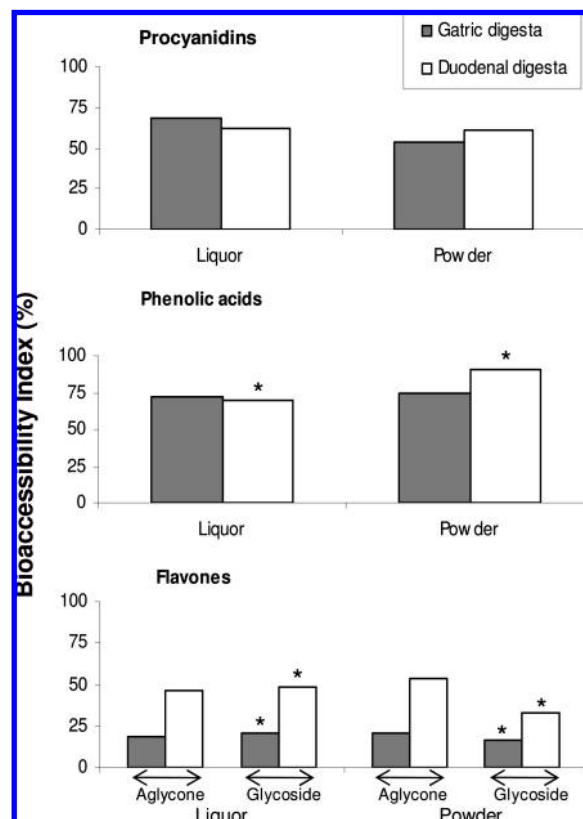

Figure 3. Bioaccessibility index of procyanidins, phenolic acids, and flavones (aglycone and glycoside forms) after the duodenal digestion step of cocoa liquor and powder. The index has been calculated according to eq 2 of the Materials and Methods. *Denotes significant differences ($p <$ 0.05) between cocoa matrices, liquor, and powder in each digestion step.

after the digestion steps (**Table 3**), the digestion allowed an increase in the concentration of glycoside forms of flavones in the digesta (chyme) mainly after the duodenal step. However, the major lipophilicity of the aglycone flavones led to a greater retention in the pellet and lower water phase solubilization (chyme), indicating a minor potential bioaccesibility.

In Vitro Static vs Continuous-Flow Duodenal Digestion. After the evaluation of the digestibility and bioaccessibility indexes after the gastric and duodenal digestion, a dialysis phase was considered for the duodenal step. The development of a dialyzed digestion method was aimed at estimating the passive diffusion of the digested phenols solubilized in the water phase (dialyzed fraction, OUT) (**Figure 1A**). For this, two models, static (DD-SD) and dynamic (DD-DD) dialysis, were considered. The static dialyzed model only reproduces the diffusion of a molecule to reach the equilibrium condition. Taking this into consideration, a dynamic continuous-flow duodenal system (**Figure 1B**) was proposed as an enhanced simulation of the duodenal conditions. In the dialysis dynamic model developed in this study, the digested matrix flows throughout the entire duodenal step.

To compare the efficiency of two dialyzed models to the estimation of the phenol digestibility, the total concentration (μ g) of each phenolic group (procyanidins, phenolic acids, and flavones) from the complete duodenal mixture (nondyalizable, IN plus dialyzable, and OUT fractions) was quantified. The total concentration (μ g) of each phenolic group from the duodenal digestion model (DD) (chyme plus pellet) without dyalisis shown in **Tables 1**−**3** was considered as the control. The content of the phenolic compounds (procyanidins, phenolic acids, and flavones, respectively) in the mixture after the duodenal digestion (**Figure 4**) did not show significant differences ($p <$ 0.05) between

the duodenal digestion model with dynamic continuous-flow (DD-DD) and the nondialyzed digestion model (DD) (**Figure 1A**). Meanwhile, the static dialyzed method (DD-SD) gave an underestimation of the digestion of phenols as compared with the other models. Thus, the total phenols quantified in the static-dialyzed mixture (IN plus OUT) (DD-SD) were significantly lower ($p < 0.05$) than the total phenols quantified in the mixtures corresponding to the dynamic dialysis (DD-DD) and nondialyzed methods (DD). The losses in the polyphenol content of the static-dialyzed mixture could be attributed to the large molecules (lipids, carbohydrates, or fiber) in the food matrices tested being held on the dialysis membrane, reducing the membrane's surface for diffusion. These results are in line with previous studies by other authors, in which the sugar content of the tested food limited the diffusion of polyphenols during digestion in a dialyzed model (*10*, *11*, *15*). Because of the continuous flow of the digested matrix in the dynamic dialyzed digestion, the possibility that phenols could be held on the dialysis membrane is reduced. As a result, the dynamic dialysis could be proposed as a better model for estimating the passive diffusion of the digested phenols during the duodenal step.

In conclusion, the in vitro digestion method coupled with the a continuous-flow dialyzed duodenal step provided a good model for examining the digestibility and bioaccessibility of polyphenols in cocoa food matrices. This could be a rapid and a simple tool evaluating the effect of the food processing on the stability and digestibility of bioactive compounds and estimating their digestibility and potential bioavailability in the formulation phase of new functional food products. Moreover, this tool could also allow the food matrix constituents to be selected to incorporate
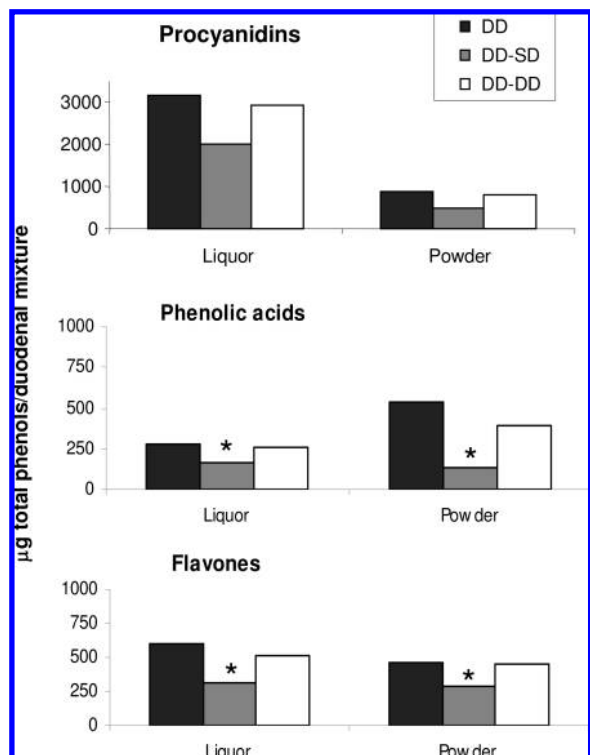
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Figure 4. Total content (μg) of procyanidins, phenolic acids, and flavones in the digestion mixture of cocoa liquor and cocoa powder after duodenal step digestion by the different models assayed: duodenal digestion without dialysis (DD), duodenal digestion with static dialysis (DD-SD), and duodenal digestion with dynamic dialysis (DD-DD). *Denotes significant differences ($p < 0.05$) between duodenal digestion models in each cocoa source, liquor, and powder.

the active compounds, thus ensuring their active and available status after human ingestion. The results obtained let us conclude that the fat content of the cocoa samples tested enhances the digestibility of some phenolic compounds, especially procyanidins, during duodenal digestion. The possible mechanism is probably related with the ability of the fat fraction to interact with the certain polyphenolic compounds following a better micellarization of the digested phenols.

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