

Flavanol and Flavonol Contents of Cocoa Powder Products: Influence of the Manufacturing Process

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Major brands of cocoa powder products present in the Spanish market were analyzed for monomeric flavanols [(+)-catechin and (–)-epicatechin] and flavonols [quercetin-3-glucuronide, quercetin-3-glucoside (isoquercitrin), quercetin-3-arabinoside, and quercetin]. In addition, the influence of the manufacturing process of cocoa powder products, in particular, the alkalization treatment (*Dutching*), on the original content of these flavonoids has been studied. (–)-Epicatechin was in the range of 116.02–730.26 $\mu\text{g/g}$, whereas (+)-catechin was in the range of 81.40–447.62 $\mu\text{g/g}$ in the commercial cocoa products studied. Among flavonols, quercetin-3-arabinoside and isoquercitrin were the major flavonols in the cocoa powder products studied, ranging from 2.10 to 40.33 $\mu\text{g/g}$ and from 3.97 to 42.74 $\mu\text{g/g}$, respectively, followed by quercetin-3-glucuronide (0.13–9.88 $\mu\text{g/g}$) and quercetin aglycone (0.28–3.25 $\mu\text{g/g}$). To our knowledge, these results are the first quantitative data in relation to the content of individualized flavonol derivatives in commercial cocoa powder products. The alkalization treatment resulted in 60% loss of the mean total flavonoid content. Among flavanols, (–)-epicatechin presented a larger decline (67%, as a mean percentage difference) than (+)-catechin (38%), probably because of its epimerization into (–)-catechin, a less bioavailable form of catechin. A decline was also confirmed for di-, tri-, and tetrameric procyanidins. In the case of flavonols, quercetin presented the highest loss (86%), whereas quercetin-3-glucuronide, quercetin-3-arabinoside, and isoquercitrin showed a similar decrease (58, 62, and 61%, respectively). It is concluded that the large decrease found in the flavonoid content of natural cocoa powder, together with the observed change in the monomeric flavanol profile that results from the alkalization treatment, could affect the antioxidant properties and the polyphenol bioavailability of cocoa powder products.

KEYWORDS: Cocoa powder; catechin; epicatechin; flavonols; quercetin; alkalization

INTRODUCTION

Cocoa (*Theobroma cacao*) and its derived products represent a very rich source of dietary flavonoids, and its consumption has increased 2.0 millions tons per year from 1960 to 2004 (1). Spain is the country that has the largest consumption of cocoa powder products per person (1668 g/person/year), followed by Norway (1647 g/person/year) and Sweden (1288 g/person/year) [reports of ACNielsen, Euromonitor International, and Caobisco Association of the Chocolate biscuit and confectionery industries of the European Union (EU)], representing approximately 28% of the total cocoa consumption in this country. Among the Spanish population, children between 7 and 14 years old are the largest consumers (Family Food Panel, Spain 2005–2006, Taylor Nelson Sofres). As recently reviewed by Ding et al. (2), outcomes from chocolate and cocoa human feeding trials are associated with a decrease in low-density lipoprotein (LDL) oxidation, oxidative stress, platelet activation, platelet function,

and an increase in high-density lipoprotein (HDL) concentration, antioxidant status, and NO bioactivity, together with an improvement in endothelial function. A lower systolic and diastolic blood pressure and an improvement in insulin sensitivity are other potential health benefits reported from cocoa consumption (2).

According to Lee et al. (3), cocoa contains a higher content of flavonoids per serving than teas or red wine. Flavonoids present in cocoa include flavanols, anthocyanins, flavonols, and flavones (4–9). Flavanols, the most abundant flavonoids in cocoa, comprise the monomeric flavanols, (+)-catechin and (–)-epicatechin, and their oligomeric and polymeric forms (procyanidins). (–)-Epicatechin has been reported as the major monomeric flavanol in cocoa, representing ca. 35% of the total phenolic content (4). Cocoa is composed of a complex series of procyanidins consisting primarily of (–)-epicatechin (10, 11). Procyanidins with a degree of polymerization (DP) up to decamer have been identified and quantified by normal-phase high-performance liquid chromatography (HPLC)/mass spectrometry (MS) (12, 13). Oligomers (procyanidins B1, B2, B5,

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and C1) and polymers account for $\geq 90\%$ of total polyphenols, and monomers account for 5–10% (4, 13). Anthocyanins identified in the cocoa bean include the -3 -arabinoside and -3 -galactoside derivatives of cyanidin and represent ca. 4% of the total polyphenol content of the cocoa bean; however, they could be hydrolyzed during the fermentation process of cocoa (4, 14, 15). The flavonol glycosides, quercetin-3-*O*-arabinoside and quercetin-3-*O*-glucoside (isoquercitrin), and quercetin aglycone have been identified in cocoa (5, 16–18). More recently, quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-glucuronide and the flavones, apigenin, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), luteolin, and luteolin-7-*O*-glucoside, have been identified for the first time in cocoa (5, 18). To our knowledge the concentration of flavonols and flavones in cocoa and its derived products has not been extensively reported (6, 7). Currently, the physiological effects derived from cocoa consumption have been ascribed only to flavanols (2). However, quercetin possesses higher free-radical scavenging properties than (+)-catechin (19) and has been shown to be one of the most effective flavonoids for the preservation of endogenous α -tocopherol in LDL cholesterol (20). In addition, quercetin and its metabolites isorhamnetin and tamarixetin produce vasodilation by means of endothelium-dependent and -independent mechanisms (7).

As occurs in other plant-derived foods, the phenolic content of cocoa-derived products is largely dependent upon the cultivar, origin, agricultural practices, and postharvest practices and processing (4, 8). Polyphenols in the cocoa beans are stored in the cotyledons. Once fermented and dried, the nib of the cocoa bean is roasted and ground, resulting in the cocoa liquor, which is the basis for chocolate manufacture. Cocoa powder is made by removing part of the cocoa butter from the cocoa liquor. Alkalinization (or *Dutching*) of the nibs, liquor, or powder can also be applied to change the color of the product, in particular, for the production of cocoa powder products (21). All of these steps, particularly fermentation and alkalinization, are assumed to lead to considerable losses of cocoa polyphenol, but scientific data in relation to this issue are still limited (4, 22–24).

This fact together with the necessity of more data related to the cocoa phenolic profile, in particular, of other antioxidant phenols, such as flavanols, have prompted us to determine the content of monomeric flavanols and novel flavanols in major brands of cocoa powder products present in the Spanish market and to study the influence of the manufacturing process of cocoa powder products, especially the alkalinization treatment, on the original phenolic content of cocoa.

MATERIALS AND METHODS

Standards. (+)-Catechin and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO); (–)-epicatechin ($>90\%$ purity) was purchased from Fluka (Neu-Ulm, Switzerland); and quercetin-3-glucoside (isoquercitrin) and quercetin were purchased from Extrasynthèse (Genay, France). Standard solutions were freshly prepared daily under dimmed light and stored in the refrigerator (4 °C) in amber glass bottles. Standards were filtered through Waters 4 mm PTFE 0.45 μm filter before injection on the column.

Samples. A total of 11 different cocoa powder products available in the Spanish market were analyzed. Different production batches of some of these products were also studied ($n = 6$ for cocoa 1, 2, and 3). To evaluate the effect of the manufacturing process of cocoa powder products on the phenolic composition of cocoa, 10 different batches of natural cocoa powder (10–12% fat) obtained from processed (fermented, dried, roasted, ground, and defatted) cocoa beans (Forastero variety of different origins) were studied. The different batches of natural cocoa powder were first submitted to alkalinization up to pH 7.2, and then both types of cocoa powders (natural and alkalinized)

were manufactured into different cocoa powder products (drink mixes) by the addition of different ingredients, such as sugar or artificial sweeteners, salt, lecithin, calcium phosphate, wheat flour, fiber, and flavoring agents. One cocoa powder product derived from natural cocoa powder, cocoa product A (20–22% cocoa), and two different products made from alkalinized cocoa powder, cocoa product B (40–42% cocoa) and cocoa product C (16–18% cocoa), were analyzed.

Sample Preparation. Phenolic compounds from natural cocoa or cocoa powder products were extracted as described by Andrés-Lacueva et al. (18). Approximately 0.5 g of each sample was mixed with deionized water (5 mL at 100 °C) and methanol (20 mL). The extract was shaken in a vortex for 1 min at room temperature and centrifuged for 10 min at 1800g. It was then concentrated under vacuum below 40 °C to a final volume of 4 mL, avoiding UV light exposure. Finally, the samples were filtered through a Waters 4 mm PTFE 0.45 μm filter, before injection on the column.

LC–DAD Analysis of Flavonoids. A Hewlett-Packard series 1050 (Palo Alto, CA) liquid chromatography system equipped with a 1050 M diode array detector (DAD) and an automatic injector coupled to a Chemstation HP Rev. Asterix. 05.02 was used. Separation (100 μL) was performed on a reversed-phase Nucleosil 120 C₁₈ (250 \times 4 mm, 5 μm) column at 40 °C. A gradient consisting of solvent A [96.6:3.4 water/glacial acetic acid (v/v)] and solvent B [20:80 solvent A/acetonitrile (v/v)] was applied at a flow rate of 1.5 mL/min as described by Andrés-Lacueva et al. (18): 0–2% B linear from 0 to 5 min, 2–4% B linear from 5 to 10 min, 4% B isocratic from 10 to 12 min, 4–8% B linear from 12 to 14 min, 8% B isocratic from 14 to 18 min, 8–20% B from 18 to 22 min, 20–25% from 22 to 26 min, 25–35% from 26 to 30 min, 35–60% from 30 to 35 min, followed by washing (solvent B) and re-equilibration of the column. DAD detection was carried out at 280 and 365 nm. The identification of flavonoids was performed by LC–MS, as described below. Flavanols and flavonols were quantified at 280 and 365 nm, respectively, using external standard calibration curves. For the quantification of quercetin-3-glucuronide and quercetin-3-arabinoside, the calibration curves of quercetin and isoquercitrin were used, respectively.

LC–MS Identification of Flavonoids. Identification of flavonoids was performed by LC–MS as described by Andrés-Lacueva et al. (18). An Alliance 2690 module from Waters (Milford, MA) chromatography system equipped with an automatic injector and a VG Platform II quadrupole mass spectrometer (Micromass, Manchester, U.K.) with an atmospheric pressure chemical ionization (APCI) interface was used. Separation was performed on a reversed-phase Phenomenex Luna C₁₈ analytical column (50 \times 2.0 mm i.d., 5 μm) (Torrance, CA). The solvent gradient described above was applied at a flow rate of 0.4 mL/min. The working conditions for the APCI were as follows: drying gas (N₂) was heated to 150 °C and introduced into the capillary region at a flow rate of 200 L/h. The capillary was heated to 400 °C, and the corona voltage was held at -3.0 kV. The extraction voltage was -40 V. Full-scan data acquisition was performed scanning from m/z 140 to 900 in centroid mode and using a cycle time of 2.0 s and an interscan time of 0.2 s. Selected ion monitoring (SIM) was used with a dwell time of 300 ms for monitoring the following ions: (–)-epicatechin and (+)-catechin (m/z 289), procyanidins (m/z 577), trimers (m/z 865), and tetramers (m/z 1153).

RESULTS AND DISCUSSION

Flavanol and Flavonol Contents of Commercial Cocoa Powder Products. (+)-Catechin, (–)-epicatechin, quercetin-3-glucuronide, quercetin-3-glucoside (isoquercitrin), quercetin-3-arabinoside, and quercetin were quantified in the different cocoa powder products (Figure and Table 1). The identification of these flavonoids has been performed by LC–MS (18) and further confirmed by LC–MS/MS as described by Sánchez-Rabáneda et al. (5).

In general, flavanols were presented in a higher concentration than flavonols in all of the products studied (Table 1). As expected, (–)-epicatechin was the most abundant monomeric flavanol in cocoa powder products (7, 22, 24). Values ranged

Table 1. Concentration ($\mu\text{g/g}$) of Flavanols and Flavonols in Commercial Cocoa Powder Products^a

Product #	(+)-Catechin	(-)-Epicatechin	Q-3-glucuronide	Isoquercitrin	Q-3-arabinoside	Quercetin
1						
Batch#1	157.61 (10.53)	322.00 (12.08)	0.91 (0.11)	9.74 (0.32)	13.00 (0.08)	0.57 (0.03)
Batch#2	174.96 (0.19)	335.28 (4.09)	1.27 (0.01)	10.92 (0.11)	9.72 (0.28)	1.58 (0.02)
Batch#3	166.45 (8.33)	344.30 (7.13)	1.48 (0.00)	11.07 (0.82)	12.26 (0.60)	2.03 (0.30)
Batch#4	237.71 (19.73)	413.58 (38.93)	2.06 (0.36)	14.88 (1.18)	15.95 (1.26)	2.87 (0.14)
Batch#5	183.45 (15.30)	331.03 (12.30)	2.38 (0.03)	13.57 (1.04)	13.22 (1.68)	2.97 (0.21)
Batch#6	184.59 (3.57)	427.40 (23.08)	1.30 (0.14)	13.44 (1.30)	14.83 (1.04)	2.46 (0.20)
Mean	184.13 (9.61)	362.26 (16.27)	1.57 (0.11)	12.27 (0.79)	13.16 (0.82)	2.08 (0.15)
2						
Batch#1	265.17 (11.53)	264.72 (26.14)	1.38 (0.01)	9.79 (0.26)	13.52 (0.95)	1.37 (0.04)
Batch#2	206.43 (16.12)	191.26 (17.42)	0.69 (0.08)	6.26 (0.67)	5.46 (0.07)	0.51 (0.03)
Batch#3	229.61 (21.21)	245.97 (25.63)	2.60 (0.16)	7.20 (0.34)	7.58 (1.06)	0.91 (0.06)
Batch#4	267.98 (1.16)	326.71 (23.86)	2.18 (0.18)	12.79 (1.17)	14.45 (1.31)	3.24 (0.44)
Batch#5	359.66 (7.79)	395.80 (19.37)	1.69 (0.10)	13.08 (0.74)	6.12 (0.43)	1.14 (0.18)
Batch#6	447.62 (22.16)	618.50 (52.94)	1.34 (0.11)	9.13 (1.15)	12.61 (0.88)	1.21 (0.16)
Mean	296.08 (13.33)	340.49 (27.56)	1.65 (0.11)	9.71 (0.72)	9.96 (0.78)	1.40 (0.15)
3						
Batch#1	124.75 (5.22)	168.45 (5.11)	1.27 (0.09)	5.42 (0.25)	5.96 (0.42)	0.28 (0.02)
Batch#2	81.40 (2.75)	116.02 (3.03)	0.13 (0.01)	3.97 (0.20)	4.36 (0.31)	0.62 (0.10)
Batch#3	150.57 (7.59)	166.48 (6.92)	0.29 (0.01)	5.22 (0.21)	6.11 (0.60)	0.56 (0.07)
Batch#4	178.56 (5.96)	280.85 (12.84)	0.55 (0.02)	6.58 (0.18)	7.91 (0.26)	0.70 (0.01)
Batch#5	171.35 (3.23)	250.57 (13.93)	1.03 (0.07)	7.72 (0.41)	9.28 (0.65)	0.68 (0.06)
Batch#6	115.91 (0.51)	212.59 (0.27)	0.86 (0.06)	6.53 (0.56)	7.43 (0.04)	0.62 (0.03)
Mean	137.09 (4.21)	199.16 (7.02)	0.69 (0.04)	5.91 (0.30)	6.84 (0.38)	0.58 (0.05)
4						
Batch#1	254.56 (15.99)	276.32 (28.70)	1.59 (0.04)	11.37 (0.64)	11.69 (1.69)	2.60 (0.24)
Batch#2	158.26 (4.17)	260.11 (23.88)	0.80 (0.11)	8.41 (0.72)	8.67 (0.26)	1.46 (0.15)
Batch#3	241.17 (16.35)	408.71 (29.75)	0.82 (0.06)	6.51 (0.41)	11.80 (0.34)	2.38 (0.17)
Batch#4	278.07 (14.15)	400.44 (31.88)	0.97 (0.07)	8.86 (1.58)	10.46 (0.73)	1.87 (0.10)
Batch#5	192.14 (5.95)	360.59 (24.93)	1.71 (0.20)	11.98 (1.06)	13.77 (0.07)	1.96 (0.23)
Batch#6	173.36 (18.45)	306.13 (2.48)	0.60 (0.05)	9.63 (0.58)	10.40 (0.50)	1.64 (0.14)
Mean	216.26 (12.51)	335.38 (23.60)	1.08 (0.09)	9.46 (0.83)	11.13 (0.60)	1.98 (0.17)
5	229.38 (24.87)	365.80 (29.41)	2.27 (0.18)	15.06 (1.02)	16.34 (0.22)	2.96 (0.50)
6	120.75 (12.21)	313.99 (30.31)	8.89 (0.62)	27.33 (0.86)	25.68 (1.80)	1.04 (0.10)
7	295.79 (18.54)	437.99 (29.14)	9.88 (0.69)	13.39 (1.23)	18.81 (1.32)	1.04 (0.07)
8	226.78 (23.74)	730.26 (60.72)	6.66 (1.17)	24.90 (2.31)	40.33 (1.36)	1.49 (0.10)
9	281.46 (19.70)	319.06 (32.31)	3.92 (0.01)	26.61 (2.88)	20.90 (1.46)	0.64 (0.05)
10	297.53 (23.41)	529.49 (34.67)	5.94 (0.47)	42.74 (3.12)	31.51 (2.21)	1.12 (0.09)
11	137.99 (10.99)	44.84 (4.30)	2.15 (0.37)	8.95 (0.15)	2.10 (0.15)	1.39 (0.08)

^a Mean ($n = 2$); standard deviation (SD).

from 116.02 to 730.26 $\mu\text{g/g}$ for (-)-epicatechin (mean = 327.91 $\mu\text{g/g}$), and from 81.40 to 447.62 $\mu\text{g/g}$ for (+)-catechin (mean = 212.61 $\mu\text{g/g}$) (Table 1). The total monomeric content ranged from 182.84 to 1066.13 $\mu\text{g/g}$ (mean = 540.52 $\mu\text{g/g}$). A range of 180–320 $\mu\text{g/g}$ (mean = 262.0 $\mu\text{g/g}$, $n = 15$) has been reported for the (-)-epicatechin content of cocoa powder products (25). In another study, (-)-epicatechin ranged from 1580 to 2580 $\mu\text{g/g}$ and (+)-catechin ranged from 610 to 900 $\mu\text{g/g}$ for natural cocoa powders ($n = 3$) (24). However, lower values were found for alkalized cocoa powders [180–380 $\mu\text{g/g}$ for (-)-epicatechin and 230–350 $\mu\text{g/g}$ for (+)-catechin ($n = 2$)] (24). Recently, Tomas-Barberan et al. (26) have reported (+)-catechin values of 6460 and 2020 $\mu\text{g/g}$ and (-)-epicatechin values of 25650 and 3300 $\mu\text{g/g}$ for a polyphenol-rich ($n = 1$) and a conventional ($n = 3$) cocoa powder, respectively.

Quercetin-3-arabinoside and isoquercitrin were the major flavonols in the cocoa powder products studied, and both were presented in very similar content [2.10–40.33 $\mu\text{g/g}$ for quercetin-3-arabinoside and 3.97–42.74 $\mu\text{g/g}$ for isoquercitrin] (Table 1). Quercetin-3-glucuronide ranged from 0.13 to 9.88 $\mu\text{g/g}$, whereas quercetin aglycone was found in very little amount (0.20–3.25 $\mu\text{g/g}$) (Table 1). The content of total flavonols quantified ranged from 9.08 to 81.31 $\mu\text{g/g}$. In general, products 6–10 were characterized by a higher concentration of quercetin derivatives than the remaining ones (products 1, 2, 3, 4, 5, and 11) (Table 1). To our knowledge, these results are the first quantitative data in relation to the content of individualized flavonol

derivatives in commercial cocoa powder products. If the different quercetin derivatives were expressed in terms of quercetin equivalents in milligrams per 100 g, which is the form currently used in nutrient databases, the cocoa powder products analyzed herein would provide a content ranging from 0.42 to 6.54 mg/100 g of quercetin equivalents. On a weight basis, cocoa powder products would provide an amount of quercetin close to that of broccoli (frozen, chopped, and unprepared) (0.91–3.52 mg/100 g, $n = 3$), apples (Golden Delicious, with peel, raw, *Malus domestica*) (1.57–4.40 mg/100 g, $n = 10$), or grapes (red, raw, *Vitis vinifera*) (0.00–3.98 mg/100 g, $n = 6$) (27). However, the amount of quercetin per serving provided by cocoa powder products (0.083–1.3 mg for a 20 g serving) is much lower than that of the aforementioned food sources [broccoli (1.82–7.04 mg for a 200 g serving), apples (with skin) (3.14–8.80 mg in a 200 g serving), and red grapes (0.00–7.96 mg in a 200 g serving)].

Finally, the variability in the total monomeric content between the different commercial products was equivalent to 37% (expressed as the coefficient of variation, %) (Table 1). In the case of total flavonols, the intervariability was equal to 60%. This high variability in the flavonoid content between products could be attributed to many factors, such as cultivar type, geographical origin, environmental factors, agricultural and postharvesting practices, and processing (4). Neimenak et al. (8) concluded that the content of (-)-epicatechin, (+)-catechin, cyanidin-3-galactoside, and cyanidin-3-arabinoside in fresh and

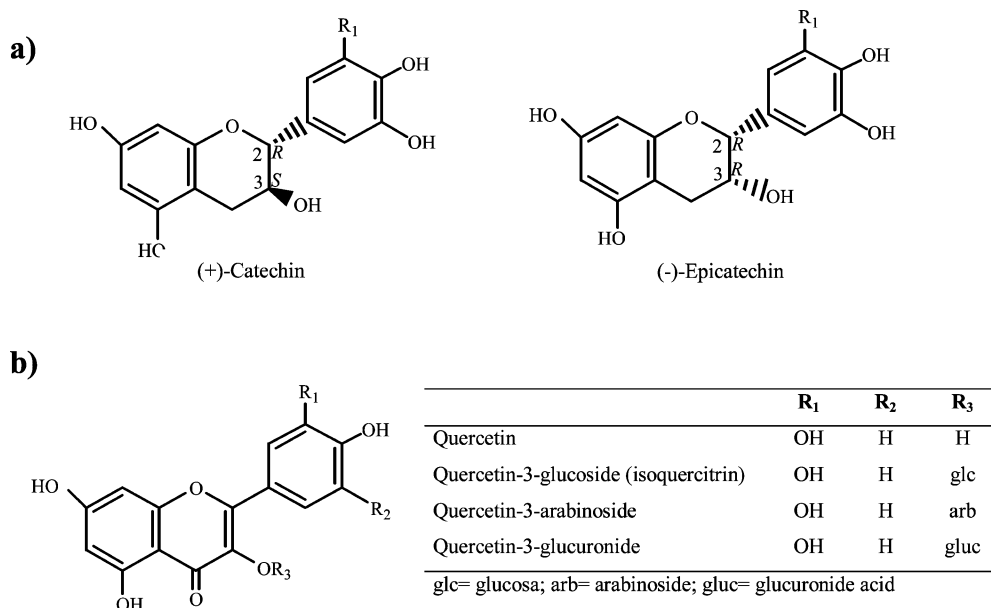


Figure 1. Chemical structure of the flavonoid compounds analyzed: (a) monomeric flavanols and (b) flavonols.

fermented-like beans was genotype-dependent. A large variation in the (–)-epicatechin content of cocoa has also been reported to occur after fermentation (22, 28).

Changes in Cocoa Flavanol and Flavonol Contents during the Manufacturing Process of Cocoa Powder Products. After fermentation and roasting at the site of cultivation, the cocoa nib was ground, resulting in the cocoa liquor. Part of the cocoa butter was then removed from the cocoa liquor, resulting in a cocoa powder containing 10–12% fat (natural cocoa powder). A fraction of this natural cocoa powder (10 different batches) was submitted to alkalization and both natural and alkalized cocoa powders were finally manufactured into different cocoa powder products as described under the Materials and Methods. Although originally performed to make the powder not to agglomerate or sink to the bottom, when it was added to milk or water-based drinks, the alkalization process (or *Dutching*) is nowadays mainly applied to modify the flavor and color of cocoa powders.

The flavanol and flavonol contents in natural and alkalized cocoa powders and in their derived cocoa powder products are summarized in **Table 2**. Natural cocoa presented a mean content of total flavonoids equivalent to 2653.13 $\mu\text{g/g}$ (–)-epicatechin, representing the highest proportion. Batches of natural cocoa powder presented a coefficient of variation (CV) between 13% (for (–)-epicatechin) and 18% (for quercetin). A similar level of variability was registered between the different batches of alkalized cocoa powder, with the exception of quercetin, which presented a 59% CV (**Table 2**).

A decrease in the concentration of all flavonoids studied was registered as a consequence of the alkalization treatment, resulting in a 60% loss of the mean total flavonoid content (**Table 2**). Among flavanols, (–)-epicatechin presented a larger decline (67%, as a mean percentage difference) than (+)-catechin (38%), resulting in a change of the original monomeric flavanol profile. Because (–)-epicatechin possesses a higher absorption than (+)-catechin, the alkalization treatment could affect the bioavailability of flavanols from cocoa products (29). A possible epimerization of (–)-epicatechin into (–)-catechin, which could explain the higher decline of the former after alkalization, has been suggested to occur during chocolate manufacturing (9), although it has not been attributed to any concrete step of the production process. These authors also found

that (–)-catechin, not naturally present in cocoa, was more abundant than (+)-catechin in 68 commercially available chocolate samples. This is another fact that could further affect the bioavailability of flavanols from cocoa products, because recently, it has been reported that (+)-catechin is more bioavailable than (–)-catechin (30).

To evaluate the effect of the alkalization treatment on cocoa procyanidins, selected ion monitoring (SIM) experiments in negative mode $[M - H]^-$ were performed at m/z 577 for dimers, m/z 865 for trimers, and m/z 1153 for tetramers (**Figure 2**). According to literature data (9), the most abundant mass peak at each m/z corresponded to dimer B2 (epicatechin-4 β -8-epicatechin) at R_t = 2.30 min, trimer C1 (epicatechin-4 β -8-epicatechin-4 β -8-epicatechin) at R_t = 3.50 min, and tetramer D (epicatechin-4 β -8-epicatechin-4 β -8-epicatechin-4 β -8-epicatechin) at R_t = 3.80 min. SIM was also performed at m/z 289 to confirm the results obtained above for monomeric flavanols, (+)-catechin (R_t = 1.80 min) and (–)-epicatechin (R_t = 2.85 min). The percentage differences between natural and alkalized cocoa powders for each peak were calculated using the SIM mass chromatogram areas. Losses registered as a consequence of the alkalization process were 36% (as a mean) for (+)-catechin, 67% for (–)-epicatechin, 69% for dimer B2, 67% for trimer C1, and 31% for tetramer D. Using reverse- and normal-phase HPLC, Gu et al. (24) have also reported lower level of flavanols (catechins and procyanidins) in alkalized cocoa powders in comparison to natural powders. However, currently, no concrete figures have been found in the literature concerning the exact influence of the alkalization treatment on the cocoa flavanol composition.

In the case of flavonols, quercetin presented the highest loss (86%) after alkalization, whereas quercetin-3-glucuronide, quercetin-3-araboside, and isoquercitrin showed a similar decrease (58, 62, and 61%, respectively) (**Table 2**). In general, changes occurring as the result of the alkalization treatment could be attributed to the oxidation of phenolic compounds under basic pH conditions, leading to brown pigments that are polymerized to different degrees. In particular, secondary reactions involving *o*-quinones previously formed during the fermentation stage by polyphenol oxidase (PPO) (31, 32) are probably involved in further reactions responsible for the browning developed during alkalization. According to Bonheví and Coll (25), the chem-

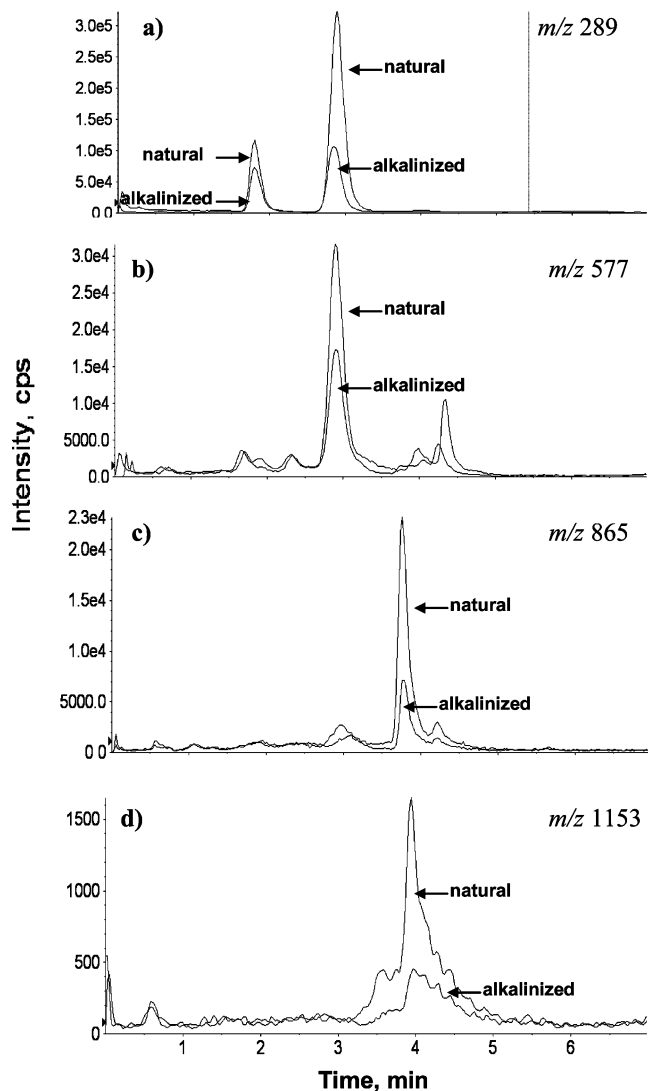


Figure 2. SIM chromatograms of natural and alkalized cocoa powders at (a) m/z 289 (monomers), (b) m/z 577 (dimers), (c) m/z 865 (trimers), and (d) m/z 1153 (tetramers).

istry of the alkalization process determines the biochemical characterization and browning behavior of cocoa beans.

Considering the cocoa powder products (products A, B, and C), as expected, the flavonoid concentration varied according to the formulation of each product (Table 2). Products A and B presented very similar mean content of total flavanols (563.27 and 530.41 $\mu\text{g/g}$, respectively). However, in the case of product A, this figure only represented ca. 20% of that of the original raw material (i.e., natural cocoa powder), whereas in the case of alkalized product B, it represented ca. 50% of the original alkalized cocoa powder. This means that, because of its lower phenolic content, a higher amount of alkalized cocoa powder in product B was needed to provide the same flavonoid content as in product A. Nevertheless, the percentage distribution of some flavonoids differed between both products. For example, the (+)-catechin/(−)-epicatechin/querceetin percentage distribution was 27:68:0.1% in product A, whereas in product B, it was equal to 50:45:0.05%. Alkalized product C presented the lowest content of all flavanols and flavonols quantified (mean total flavonoids: 223.47 $\mu\text{g/g}$; 20% of the alkalized raw material), with the querceetin levels being under the limit of quantification (2) (Table 2). However, this later product is a special formulation in which the nutritional quality of the product is balanced by the addition of cocoa fiber.

Finally, the variability in the total content of flavonoids between the different production batches of each cocoa product was relatively low, 21, 19, and 16% CV, for cocoa powder products A, B, and C, respectively, indicating a well-standardized manufacturing process (Table 2). Isoquercetrin and querceetin were the compounds that presented the highest variation in products A and B, whereas for product C, this was found for (−)-epicatechin and querceetin-3-glucuronide.

CONCLUSIONS

This paper provides for the first time quantitative data of individualized flavanol derivatives in cocoa powder products in a wide range of commercial products available in the Spanish market. Together with the monomeric flavanol content also given, this data is very useful for the calculation of daily flavonoid intake and its correlation with disease incidence or early markers in epidemiologic and clinical studies. The results found herein in relation to the alkalization process indicate that the dramatically decrease found in the flavonoid content of natural cocoa powder together with the observed change in the monomeric flavanol profile negatively affect the content of antioxidant polyphenols of cocoa and probably their bioavailability. Considering that cocoa powder products have a lower level of saturated fats than chocolate bars, it seems necessary to establish a compromise between color and phenolic content, especially for cocoa powder products derived from alkalized cocoa powder, which is a more expensive raw material but markedly reduced in polyphenol content.

LITERATURE CITED

- (1) International Cocoa Association. Annual Report 2003–2004. London, U.K., <http://www.icco.org/anrep/anrep0304english.pdf>.
- (2) Ding, E. L.; Hutfless, S. M.; Ding, X.; Girotta, S. Chocolate and prevention of cardiovascular disease: A systematic review. *Nutr. Metab.* **2006**, *3*, 1–12.
- (3) Lee, W. K.; Kim, Y. J.; Lee, H. J.; Lee, C. Y. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *J. Agric. Food Chem.* **2003**, *51*, 7292–7295.
- (4) Wollgast, J.; Anklam, E. Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Res. Int.* **2000**, *33*, 423–447.
- (5) Sánchez-Rabaneda, F.; Jáuregui, O.; Casals, I.; Andrés-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventós, R. M. Liquid chromatographic/electrospray ionization mass spectrometry study of the phenolic composition of cocoa (*Theobroma cacao*). *J. Mass Spectrom.* **2003**, *8*, 35–42.
- (6) Lamuela-Raventós, R. M.; Andrés-Lacueva, C.; Permanyer, J.; Izquierdo-Pulido, M. More antioxidants in cocoa. *J. Nutr.* **2001**, *131*, 834.
- (7) Lamuela-Raventós, R. M.; Romero-Pérez, A. I.; Andrés-Lacueva, C.; Tornero, A. Review: Health effects of cocoa flavonoids. *Food Sci. Technol. Int.* **2005**, *11*, 159–176.
- (8) Niemenak, N.; Rohsius, C.; Elwers, S.; Ndoumou, D. O.; Lieberei, R. Comparative study of different cocoa (*Theobroma cacao* L.) clones in terms of their phenolic and anthocyanins contents. *J. Food Compos. Anal.* **2006**, *19*, 612–619.
- (9) Cooper, K. A.; Campos-Giménez, E.; Jiménez Alvarez, D.; Nagy, K.; Donovan, J. L.; Williamson, G. Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationship of their concentration in chocolate. *J. Agric. Food Chem.* **2007**, *55*, 2841–2847.
- (10) Porter, L. J.; Ma, Z.; Chan, B. G. Cacao procyanidins: Major flavonoids and identification of some minor metabolites. *Phytochemistry* **1991**, *20*, 1657–1663.

- (11) Rigaud, J.; Escribano-Bailón, M. T.; Prieur, C.; Bouquet, J. M.; Cheynier, V. Normal-phase high-performance liquid chromatography separation of procyanidins from cacao beans and grape seeds. *J. Chromatogr., A* **1993**, *654*, 255–260.
- (12) Hammerstone, J. F.; Lazarus, S. A.; Mitchell, A. E.; Rucker, R.; Schmitz, H. H. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **1999**, *47*, 490–496.
- (13) Adamson, G. E.; Lazarus, S. A.; Mitchell, A. E.; Prior, R. L.; Cao, G.; Jacobs, P. H.; Kremers, B. G.; Hammerstone, J.; Rucker, R. B.; Ritter, K. A.; Schmitz, H. H. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* **1999**, *47*, 4148–4188.
- (14) Forsyth, W. G. C.; Quesnel, V. C. Cacao glycosidase and colour changes during fermentation. *J. Sci. Food Agric.* **1957**, *8*, 505–509.
- (15) Nicholas, J. J. Phytochemicals and phenolics. In *Chocolate and Cocoa. Health and Nutrition*; Knight, I., Ed.; Blackwell Science, Ltd.: Oxford, U.K., 1999; pp 119–142.
- (16) Jalal, M. A. F.; Collin, H. A. Polyphenols of mature plant, seedling and tissue cultures of *Theobroma cacao*. *Phytochemistry* **1977**, *16*, 1377–1380.
- (17) Sanbongi, C.; Osakabe, N.; Midori, N.; Takizawa, T.; Gomi, S.; Osawa, T. Antioxidative polyphenols isolated from *Theobroma cacao*. *J. Agric. Food Chem.* **1998**, *46*, 454–457.
- (18) Andrés-Lacueva, C.; Lamuela-Raventós, R. M.; Jáuregui, O.; Casals, I.; Izquierdo-Pulido, M.; Permanyer, J. An LC method for the analysis of cocoa phenolics. *LC:GC Europe* **2000**, 902–904.
- (19) Dávalos, A.; Bartolom, B.; Gómez-Cordovés, C. Inhibition of methyl linoleate autoxidation by phenolics and other related compounds under mild oxidative conditions. *J. Sci. Food Agric.* **2004**, *84*, 631–638.
- (20) De Whalley, C. V.; Rankin, S. M.; Hoult, J. R. S.; Jessup, W.; Leake, D. S. Flavonoids inhibit the oxidative modification of LDL by macrophages. *Biochem. Pharmacol.* **1990**, *39*, 1743–1750.
- (21) Beckett, S. T. *The Science of Chocolate*; Royal Society of Chemistry (RSC) Paperbacks: Cambridge, U.K., 2000.
- (22) Kim, H.; Keeney, P. G. (–)-Epicatechin content in fermented and unfermented cocoa beans. *J. Food Sci.* **1984**, *49*, 1090–1092.
- (23) Kealey, K. S.; Snyder, R. M.; Romanczyk, L. J.; Geyer, H. M.; Myers, M. E.; Withcare, E. J.; Hammerstone, J. F.; Schmitz, H. H. Cocoa components, edible products having enhanced polyphenol content, methods of making same and medical uses. Patent Cooperation Treaty (PCT) WO 98/09533, Mars Incorporated, McLean, Virginia, 1998.
- (24) Gu, L. W.; House, S. E.; Wu, X. L.; Ou, B. X.; Prior, R. L. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J. Agric. Food Chem.* **2006**, *54*, 4057–4061.
- (25) Bonvehi, J. S.; Coll, F. V. Evaluation of bitterness and astringency of polyphenolic compounds in cocoa powder. *Food Chem.* **1997**, *60*, 365–370.
- (26) Tomarints-Barberán, F. A.; Cienfuegos-Jovellanos, E.; Marín, A.; Muguera, B.; Gil-Izquierdo, A.; Cerdá, B.; Zafrilla, P.; Morillas, J.; Mulero, J.; Ibarra, A.; Pasamar, M. A.; Ramón, D.; Espín, J. C. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J. Agric. Food Chem.* **2007**, *55*, 3926–3935.
- (27) United States Department of Agriculture (USDA), Agricultural Research Service. USDA Database for the Flavonoid Content of Selected Foods. Release 2.1. 2007. Retrieved from the Nutrient Data Laboratory Home Page: <http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html>.
- (28) Counet, C.; Ouwerx, C.; Rosoux, D.; Collin, S. Relationship between the procyanidins and flavor contents of cocoa liquors from different origins. *J. Agric. Food Chem.* **2004**, *52*, 6243–6249.
- (29) Baba, S.; Osakabe, N.; Natsume, M.; Muto, Y.; Takizawa, T.; Terao, J. In vivo comparison of the bioavailability of (+)-catechin, (–)-epicatechin and their mixture in orally administered rats. *J. Nutr.* **2001**, *131*, 2885–2891.
- (30) Donovan, J. L.; Crespy, V.; Oliveira, M.; Cooper, K. A.; Gibson, B. B.; Williamson, G. (+)-Catechin is more bioavailable than (–)-catechin: Relevance to the bioavailability of catechin from cocoa. *Free Radical Res.* **2006**, *40*, 1029–1034.
- (31) Reeves, S. G.; McDowell, I.; Behn, K.; Dench, J. Biochemical studies of cocoa bean *o*-diphenol O₂ oxidoreductase (catechol oxidase). *Food Chem.* **1988**, *29*, 209–219.
- (32) Guyot, S.; Vercauteren, J.; Cheynier, V. Colourless and yellow dimers resulting from (+)-catechin oxidative coupling catalysed by grape polyphenol-oxidase. *Phytochemistry* **1996**, *42*, 1279–1288.

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