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Valorisation of Compounds with High Nutritional Value from Cocoa By-Products as Food Ingredients and Additives

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Abbreviations

AMX: amoxicillin

ANOVA: analysis of variance

BHI: brain heart infusion

CBS: cocoa bean shell

CE: catechin equivalents

DVB/CAR/PDMS: divinylbenzene/carboxen/polydimethylsiloxane

DW: dry weight

E-nose: electronic nose

FBS: fetal bovine serum

FLC: fluconazole

GAE: gallic acid equivalents

GD: grinding degree

GID: gastrointestinal digestion

GTF: glucosyltransferase

HBSS: Hank‘s balanced salt solution

HS-SPME/GC-qMS: headspace solid-phase micro-extraction coupled with gas chromatography-quadrupole mass spectrometry

IDF: insoluble dietary fiber

ISTD: internal standard

LC-HRMS: liquid chromatography–high-resolution mass spectrometry

MEM: minimum essential medium

MIC: minimal inhibitory concentration

NYS: nystamine

OTA: ochratoxin

PB1: procyanidin B1

PB2: procyanidin B2

PCA: principal component analysis

PCA: procyanidin A-type

PCB: procyanidin B-type

PEN G: penicillin G

PEN V: penicillin V

PLS-DA: partial least squares discriminant analysis

Q-3-G: quercetin-3-O-glucoside

RP-HLPC-PDA: reversed-phase high-pressure liquid chromatography coupled with photodiode array detector

RSA: radical scavenging activity

SDF: soluble dietary fiber

TDF: total dietary fiber

TE: trolox equivalents

TDF: total dietary fiber

TFC: total flavonoid content

TPC: total phenolic content

TTC: total tannin content

TS: tryptic soy

VOC: volatile organic compound

W4M: Workflow4Metabolomics

YPD: yeast extract-peptone-dextrose

Abstract

This doctoral thesis was conducted in the framework of a co-supervised PhD between the Department of Agriculture, Forestry and Food Sciences of the University of Turin and the Unit of Pharmacognosy, Bioanalysis and Drug Discovery of the Faculty of Pharmacy at the Université libre de Bruxelles. The present manuscript was conceived as a thesis of articles and is composed of 9 different scientific publications. The general introduction of the work was issued from a published narrative review, while the result and discussion part is composed by eight chapters based on different scientific articles issued from the PhD project.

The cocoa bean shell (CBS) is the external tegument that covers the cocoa bean, and is one of the major by-products in cocoa industry. It is normally discarded or underutilized, which could result in economical and environmental issues. However, CBS represents a notable source of polyphenols and methylxanthines (theobromine and caffeine) which can give it different biofunctionalities such as antioxidant and antidiabetic properties, among others. It also contains high amounts of dietary fiber (about 50% *w/w*), minerals, vitamins, and proteins. CBS has low-fat content, and interesting cocoa-aroma compounds. All this could make CBS useful as a food ingredient, and source of biofunctional compounds.

The first part of the experimental work of this thesis is devoted to the chemical characterization of CBS and the establishment of its polyphenolic and volatile organic compound (aroma) profiles. The utilization of such profiles, determined by both complete characterization methods and screening methods, was also proposed for authentication purposes of CBS depending on its geographical origin and variety.

In a second step, the utilization of CBS as a low-cost food ingredient for functional food production was envisaged. CBS-based beverages and biscuits were proposed as model foods. The influence of the CBS addition to the model foods was evaluated from both technological and nutritional points of view. Changes on the physicochemical characteristics of the model foods were assessed as well as their content in compounds of interest and potential biofunctionalities. Moreover, these studies served also to evaluate the effect of the different food matrices on the bioaccessibility and intestinal permeability of the bioactive compounds contained in CBS.

In the third and last part of this work, a different utility was given to the study of the cocoa by-product. The antimicrobial potential of CBS was assessed against different bacterial and fungal strains and a metabolomic strategy was applied in order to individualize the putative active compounds against the *Streptococcus mutans* proliferation.

This work was a contribution for the valorization of a high add-value product such as the CBS, and a step towards a zero-waste cocoa industry within the frame of sustainable circular economy.

GENERAL INTRODUCTION

The following general intoduction focusses on the cocoa bean shell, its production, chemical composition and main uses and biofunctional potentials.

This section was issued and adapted from a published literature review paper:

Cocoa Bean Shell – A By-Product with Nutritional Properties and Biofunctional Potential

* Olga Rojo-Poveda, Letricia Barbosa-Pereira, Giuseppe Zeppa, and Caroline Stévigny

Published in *Nutrients*, 2020, *12(4)*, 1123, doi: 10.3390/nu12041123

If the reader desires it, the original paper can be consulted in this link

1. The cocoa bean shell

A part of the fruit from the plant *Theobroma cacao L.* is the well-known cocoa bean, which is the main raw material for chocolate manufacturing. Cocoa bean production takes place mainly in tropical areas, and it reaches more than 4.7 million tons per year worldwide, from which 76.3%, 17.4%, and 6.3%, were estimated to be produced in Africa, America, and Asia and Oceania, respectively, during the harvest season of 2018/2019 (Figure 1A). Cocoa bean exportation constitutes about 71% of the total produced volume [1], and, although Europe is not a producing continent, its processing of cocoa beans reaches 1.7 million tons, leading the statistics over other continents [2] (Figure 1B). After being harvested, cocoa beans are firstly separated from their pods, then they are subjected to fermentation, followed by a drying phase. At this point, cocoa beans are transferred to the chocolate production industries, where they are roasted and winnowed in order to separate them from their shells, since no more than a 5% of shell is allowed on cocoa products according to the Codex Alimentarius [3-5].

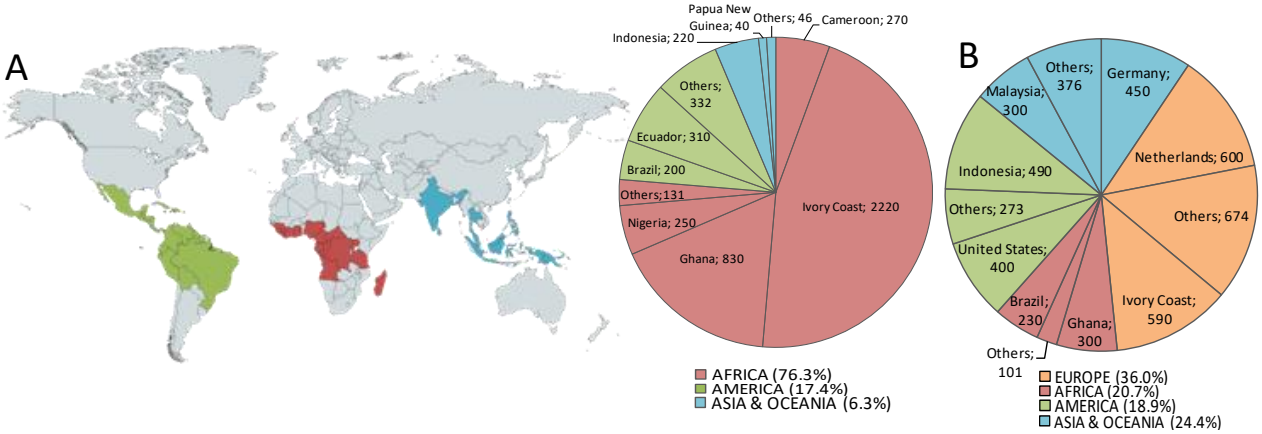

Figure 1. Forecast of global cocoa bean production (**A**) and global cocoa grinding (**B**) during the season of 2018/2019. Adapted from [2].

Cocoa production generates substantial quantities of waste. Indeed, only 10% of the total cocoa fruit weight is used for its commercialization, while the remaining 90% is discarded as waste or by-products [6,7]. One of these by-products is the external tegument that cover the cocoa beans, also known as cocoa bean shells (CBS, Figure 2), which are generated during the cocoa bean roasting process, as already mentioned. CBS constitute about 10-17% of the total cocoa bean weight [8] and some studies have revealed that these percentages are likely to vary depending on the fermentation type of cocoa beans [9].

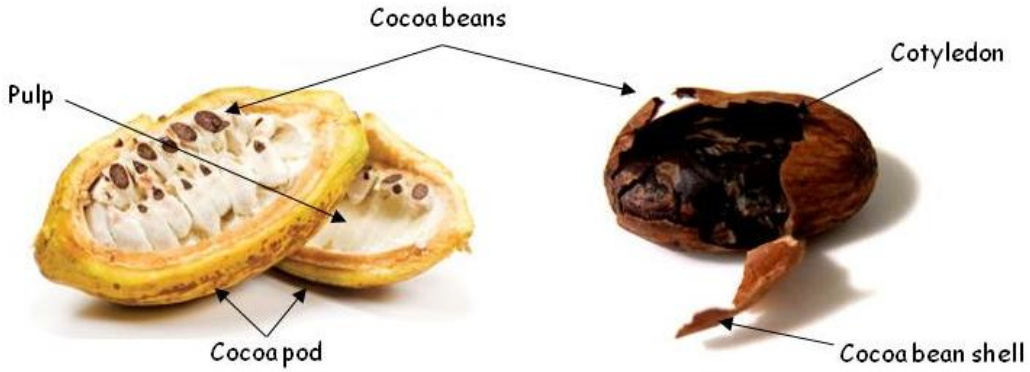

Figure 2. Cocoa beans and their processing by-products. Adapted from [10,11].

Taking into account the weight percentage of CBS and the abovementioned cocoa production data, this would mean that more than 700 thousand tons of CBS waste is produced worldwide, from which more than 250 thousand tons is only produced in Europe. To give an idea of this, the production of one kg of chocolate would produce an output of 98 g of CBS [12]. The increasing demand for cocoa beans has led to an accumulation of this by-product, representing a serious disposal problem that could be aggravated by legal restrictions [7]. Indeed, the disposal of CBS could carry important economic and environmental issues [13], since they contain polyphenols with potential phytotoxic activity [14] and considerable amounts of theobromine, which has been reported to be toxic for some non-human mammals [15]. Besides, its toxicity in aquatic animals has also been reported [16].

Despite being considered a by-product, the nutritional composition of CBS does not differ hugely from that of cocoa beans, except for fats, which are much more present in cocoa beans, while fibers predominate the shells [17]. Besides, CBS also contains considerable quantities of interesting bioactive compounds, such as polyphenols, which are known to be responsible for the different nutrition-related health benefits provided by cocoa [18].

Recently, the bioconversion of food processing residues into valuable products has begun to receive increasing attention, and, as a result, industrial countries are preparing strategic policies to develop a bio-based circular economy [19,20]. Due to all the above-mentioned reasons, valorization strategies for CBS have appeared in different fields and several studies have been carried out in order to find new applications for this by-product. Among these applications, new uses in the food industry field, feedstuff for livestock, or utilization by industry as a biofuel, absorbent or composite, among others, could be considered as the most common applications. However, in the last few years, other types of applications focusing on the biofunctionality and bioactivity of this cocoa by-product have also appeared. In this introduction, the current knowledge and latest advances of CBS different uses and

applications for human health from a nutritional and biofunctional point of view will be reviewed.

2. Nutritional and chemical composition

2.1. Proximate composition – Moisture, ashes, proteins, fats and carbohydrates

The proximate composition of CBS has been reported by several authors and this is summarized in Table 1. CBS proximate composition comprises proteins, fats, sugars, moisture, and ashes [21], and has been described to be similar to that of cocoa beans. However, CBS present a much lower percentage of fats compared to cocoa beans, which is substituted by a much higher amount of fibers [17]. CBS also have a higher content of proteins, fats, and carbohydrates when compared with other cocoa by-products, such as cocoa pods [22].

However, the proximate composition of CBS can significantly vary, since, as a vegetable product, its composition is subjected to several variable factors, such as the climatic conditions of the farming area, the cocoa variety, processing conditions (fermentation, drying, roasting temperature), etc. [23].

The values found for the moisture of CBS range from 3.60% to 13.13%, which highly depends on whether the CBS are roasted or not [24]. Bonvehí et al. [25] obtained values between 3.6% and 7.8% for moisture and affirmed that this is an acceptable range for stable CBS storage. Nonetheless, CBS have been reported to be considerably hygroscopic, and, therefore, molds could appear if stored at higher moisture levels [13,26].

Ash content was established to be comprised between 5.96 and 11.42 g/100 g of CBS according to the literature, once again being influenced by the roasting process, which increases this value by about 15% according to Agus et al. [24]. Osundahunsi et al. [26] reported that the main components found in CBS ash are sodium and potassium (7.2 g and 3.1 g per 100 g of CBS ash, respectively). Of the total calculated ash, Gónzalez et al. [1] reported that 30.4% of it would be water-soluble ash while about 38.4% would be acid-insoluble ash (mainly silica derivates such as sand and siliceous earth) [27].

Table 1. Nutritional and chemical composition of cocoa bean shells (CBS)

Parameter	Amount [a]	References
Energy (kcal/100 g)	122.00	[15]
Moisture (%)	3.60–13.13	[13,24,25,28-36]
Ash (g/100g)	5.96–11.42	[13,28,31,33-39]
Proteins (g/100g)	10.30–27.40	[13,24,25,28,30,32-36,38-42]
Fats (g/100g)	1.50–8.49	[13,28,30,31,33,35,36,38-43]
Carbohydrates (g/100g)	7.85–70.25	[13,15,30,33,36,40]
- Starch (g/100g)	0–2.80	[22,25,37,44,45]
- Soluble sugars (g/100g)	0.16–1.66	[25,30,38]
Dietary fiber (g/100g)	39.25–66.33	[13,17,28,31,33,36,38,41,46]
- Soluble fiber (g/100g)	7.03–16.91	[13,17,31,33,34,36,38,39]
- Insoluble fiber (g/100g)	28.34–50.42	[13,17,31,33,34,36,38,39]
Pectin (g/100g)	7.62–15.59	[28,47-49]
Minerals		
- Calcium (g/100g)	0.23–0.44	[37,50]
- Phosphorus (g/100g)	0.58–1.00	[37]
- Magnesium (g/100g)	0.48–1.29	[37,50]
- Potassium (g/100g)	1.25–1.82	[37,50]
- Sodium (mg/100g)	16.00–192.20	[37,50]
- Iron (mg/100g)	27.60–80.50	[37,50]
- Manganese (mg/100g)	4.53	[50]
- Copper (mg/100g)	2.35–6.62	[37,50]
- Selenium (mg/100g)	0.21	[50]
- Cobalt (mg/100g)	0.10	[50]
- Zinc (mg/100g)	2.75–19.00	[37,50,51]
- Chromium (mg/100g)	0.67–4.86	[37,50]
Vitamins		
- B1 (μg/g)	0.70–3.10	[37]
- B2 (μg/g)	0.90–3.10	[37]
- B6 (μg/g)	tr	[37]
- D (μg/g)	tr–0.53	[37,52]
- E (μg total tocopherols/g CBS fat)	1.02	[34]
Polyphenol content		
- Total phenolic content[b]	3.12–94.95	[13,38,39,53-59]
- Total flavonoid content[c]	1.65–40.72	[13,39,57]
- Total tannin content[c]	1.70–25.30	[13,37,39,57]
Flavanols		
- Epicatechin (mg/g)	0.21–34.97	[57,60-63]
- Catechin (mg/g)	0.18–4.50	[60-63]
- Procyanidin B1 (mg/g)	0.55–0.83	[63]
- Procyanidin B2 (mg/g)	0.23–1.38	[62,63]
Methylxanthines		
- Theobromine (g/100g)	0.39–1.83	[28,35,37,45,55,57,60,62,64,65]
- Caffeine (g/100g)	0.04–0.42	[37,45,55,57,61,62,64]
Volatile organic compounds (aromatics) (μg/g)	4.92–16.10	[29,66]

[a] Data are referred to a CBS dry weight basis unless indicated differently. Intervals have been created, comprising all the values from the cited literature;
[b] mg of gallic acid equivalents/g of dried CBS;
[c] mg of catechin equivalents/g of dried CBS;
CBS: cocoa bean shell; tr: Traces.

Concerning the protein content, the reviewed literature establishes that proteins constitute between 10.30% and 27.40% of the CBS dried weight. This is a remarkable quantity and, therefore, some researchers have considered this by-product as a source of extractable protein [34,40]. In addition, a study found that fermenting the shells with *Pleurotus ostreatus* spawn could produce an increase of up to 25.2% in the protein content [67]. However, it has been shown that the roasting process normally has an unfavorable effect on this quantity, since Agus et al. [24] reported a decrease from 27.43% to 25.07% of crude protein for CBS after roasting. Pérez et al. [22] found that CBS proteins have 78.04% digestibility, which is not far from the 68% reported by Bonvehí et al. [25]. CBS contain all the essential amino acids, representing 44.7% of the total amino acids [25,50]. However, a small percentage of the total amino acids are D-amino acids, and their relative quantity with respect to L-amino acids increases during the roasting phase. D-amino acids provide low nutritional value, since they are not digested like their chiral counterparts. Nevertheless, they still contribute to flavor formation during fermentation and roasting [68].

The fat content accounts for 1.50–8.49% of dried CBS and is therefore considered a minor component of the by-product when compared to the approximate 50% fat content in cocoa beans [25]. However, the fat in CBS has also received interest from researchers that have optimized methods for its extraction [69]. As reported for the protein content, the roasting process could also entail a decrease of about 36% of the fat in CBS [24]. CBS fat is highly acidic and richer in the unsaponifiable matter than cocoa bean fat, and, for this reason, it is not often considered as cocoa butter [43,58]. Indeed, some CBS fat compounds differ considerably from those of cocoa butter, and, in some cases, these differences have been used in order to estimate the shell content of cocoa powder [70] or cocoa butter [71]. Nevertheless, oleic, palmitic, capric, and stearic acids are the main fatty acids in both CBS and cocoa fats when considering the saponifiable fraction [43] [55,72]. Regarding this fraction, Lessa et al. [73] found that it is comprised of 34.7% unsaturated fatty acids and 64% saturated fatty acids for non-fermented CBS, and that these percentages vary to 51.2% and 48%, respectively, after fermentation. These values are in accordance with the 0.66–0.74 unsaturated/saturated mass ratio reported by Okiyama et al. [34]. Also, phytoprostanes and phytofurans, which are isoprostanoids derived from the peroxidation of α-linoleic acid, have been detected in CBS in quantities of 474.3 and 278.0 ng per gram of dried CBS, respectively [74]. The unsaponifiable fraction of the CBS fat is formed by compounds such as phytosterols, of which stigmasterol would be the predominant one, while cholesterol concentrations are almost insignificant when compared to those of cocoa beans [24].

According to the literature, carbohydrates constitute 7.85%–70.25% of the CBS dry weight. These values differ considerably depending on whether fiber content is taken into consideration or not, and also because they are often calculated by subtraction, which entails added variability [33]. Concerning digestible carbohydrates, CBS contain none or small quantities of starch, mostly available starch [22], and a very small quantity of soluble sugars, considered negligible in some studies [25,38]. Regarding the non-digestible fraction, this is formed by pectic polysaccharides (45%), hemicelluloses (20%), and cellulose (35%) and constitutes the dietary fiber [75], which will be explained in detail in Section 2.2. below. Glucose is the main monosaccharide in CBS and accounts for almost half of the carbohydrate fraction, followed by galactose, mannose, rhamnose, arabinose, and xylose in a decreasing order [30,50,72,75].

2.2. Dietary fiber

The dietary fiber of CBS is composed of structural carbohydrates, also known as non-starch polysaccharides. It is constituted by residues of plant cell walls and is not digestible by human enzymes, and, therefore, it provides no energy value [30,31]. Consumption of dietary fiber is important due to its contribution to proper intestinal transit. Besides, some authors have also reported that the dietary fiber contained in CBS possesses several other biofunctions, such as reducing cardiovascular risks by reducing cholesterol and triacylglycerol levels or reducing diabetes effects by retarding glucose absorption, as reviewed in Section 4 of this general introduction [76,77]. On the other hand, CBS dietary fiber also adsorbs important concentrations of polyphenols, which could give it antioxidant properties and contribute to decreasing oxidative stress and inflammation processes in the intestine [7,78].

The total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) values of CBS reported in the literature are shown in Table 1. Noteworthy differences for these values are due to the way CBS fiber has been determined in the different studies. Gravimetric methods that are usually used for fiber analysis comprise both the non-starch polysaccharide fraction and the fraction known as the ‘Klason lignin’ fraction, which is in some cases formed not only by lignin, but also by Maillard products and complexes formed by tannin and protein interactions [75]. Redgwell et al. found that gravimetrically determined fiber on CBS (including the Klason fraction) accounts for 63.6% of the CBS dried weight, while fiber determined as total polysaccharides would be just 38.2% [75]. Similarly, Lecumberri et al. found values of 60.5% and 28.1%, respectively [38]. Excluding the Klason

fraction, CBS fibre would be composed of about 45% pectic compounds, 35% celluloses, and 20% hemicelluloses. As mentioned in Section 2.1, glucose would be the main monosaccharide composing CBS fiber. Calculations of the IDF and SDF fractions on CBS vary between authors, however, IDF is always the most abundant, with the IDF/SDF ratio ranging between 2.2 and 4 [33,36,75]. Compared to other cocoa by-products such as the cocoa pod, CBS possess equivalent quantities of total dietary fiber, but with a higher percentage of SDF, which would be the one providing more interesting biofunctional properties, as described by Matínez et al. [33].

Particularly, the pectin fraction of CBS fiber formed mainly by galacturonic acids has attracted the attention of many researchers, mainly because of its interesting gelling properties, which are very useful in fields such as the food, pharmaceutical, or cosmetic industries [28,47]. Pectins are present in both SDF and IDF in the form of high methoxyl pectins for the former and low methoxyl pectins for the latter [17]. CBS pectin is sometimes considered a 'low quality pectin' when compared to other commercial pectins [49,79] and is present in lower concentrations than that of citrus or apples (about 9% against 15% and 30% dry weight, respectively) [48]. However, optimizations for the pectin extraction process from CBS have been proposed [47,80].

2.3. Phenolic compounds

Polyphenols are, together with fiber, the most interesting and studied compounds in CBS and are the main compounds responsible for the biofunctional properties attributed to this cocoa by-product. These compounds are present in all vegetable origin foods and they are well known for producing several biological activities. A special group of polyphenols are flavonoids, among which, flavanols are the main group in cocoa [81]. They are not essential for short-term well-being, but there is growing evidence suggesting that a modest long-term intake of polyphenols could give several health benefits, since they possess antioxidant properties, act as free-radical scavengers, and reduce oxidative stress. They can take part in anti-inflammatory processes, exert antidiabetic properties, or reduce the risk of several diseases such as cancer, chronic diseases, cardiovascular disease, or even neurodegenerative disorders [78,82-86]. In addition, the intake of dietary flavanols has been reported to improve cognitive function and task performance [87].

The total phenolic content (expressed as mg of gallic acid equivalents/g of dried CBS), total flavonoid content (expressed as mg of catechin equivalents/g of dried CBS), and total tannin content (expressed as mg of catechin equivalents/g of dried CBS) reported by several

researchers are gathered in Table 1 and range between 6.04–94.95, 1.65–40.72, and 1.70–25.30, respectively. Again, these values show great variability depending on the research work, mainly due to the polyphenolic extraction conditions and the employed solvents, although the total flavonoid content (TFC) and total tannin content (TTC) are in general well correlated with the TPC values [13,57]. Indeed, several authors have taken interest in this fact and have studied several possibilities in order to optimize different types of CBS polyphenol extraction, using techniques such as supercritical CO_2, water extraction [58,59,61,88,89], pulsed electric fields [57], high-voltage electric discharges [56,64], pressurized ethanol [34,58,62], or ultrasound techniques [90]. Macroporous resins have been used to increase the total polyphenol content of a CBS extract from 2.23% to 62.87% w/w CBS [91]. The polyphenolic content has been demonstrated to significantly vary depending on the geographic origin, variety, plant genotype, and even the harvest season [57,60]. Bruna et al. attributed a higher polyphenol content to stress situations of the cocoa tree [92]. Other factors affecting polyphenolic quantities could be the type of fermentation and fermentation time, which has been reported to give optimal TPC values after 24 h and then decrease afterwards [73,93]. Light-exposed and high-temperature processes during cocoa manufacturing, such as sun-drying or the roasting process, could imply polyphenol degradation [81]. In order to minimize this kind of degradation and maintain the polyphenolic integrity and activity of CBS, some studies have proposed strategies such as extract encapsulation [40,63].

When compared to cocoa beans, the TPC values of CBS are similar to those between 5.77 and 49.56 mg gallic acid equivalents /g of cocoa beans, as indicated by Hernández-Hernández et al. [94]. Concerning other types of cocoa by-products, it has been found that the TPC values of cocoa pods are slightly higher than those of CBS, while the TFC values are almost two-fold higher in CBS than in cocoa pods [95]. The antioxidant activity of CBS also seems to be correlated with the total phenolic content of the by-product and some authors have maintained that this activity is mainly due to the flavonoid content of CBS [96], also being influenced by temperature during cocoa processing [97]. However, the TPC, TFC, TTC, and the antioxidant activity are values obtained by screening spectrophotometric methods with several interferences that could vary the obtained quantities, which could also be a reason for the huge ranges of the values that have been found [98].

More specific analyses, such as high-performance liquid chromatography (HPLC) coupled to UV or mass spectrometry detection, aiming to find specific polyphenolic compounds on CBS, have shown that procyanidins and catechins are the main polyphenols

present in this by-product. In particular, (-)-epicatechin is the most abundant and commonly reported flavan-3-ol contained in CBS, followed by (+)-catechin and their dimmers, procyanidin B1 (epicatechin-(4β→ 8)-catechin), and procyanidin B2 (epicatechin-(4β→ 8)-epicatechin) (Figure 3A, B, C, D, respectively), which were found in quantities that ranged from 0.21 to 34.97 mg epicatechin/g of CBS, from 0.18 to 4.50 mg catechin/g of CBS, from 0.55 to 0.83 mg procyanidin B1/g of CBS and from 0.23 to 1.38 mg procyanidin B2/g of CBS, as shown on Table 1. Other polyphenols, such as protocatechuic acid, quercetin, and quercetin derivates, caffeic acid, procyanidin dimers, trimers, and tetramers, among others, have also been found in CBS [13,55,57,78,99,100].

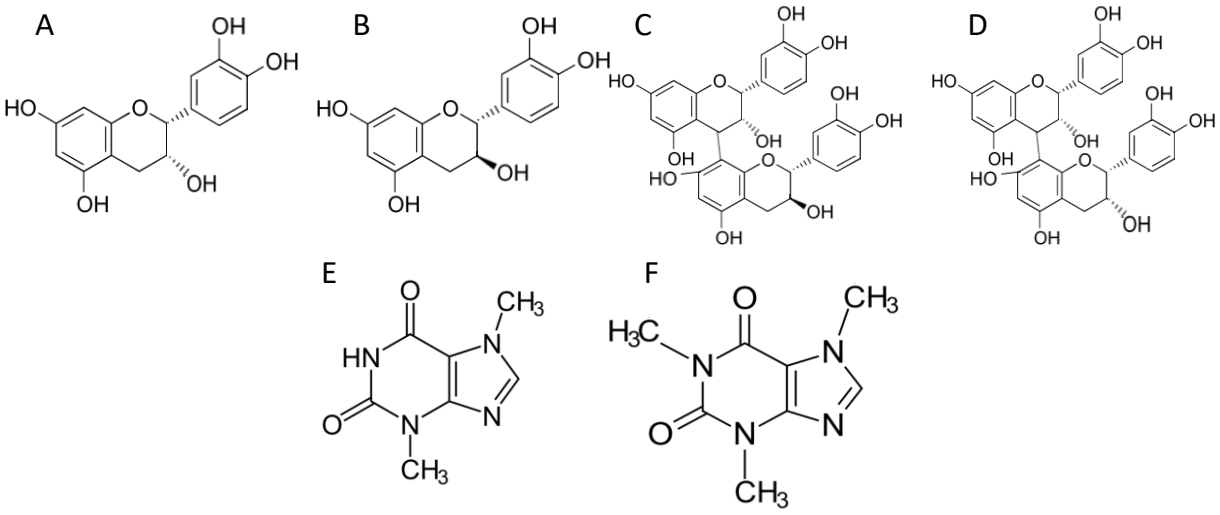

Figure 3. Main polyphenols (epicatechin, A; catechin, B; procyanindin B1, C; and procyanidin B2, D) and methylxanthines (theobromine, E; and caffeine, F) contained in CBS.

2.4. Methylxanthines

The main methylxanthines found in CBS are theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-dimethylxanthine) (Figure 3E and 3F, respectively). Both are alkaloids that are characteristic of cocoa, although theophylline has also been detected, mostly at trace level, however [61]. Both theobromine and caffeine are known for acting on the central nervous system and influencing mood positively, being one of the reasons for the high cocoa acceptance between consumers. Both methylxanthines have been related to several beneficial effects on human health, such as acting as diuretic, anticarcinogen, or anti-obese agents, among other effects [101]. Caffeine is commonly added to soft drinks as a flavoring agent, and it is also used in pharmaceutical formulations. However, its high consumption has been related to some disorders, such as kidney dysfunction, tachycardia, excessive gastric acid secretion, or even seizures and delirium [102]. On the other hand, theobromine, which is also

a caffeine metabolite, is colorless and odorless, with a slightly bitter taste, characteristic of chocolate. Theobromine has a much weaker action on the central nervous system since it has a 2- to 3-fold lower affinity for adenosine receptors than caffeine. Theobromine also possesses myorelaxant and cardiac stimulation properties and has been used as a coronary artery dilator or bronchodilator for asthma treatment [13,103-105].

Methylxanthines are mainly synthesized via the cotyledons of cocoa beans [106] and they have been demonstrated to migrate to the shell during cocoa fermentation [107,108]. Indeed, Hernández-Hernández et al. [94] found that theobromine concentrations in raw cocoa beans and raw CBS were 18.07 mg/g of cotyledon and 3.90 mg/g of CBS, respectively, while the concentrations on their fermented counterparts were 9.79 mg/g of cotyledon and 12.00 mg/g of CBS, respectively. The amounts of theobromine in CBS have been reported to be 5–7 fold higher than caffeine [13]. Concretely, these values were 0.39–1.83 mg/100 g of dried CBS for theobromine and 0.04–0.42 mg/100 g of dried CBS for caffeine. As well as polyphenols, several techniques for methylxanthine extraction optimization from CBS have been proposed [55,56,61,64,65].

Because of these reasons, also taking into consideration the moderate concentration of caffeine, methylxanthines contained in CBS may also exert interesting bioactivities on human health and could give added value to CBS as a biofunctional ingredient. Besides, an interaction between cocoa flavanols and methylxanthines has been reported, where methylxanthines help to increase epicatechin levels in plasma, enhancing the vascular effects of flavanols [109].

2.5. Minerals and vitamins

CBS is expected to be rich in minerals because of its considerable quantity of ashes, which represent an index of mineral content in vegetable samples [110]. The mineral amounts found for CBS are reported in Table 1. Potassium, magnesium, calcium, and phosphorous are the most abundant minerals contained in the by-product, followed by smaller quantities of sodium and iron, among others. These elements tend to accumulate in the outer parts of the cocoa bean, and hence they are found in CBS in much higher quantities than in cocoa nibs. Bentil et al. [67] showed that the solid-state fermentation of CBS with *Pleurotus ostreatus* spawn and *Aspergillus niger* significantly increased the concentrations of calcium, phosphorus, and potassium. Nevertheless, mineral content in CBS could present great variability, mostly related to the cocoa's geographic origin, since mineral absorption by the

plant is highly dependent on mineral availability in the ground, and, therefore, dependent on the soil type and quality of the area [111,112].

Concerning vitamins, CBS have been reported to be a source of vitamin D [113], although studies reporting its concentrations are very old (dating from 1935) and new ones would be necessary. Knapp et al. [52] found quantities up to 21 IU (international units) per gram of CBS (equivalent to 0.53 μg/g of CBS), which is 20-30 the potency of dairy butter, but only when these were obtained from fermented and sundried cocoa beans, sustaining that vitamin D is probably formed by the light activation of a precursor present in fermentation molds, namely ergosterol. Kon et al. [114] took advantage of this fact and observed that when feeding cows with CBS, vitamin D levels of their butter fat were higher. They also observed that vitamin D was mainly concentrated in CBS fat, which contains 40% of the total vitamin D activity in CBS. Bonvehí et al. [37] found considerable quantities of vitamins B1 and B2 in CBS (shown on Table 1), close to the 15% of the recommended dietary allowance, while vitamins B6 and D were just detected at trace levels and vitamin C was not found in CBS. Also, α-tocopherol, (β+γ)-tocopherol, and δ-tocopherol, which act as vitamin E, were found at a total quantity of 1.02 mg per g of CBS fat.

3. Applications

31. Food applications

Among its multiple applications, CBS have been largely proposed as clean label ingredients and/or additives because of the nutraceutical character that their high fiber and polyphenol contents provide. Besides, some studies have shown that CBS possess between 10% and 20% of the total amount of volatile organic compounds found in roasted cocoa beans (Table 1), many of them being key-aroma compounds for cocoa and chocolate [29,66]. This makes CBS a very interesting, low-cost ingredient for cocoa substitution or cocoa flavoring. For these reasons, CBS have been mainly employed in baked products such as biscuits and bread, in order to increase their fiber content and give them antioxidant properties [46,115,116]. CBS are normally added as directly ground as cocoa flour, or as fiber extracts obtained after enzyme treatment. CBS have also been proposed as a fat replacer, replacing up to 50% and 70% of vegetable oil in functional cakes and chocolate muffins, respectively [117,118], with a generally good consumer acceptance in all cases. Another extended use of CBS in the food field has been to create beverages, such as carbonated soft drinks [119], preparations for home-made functional beverages [13], or a dairy drink made with CBS together with other by-products from coffee and oranges [120]. CBS have also been proposed

as an extra ingredient to nutritionally fortify extruded snack products, slightly lowering their physical properties, but still within the consumer acceptance range [121].

However, in order to obtain polyphenol-rich products with potential antioxidant capacities, there is concern over the stability of such compounds with temperature, time, or even during the digestion of CBS foods. That is why CBS encapsulation strategies applied to the food field have also been suggested [122]. Altin et al. [123] proposed CBS encapsulation with chitosan-coated liposomes for drinking yogurt preparation that allowed the stabilization of the phenolic content during storage and increased *in vitro* bioaccessibility in terms of the TPC, TFC, and antioxidant activity, while Papillo et al. [63] used CBS microencapsulation by spray-drying with maltodextrins as stabilizing agents in order to stabilize CBS polyphenols in baked products, obtaining biscuits with an invariable polyphenol content after going through the baking process and with up to 90 days of storage.

As a food additive, several authors have taken advantage of CBS antioxidant properties in order to avoid lipid oxidation. To this end, Ismai and Yee [124] added CBS and roselle seed extracts to beef, avoiding lipid oxidation to a greater extent than with synthetic antioxidants such as butylated hydroxytoluene (BHT) and β-tocopherol. Manzano et al. [53] proposed to improve the stability of soya cooking oil by adding a CBS polyphenolic extract, obtaining oils with lower free fatty acids and peroxide generation indices after repeated uses. A similar application was also given by Hernández-Hernández et al. [125], who added an encapsulated CBS polyphenol extract to olive oil jam in order to prevent it from becoming rancid. CBS have also been proposed for the production of liquid smoke additives, which are extensively used for their antioxidant, antibacterial, anti-fungal, anti-termite, and food preservative properties [126]. A different use was proposed by Osundahunsi et al. [26], who suggested to use CBS ashes as an alkalizing agent for cocoa beans, reintroducing the CBS into the chocolate production process. The antioxidant properties of CBS were also used by incorporating them into bioelastomers in order to create active packaging that could preserve foodstuffs for longer [127].

3.2. Utilization as feedstuffs

The use of CBS as a feedstuff has been largely proposed for a long time, as this is common for food processing by-products. CBS have considerable amounts of proteins, minerals, and vitamins that make it an interesting and inexpensive material for livestock feed. However, CBS also contain great quantities of tannins and theobromine, which could act as anti-nutrients in some animals, blocking some essential nutrients during digestion and

reducing their bioavailability [128]. Theobromine can also cause different toxic effects in some animals, such as liver and thyroid malfunction in horses [103] or even death in dogs when ingested in high quantities [129]. The exact reasons for theobromine toxicity species-dependence are unclear, but it may mainly lay on the inter-species differences in the rate of theobromine catabolism. Although the catabolic pathway in human is similar to that described for many animals, there are high differences in the rates in which this catabolism occurs. Indeed, while theobromine half-life in plasma ranges from 15.5 to 21 hours in livestock, this rate is only 7-12 hours for humans. This relatively low rate of degradation is therefore believed to be the main responsible for the adverse effect of theobromine in non-human mammals and other animals [15].

Despite the presence of theobromine, studies have revealed positive effects when using diets fortified with the right quantity of CBS for poultry, rabbits, ruminants, or pigs. Adeyemo et al. [128,130] fixed the maximum maize and soybean meal substitution with CBS at 10%, where they observed internal organs reduced in weight in broiler birds, while gut morphology was improved, showing enhanced villous and crypt dimensions. Emiola et al. [131] observed the same mentioned negative changes in laying hens with over 15% CBS substitution. Regarding egg quality, maximums of 10–25% of maize substitution by CBS in laying hens diet was proposed as the limit, beyond which egg weight and quality would be compromised [132,133]. However, an important remark concerning broiler feed with CBS was made by Day and Dilworth [134], who found that equivalent quantities of pure theobromine were more toxic for broilers than those furnished by CBS meal. For rabbits, a maximum substitution of 10% was proposed, since above that value, a decrease in the packed cell volume (nutritional deficiencies) and an increase in white blood cells (nutritional stress) was observed, although weight loss was only observed with above 20% CBS meal replacement [41]. Other studies suggested the inclusion of 200 g of CBS per kg of rabbit body weight as the value for the optimal benefit–cost ratio [135,136]. Concerning cattle, a high fiber content supposes an added value for CBS as a feed material, and diets containing up to 40% CBS have been shown to produce positive effects on daily body weight gain and feed efficiency [137]. Used as a bedding material, it was shown that CBS increased milk yield for cows due to an increase in their laying time, besides, it also decreased bacterial counts on the teat and cortisol levels in cows (which is a stress marker), as well as ammonia concentrations in the barns [138]. Magistrelli et al. [139] showed that a diet with up to 7.5% CBS would not affect growth for pigs and would enhance their microbiota by increasing the levels of bacteria that produce short-chain fatty acids, such as butyrate, known for its anti-inflammatory effects. In

aquaculture, it was found that feeding Nile tilapia with a 23% CBS diet resulted in a 35.6% feeding cost reduction and an enhanced weight gain and feed conversion ratio [140]. Other studies have shown that the addition of more than 2315 mg of CBS ethanolic extract per liter of water could cause acute toxicity in Mango tilapia [16].

It has been proven that occurrence of theobromine has limited the direct use of CBS in animal feed, and the European Food Safety Authority (EFSA) has lately established 300 mg/kg as the maximum level of theobromine in feedstuff, with the exception of 700 mg/kg for adult cattle complete feedstuff [103]. For these reasons, several theobromine remediation strategies have appeared in order to increase CBS use as a feeding material. Among these strategies, physicochemical treatments have been proposed, such as the boiling of CBS [141,142] or hydrotropic extraction [142]. Also, several studies have proposed fungi fermentation treatments of CBS for bio-detheobromination, showing that species such as *Aspergilllus niger*, *Talaromyces*, or *Pleurotus ostreatus* spawn are capable of metabolizing theobromine, obtaining up to a 78.13% theobromine content reduction in CBS [67,128,143,144].

3.3. Uses in industry and other applications

Besides food applications and uses as a feedstuff supplement, CBS applications are numerous and varied. Among other uses, we can find CBS being used for biofuel production, activated carbon preparation, bioadsorbents, mulch, fertilizer, etc.

CBS have been employed in industry as biomass for fuel production because of its high calorific value that ranges between 7400 and 8600 BTU (British termal unit), being slightly higher than that of wood [45]. Mancini et al. [145] produced biomethane from CBS, obtaining up to 199 mL CH_4/g volatile solids and increased this value by 14% when pretreating CBS with *N*-methylmorpholine-*N*-oxide. Ilham and Fazil [146] obtained biogas by performing an anaerobic co-digestion of cow manure and CBS, producing 10-fold higher quantities of biogas than with the anaerobic digestion of cow manure alone. Awolu and Oyeyemi [147] used CBS for bioethanol production, using acid hydrolysis and fermentation with *Saccharomyces cerevisae*.

CBS have also been widely used in diverse material production processes, taking advantage of both the chemical and physical properties of CBS. CBS have been incorporated into bioplastics to give them antioxidant properties, biodegradable characteristics, and enhanced physical properties with minimal compound migration (less than the 10 mg/dm^2 allowed by the European Union for bioplastics) for use in food packaging, cosmetic, or

biomedical devices [127,148,149]. Lik et al. [150] developed particleboard, adding up to 60% CBS, while other studies have used the by-product for asbestos substitution in the fabrication of composite brake pads, obtaining good quality materials [151,152]. Also, the addition of CBS to aluminium has been carried out, providing enhanced hardness to the material, although both the tensile strength and ductility were compromised in this case [153].

Due to its particular macromolecular composition, which is rich in lignin, CBS employment for active carbon production has also been extensively proposed. Plaza-Recobert et al. [154] prepared activated carbon monoliths from CBS without a binder, thanks to the by-product's composition of lignocellulosic molecules, gums, pectin, and fats. Other studies have managed to control the active carbon mesoporosity by employing CBS as a lignocellulosic precursor [155]. Ahmad et al. [156] used CBS-based activated carbon as an efficient cationic dye (methylene blue) absorber. Indeed, several authors have proposed CBS, with or without modifications, as a bioadsorbent for textile dyes, gas pollution, heavy metals, or even protein immobilization [157-159].

The utilization of CBS as organic mulch or fertilizer is also very common [160]. Although some authors claim that CBS is too light and that it could affect soil properties if used in large quantities, others consider that its content in nitrogen, phosphate, and potassium could add quality to soil when used as a mulch or fertilizer [121,161]. Besides, this cocoa by-product acts as a humus-forming base, since it does not decompose rapidly, which makes it optimal for use as a fertilizer. Indeed, it has been widely used as a support for fungi cultivation [162-164].

Other examples of alternative CBS uses are utilization for endoglucanase production by fermenting it with *Penicillium roqueforti*, which could be of large importance, since these cellulase enzymes are widely used in different fields, such as the food industry for the extraction of fruit and vegetable juices, in bioethanol production, in paper and cellulose production, or in textile and laundry [165]. Tu et al. [166] developed a natural dye from CBS that was able to give UV protection properties to cotton fabric and Fontes et al. [167] valorized CBS ashes for cement replacement in concrete, obtaining an acceptable and durable material, although its mechanical strength was reduced as a consequence.

4. Biofunctionality and potential health benefits

Since first being consumed by humans, several health benefits and different beneficial properties have been attributed to the fruit of the cocoa tree, mainly because of the high content of polyphenols, mostly flavonoids. As mentioned before, these cocoa phytochemicals

have been largely reported to give different biofunctionalities to cocoa products, such as anti-cancer activity [168-170], effects against diabetes [84,169], effects against neurodegenerative disorders [82], benefits on cardiovascular health [169], action as antimicrobial agents [171], or properties of inflammatory mediators [172]. Also, cocoa polyphenols are well known for their antioxidant properties, which are in many cases responsible in part for all the previously mentioned functions [173,174], together with their particular structures, which makes them resemble several inhibitors and receptor agonists or antagonists of many cell signaling pathways [82]. As could be expected, these properties could probably be extended to other products coming from the cocoa tree, such as the different cocoa by-products. Concretely, for CBS, the by-product that interests us in the present work, in the last few years, several studies have proposed alternative uses in both the food and pharmaceutical industries because of the benefits that could be provided. More specifically, antibacterial and antiviral properties, benefits against cardiovascular diseases, anticarcinogenic effects, antidiabetic activities, neuroprotective potential, and anti-inflammatory effects have been reported. These biofunctionalities and their mechanisms of action are summarized in Table 2 and explained below.

4.1. Antibacterial activity and anticariogenic effects

The widespread use of antibiotics for both livestock and the human population has led to problems such as bacterial resistance or the reduction of economic profits for farmers. For these reasons, products such as CBS have attracted attention because of their antimicrobial capacities, and, therefore, their potential to be used as antibiotic substitutes [175].

For CBS, weak antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, and *Bacillus cereus* has been reported when using acetone, ethanol, methanol, and water extracts, with minimal inhibitory concentrations that ranged between 0.468 and 3.750 mg dry extract/mL [39]. The acetone extract seemed to be the most active, while the water extract was the one with higher minimal inhibitory concentrations. However, the authors stated that no direct correlation was found between the polyphenol concentration and antibacterial activity of the different extracts.

Nevertheless, CBS have been shown to possess stronger antibacterial activities against some type of *Streptococci* bacteria, namely *Streptococcus mutans*, a strain involved in the development of dental caries. Ooshima et al. [176] found a considerable reduction of both oral *Streptococci*, *S. mutans*, and *S. sobrinus*, growth rates with ethanolic extracts of CBS and stated a reduction of plaque deposition. The latter effect was due to the inhibition of bacteria

glucosyltransferases (GTF) by the CBS extract, and, therefore, a reduction of their sucrose-dependent cell adherence [177,178]. The works done by Matsumoto et al. and Osawa et al. [179,180] have also reported the cariostatic effects of CBS due to GTF inhibition, proposing that such inhibition could be caused by high-molecular weight polyphenols, concretely by polymeric epicatechins with C-43 and C-8 intermolecular bonds, estimated to have a molecular weight of 4636 in their acetylated form. Another proposed cause for the inhibition of plaque deposition was the reduction of the hydrophobicity on the cell surface of *S. mutans* caused by polyphenols. Activity against *S. mutans* due to the fatty acids contained in CBS has also been proposed, mainly due to oleic and linoleic acids.

Other authors have taken advantage of the CBS anticariogenic effects to develop different oral hygiene products, such as a toothbrush disinfectant capable of reducing up to 32.25% of the total *S. mutans* [181], or mouthwashes able to reduce the occurrence of these bacteria in saliva to the same levels as 0.2% chlorhexidine, a well-known antiseptic agent [182]. Kwon et al. [183] patented a chewing gum containing a 0.1-1.0% freeze-dried CBS ethanolic extract, aiming to prevent tooth decay.

Table 2. Preparations, applications, and mechanisms of action of the main CBS biofunctionalities and benefits for human health reported in the literature.

Extract/Fraction (Dose)	Application	Mechanism of Action	References
Acetone, ethanol, methanol (80%), and water extracts of defatted CBS (100 mg dry CBS/mL)	Antibacterial	Weak antibacterial activity against *E. coli*, *S. aureus*, *Salmonella*, and *B. cereus* (acetone > ethanol = methanol > water) when compared to a positive control (cephadex) with the inhibition zone diameter technique. Minimum inhibitory concentrations (MIC) of 0.468-3.750 mg/mL when using microdilution technique.	[39]
CBS ethanol, methanol, and acetone (50%, v/v) dried extracts (0.1 g/mL)	Antibacterial (anti-cariogenic)	Inhibitory activity against glucosyltransferase (GTF) from *S. mutans*.	[177]
Cellulase-treated CBS extracted with 30% ethanol, re-dissolved with1% ethanol (1.0 mg dry extract/mL) – *in vitro* CBS dissolved in 20% ethanol (20mg/mL), diluted with water to 1 mg/mL in 1% ethanol (mouth rinses) – *in vivo*	Antibacterial (anti-cariogenic)	*In vitro*: Avoid plaque deposition by inhibiting the adherence of *S. mutans* to saliva-coated hydroxiapatite (inhibition of glucosyltransferase due to epicatechin polymers) and reducing *S. mutans* in plaque (antibacterial activity of unsaturated fatty acids on CBS). *In vivo*: CBS mouth rinse more efficient than just 1% ethanol mouth rinse. Inhibition of plaque deposition and *S. mutans* in saliva.	[180]
Cellulase-treated CBS extracted with 50% ethanol and fractionation of the dried extract by chromatography	Antibacterial (anti-cariogenic)	Antibacterial activity due to oleic and linoleic acids on CBS. Inhibition of bacterial adhesion due to the glucosyltransferase inhibition by polymeric epicatechins with C-43 and C-8 intermolecular bonds estimated to be 4636 in molecular weight in an acetylated form.	[179]
0.1% CBS extract mouth rinse prepared as in [180]	Antibacterial (anti-cariogenic)	*In vivo* reduction of *S. mutans* in saliva significantly similar to that obtained with 0.2% chlorexidine.	[182]
Cellulase-treated CBS extracted with 30% ethanol and resuspended on water (1 mg/mL)	Antibacterial (anti-cariogenic)	*In vitro* and *in vivo* reduction of oral *Streptococci* (*S. mutans and S. sobrinus*) growth rate and reduction of plaque deposition by decreasing sucrose-dependent adherence (inhibition of GTF). Minimum cariostatic concentration of 1.0 mg dry extract/mL.	[176]
Cellulase-treated CBS extracted with 50% ethanol and resuspended in water (1 mg/mL)	Antibacterial	CBS extract used as toothbrush disinfectant, reducing up to 32.25% of bacterial contamination by *S. mutans*.	[181]
Lignin fractions of CBS extracted with 1% NaOH and precipitated with acetic acid and ethanol	Antiviral (HIV and influenza) Anti-carcinogenic	Carbohydrate-rich fractions showed a high selectivity index against HIV (SI = 30-10000). Inhibition of cytopathic effects produced by the influenza virus against MDCK cells. Enhanced radical scavenging activity synergistically with vitamin C. 100-1000 μg/mL of the CBS lignin fraction stimulates the proliferation of human normal gingival fibroblasts (HGFs), but not that of the human oral squamous cell carcinoma cell line (HSC-2).	[184,185]

Table 2 (continuation). Preparations, applications, and mechanisms of action of the main CBS biofunctionalities and benefits for human health reported in the literature

CBS extracted with 0.1 N NaOH, then lyophilized and fractionated according to the molecular weight	Antiviral (HIV)	Anti-HIV activity via inhibition of virus adsorption, and, therefore, inhibition of the cytopathic effect on MT-2 and MT-4 cells (highly sensible to HIV-1) when treated with 31.2-250 μg DW CBS/mL. Inhibition of syncytium formation between uninfected and HIV-infected MOLT-4 cells.	[186]
Ground CBS (60% of total dietary fiber)	Effects on cardiovascular health	Hypolipidemic and cholesterol reducing action *in vivo*: Reduction of total and low-density lipoprotein cholesterol and reduction of the lipid peroxidation in serum and liver. Mostly soluble fraction of the dietary fiber.	[76]
Fractionated (20, 40, 60, and 80% ethanol) CBS freeze-dried extracts obtained with 50% methanol, ethanol, and acetone (0.1 g/mL)	Anti-carcinogenic	CBS polyphenolic fractions reduce the DNA synthesis of cancer cells and the inhibition of the gap-junction intracellular communication (GJIC).	[187]
Dried methanolic extract of CBS (5 g/200 mL)	Anti-carcinogenic	Anti-proliferative action against breast, liver, colon, lung, and cervical cancer cell lines.	[54,188,189]
Soluble dietary fiber (SDF), insoluble dietary fiber (IDF), and total dietary fiber (TDF) powders from CBS	Anti-carcinogenic Effects on cardiovascular health Antidiabetic	Binding capacity for bile acids (potential carcinogens) resulting in their detoxification. Binding capacity for oil and cholesterol, decreasing their bioavailabilities. Absorption of glucose retarding its diffusion and α-amylase inhibition.	[77]
Aqueous extracts of CBS	Antidiabetic	Inhibition of α-glucosidase enzyme close to that of acarbose 0.5 mM.	[13]
Desugared ground CBS	Antidiabetic	Sugar retention on the SDF fraction.	[38]
Supercritical CO_2 extracts of CBS dissolved in acetone	Neuroprotective action	Protective action against ischemic oxidative damage in neuronal phenotype differentiated cells.	[88]
Dried ground CBS	Neuroprotective action and anti-inflammatory	Great content on phytoprostanes (474.3 ng/g DW CBS) phytofurans (278.0 ng/g DW CBS) with cytoprotective activity in immature brain cells and involved in anti-inflammatory processes.	[74]
Methanol and acetone CBS fractions of ethanolic extract (rich in epicatechin and tannins) (5 mg dried extract/mL)	Anti-inflammatory	Prevention of oxysterol mixture-induced IL-8 release (pro-inflammatory cytokine) on Caco-2 intestinal cell models and prevention of exaggerated toll-like receptor 2 and 4 (TRL2 and TRL4) responses, which may contribute to induce oxysterol-dependent intestinal inflammation.	[78,190]
Freeze-dried CBS aqueous extract (0.02 g/mL)	Action against obesity and inflammation-related disorders	Adipogenesis modulation and inhibition of adipokine production (responsible for inflammation processes and insulin resistance).	[100]

4.2. Antiviral properties

The antiviral effects of CBS, mainly against HIV and influenza, have been reported to occur mostly due to the lignin-carbohydrate complexes, rather than other polyphenols with a lower molecular weight, such as tannins or flavonoids [185]. Indeed, Sakagami et al. [184] obtained selectivity indices (SI = 50% cytotoxic concentration, CC_{50}/50% effective concentration, EC_{50}) of CBS lignin fractions against HIV that ranged from 30 to 10,000, which are surprisingly high values when compared to those of tannins (SI = 1–10), flavonoids (SI = 1), or lignin fractions obtained from other sources, such as cocoa mass (SI = 10–100). These values obtained for CBS were in some cases comparable to those of reverse transcriptase inhibitors. Unten et al. [186] found out that CBS action against HIV was at a maximum when extracts were added to cells at the same time as virus adsorption, and, therefore, the action is not directly related to the virus replication after infection, but that it was instead mostly due to the inhibition of virus absorption. Indeed, they observed that the cytopathic effect on highly HIV susceptible cells was inhibited when treated with 31.2–250 μg dried CBS extract/mL. Besides, they also observed the inhibition of syncytium formation between infected and uninfected cells, avoiding HIV replication in two different ways. Similarly, it was found that the same CBS lignin fractions were able to inhibit the cytopathic effects produced by the influenza virus [184], an effect that was already reported for condensed tannins [171]. These fractions presented a selectivity index of 155 against the influenza virus and could act synergistically with vitamin C, enhancing its activity.

4.3. Action on cardiovascular health

Cocoa flavanols have been related to cardiovascular disease prevention thanks to different aspects, such as their antioxidant activity on plasma, reducing platelet reactivity, or their anti-inflammatory properties that could decrease the risk of arteriosclerosis or thrombosis [169,191,192]. However, the *in vivo* bioavailability of these compounds when obtained with diet is generally low, and, therefore, an important part of the CBS contribution to cardiovascular health takes part in the digestive system. Indeed, some authors have related the beneficial effects of CBS on cardiovascular health with the reduction of atherogenic fat absorption. Thus, Nsor-Atindana et al. [77] found that CBS fiber has a considerably high adsorption capacity for oil and cholesterol, decreasing their bioavailability during the gastrointestinal digestion process. On the other hand, Lecumberri et al. [76] reported *in vivo* hypolipidemic and cholesterol reducing effects of CBS in rats, mainly due to the SDF

fraction. Indeed, they found significant reductions of the total and low-density lipoprotein cholesterol when consuming CBS after having followed a cholesterol-rich diet. Besides, the consumption of CBS fiber also reduced lipidic peroxidation in the serum and liver, probably because of the polyphenolic compounds.

4.4. Anticarcinogenic action

Cocoa polyphenols are known to possess anticarcinogenic properties, mainly because of their potential to reduce excessive oxidative stress, which is characteristic of all the different stages of cancer development and is highly involved in DNA damage, leading to mutation [169]. Flavanols and procyanidins from cocoa have also demonstrated to be implicated in the regulation of different cancer-related signal transduction pathways regarding mutagenesis, tumorigenesis, angiogenesis, or metastasis, among others [170,193,194]. Concerning the anticarcinogenic effects of CBS, some studies have revealed antiproliferative effects of fermented CBS methanolic extracts against breast, liver, colon, lung, and cervical cancer cell lines, although unfermented CBS have not shown such promising results [54,188,189]. However, these studies did not show any positive correlation between the antiproliferative effects and the total phenolic content, which may suggest that antiproliferative activity is subject to just some of the compounds contained within CBS extracts. Indeed, Nsor-Atindana et al. [77] found out that dietary fibers from CBS have the capability of adsorbing and detoxifying bile acids, which are known to cause injury in gastric mucosal epithelial cells, resulting in DNA damage, and, therefore, can act as potential carcinogens. Sakagami et al. [185] observed that a 100-1000 μg/mL CBS lignin fraction is able to stimulate the proliferation of human normal gingival fibroblasts (HGF), but not those of the human oral squamous cell carcinoma cell line (HSC-2). On the other hand, Lee et al. [187,195] discovered that polyphenolic extracts and fractions of CBS have inhibitory effects on carcinogenesis. These fractions have been shown to inhibit the proliferation of liver, stomach, and colon cancer cells by suppressing their DNA synthesis, namely, 4-fold higher than vitamin C, which is a well-known chemopreventive dietary compound. Besides, the same fractions had a 10-fold higher ability to reduce the inhibition of the gap junction of intracellular communication (GJIC) than vitamin C, an inhibition that is characteristic of carcinogenesis and usually used as a key biochemical index for this phenomenon. This reduction seemed to increase proportionally with the polyphenolic content.

4.5. Antidiabetic activity

Cocoa flavanols have already been proven to act as chemopreventive agents by helping the prevention or treatment of type 2 diabetes mellitus. They can regulate insulin secretion and protect β-pancreatic cells. They also have an insulin-like activity, and thus, cocoa polyphenols can enhance insulin sensitivity by improving glucose transport to tissues such as skeletal muscle, liver, or adipose tissue, resulting in glycemic control, besides protecting these tissues from the oxidative and inflammatory damages that are associated to diabetes [84]. Regarding CBS, Rojo-Poveda et al. [13] developed CBS functional beverages with a potential antidiabetic capacity, since they were able to inhibit α-glucosidase, an enzyme involved in glucose degradation. Some of those beverages displayed an effect close to that of acarbose 0.5 mM, which is a drug used to treat diabetes. It has also been found that dietary fiber fractions of the by-product can inhibit α-amylase (another enzyme involved in glucose degradation) and adsorb glucose, retarding its diffusion through the intestinal wall, which could lead to antidiabetic properties during the passage of CBS through the human gut when consumed with diet [38,77].

4.6. Other biofunctional properties

In addition to the already mentioned biofunctions, CBS also present neuroprotective, anti-inflammatory, and anti-obesity properties, among others. Supercritical CO_2 CBS extracts have been shown to protect human neuroblastoma cells against ischemic damage, although the more active fractions were not the ones presenting the higher antioxidant activity [88]. Also, it was found that CBS contain considerable quantities of phytoprostanes and phytofurans, which are believed to be involved in brain and central nervous system development, since they present cytoprotective activity in immature brain cells. Besides, these molecules are likely to play a role in the prevention of metabolic disorders and can exhibit anti-inflammatory, immune function regulation, and apoptosis-inducing activities [74].

Likewise, Rossin et al. [78,190] found that CBS could play an important role in the treatment of inflammatory bowel diseases (IBD), since phenol-rich CBS fractions are able to inhibit the inflammatory effects caused by oxysterols on intestinal cells (Caco-2). These fractions, with the presence of high amounts of (-)-epicatechin and tannins, fully avoid the production of IL-8, which is a pro-inflammatory cytokine and prevent exaggerated toll-like receptor (TLR)-mediated immune and inflammatory responses.

An action against obesity and inflammatory-related disorders was also proposed by Rebollo-Hernanz et al. [100]. They found that a freeze-dried aqueous extract of CBS, mainly containing polyphenols, was able to inhibit lipid accumulation and lower pro-inflammatory

cytokine production. Besides, it attenuates inflammation in adipose tissue macrophages and inhibits their activation, and, therefore, regulates adipokine secretion, which could otherwise cause mitochondrial dysfunction and insulin resistance.

In another study, 10 μg/mL of CBS carbohydrate-rich fractions inhibited the cytotoxicity caused by cigarette smoke on HGF and HSC-2 cells, although higher concentrations seemed to increase it [185].

5. Safety aspects

As a by-product of the cocoa tree, CBS could be subjected to several contamination sources during the whole farming, production, and manufacturing processes, acquiring different chemical compounds that could be harmful to human health. During farming, CBS, as well as the whole cocoa plant, can be exposed to natural contamination coming from the soil in which it the cocoa tree grows, where the main concern is heavy metal absorption. It can also be exposed to contamination by insecticides used during cultivation, which are largely used to reduce the 30–40% of global cocoa production that is annually lost to insects and diseases [196]. Unfortunately, insecticides, such as neonicotinoids, mainly concentrate on the outer part of the cocoa bean, i.e., the CBS, but some studies have developed methods for their assessment and clean-up, also recommending a greater efficiency of insecticide application to avoid accumulation to unsafe levels. [12,197]. After harvesting and during the journey to chocolate manufacturing locations, CBS could also be exposed to another kind of contamination, such as polycyclic aromatic hydrocarbons (PAHs), due to inappropriate drying processes near smoke [198], or mold and mycotoxin formation during storage and transport. With this, because of the occurrence and importance of these risk factors, a special focus must be given to heavy metals and mycotoxin accumulation when using CBS.

5.1. Heavy metals

Heavy metals generally consist of non-essential metals with toxic potential for human health. As a plant, the cocoa tree absorbs these metals from contaminated soil and accumulates them, mainly in the fruit, and, therefore, also on the outer part of the cocoa bean. Being a protective barrier for the bean, CBS absorb higher quantities of heavy metals, mostly lead and cadmium [111,199,200]. Indeed, some authors have studied the possibilities of using CBS as an adsorbent for heavy metal removal from acidic soils [201,202]. Soil is considered an important metal source, since big parts of cocoa crops are located in volcanic soils, though, atmospheric contamination during cocoa treatment and shipping is believed to be the main

source in the case of lead contamination [203]. This contamination is caused by leaded gasoline combustion, which is still commonly used in several cocoa-producing countries, whose emissions could be in direct contact with the cocoa shell during the fermentation, sun-drying, and shipping processes of cocoa beans [204]. Assa et al. [205] found lead concentrations in CBS ranging from 5.80 to 11.15 mg/kg, which were 100-fold higher than those observed inside cocoa beans and significantly higher than the 1.00 mg/kg of lead contamination allowed by the European Union in cocoa powder, although these values are highly variable between different studies. Hence, it is of great importance to control the lead content of CBS, especially when using them as a food ingredient, and to concentrate efforts on the mitigation of the primary sources. Concerning cadmium, Lewis et al. [206] found that 18-56% of its total content on the entire cocoa bean is concentrated in the shell, although they claimed that these values could vary depending on the plant genetics and that a genetic strategy could be used in order to mitigate cadmium concentrations. However, levels of 0.05-0.10 mg per kg of CBS have been found for Cd, which are still far from the 1.5 mg/kg maximum allowed by the Codex Alimentarius for cocoa powder [205,207]

5.2. Mycotoxins

Mycotoxins are one of the major safety concerns in cocoa and CBS. These are low-molecular weight toxins that are present in a wide variety of food products, produced by some fungi species from the *Aspergillus* and *Penicillium* genera, which can contaminate cocoa during fermentation, drying, and storage and are generally thermostable, so they are not completely eliminated during roasting [208,209].

One of the main mycotoxins found in cocoa products is ochratoxin A (OTA), which has been related to nephrotoxic, teratogenic, and immunosuppressive activities, and is classified as a 2B carcinogen (possible human carcinogen) [210]. Studies have demonstrated that the major OTA percentage of the total cocoa bean is contained in CBS, typically about 50–95%, with concentrations that generally range between 0.13 and 2.01 μg of OTA per kg of CBS, which is still within the acceptable values determined by the European Commission for cocoa beans (less than 2 μg/kg) [34,209-211], although other authors have reported values of up to 23.1 μg of OTA per kg of CBS [212]. Aflatoxins have also been detected in cocoa products. Similar to OTA, aflatoxins can produce hepatotoxic, teratogenic, mutagenic, and carcinogenic effects in humans. Concretely, aflatoxins B1, B2, G1, and G2 have been detected in CBS in concentrations that range between 0.01–1.00 μg/kg, <0.03–0.02 μg/kg, <0.03–0.44 μg/kg and <0.03–0.06 μg/kg, respectively, which are still considered as safe values [34,213]. Another

mycotoxin detected on CBS was deoxynivalenol, also known as vomitoxin, since it creates nausea, which again was found to be concentrated in CBS more than in cocoa nibs, but still in concentrations without posing a risk to human health [214].

Mycotoxins have been demonstrated to be present in CBS in generally acceptable concentrations. However, these levels can sometimes increase because of different factors. For these reasons, several studies have focused their attention on mycotoxin remediation. Amezqueta et al. [208] eliminated up to 98% of the OTA contained in CBS with a simple chemical method, employing sodium carbonate and potassium carbonate at low concentrations, while Arlorio et al. [28] conducted a Soxhlet extraction with 2-propanol eliminating, 70.2% of the OTA contained in the CBS. Aroyeun and Adegoke [215] found an OTA reduction efficiency of 64.3–95% by employing essential oils, while Manda et al. [216] observed an average OTA decrease of 23.8% with just a controlled roasting process at 140 °C for 30 minutes. Oduro-Mensah et al. [144] proposed a method employing filamentous fungi that degraded 31-74% of the OTA contained in cocoa pods. Besides, CBS have been affirmed to be especially likely to fungi spoilage, due to the pulp residues that can remain on it after bean separation from the pods and fermentation, so the occurrence of mycotoxins could also be partially prevented if special attention is directed to bean cleanliness after fermentation [217].

CONTEXT AND AIMS OF THE WORK

In a world of increasing population growth and emerging healthy eating trends and concerns, food markets are becoming more and more competitive and new challenges such as sustainable production are arising in this context. However, food waste remains one of the main concerns in food industry. For this reason, importance has been given in recent years to the utilization and revalorization of food by-products. Specifically, in the cocoa industry, several by-products exist. Only a small part of the cocoa fruit is used for chocolate and cocoa product production, while the rest is normally discarded or underutilized. The cocoa bean shell (CBS) is one of these cocoa by-products and constitutes 10 – 17% of the total cocoa bean weight. Nevertheless, contrary to other cocoa by-products, CBS normally gets to the final steps of cocoa manufacturing process before its disposal, with the added costs and complications that this can generate. At that point, CBS is normally discarded or reutilized in ways in which its main and most interesting properties are not well taken advantage of. Indeed, it has been found that CBS possesses several qualities and compounds able to give it different biofunctionalities and to promote human health and well-being.

The general aim of this thesis was to address the valorization of the CBS from a broad point of view, considering applications and combining expertise in both food technology and pharmaceutical fields. In the framework of this approach, CBS could be recycled by utilizing the concept of circular economy for a high add-value product. The present PhD work is a continuation of the COVALFOOD project "Valorisation of high added-value compounds from cocoa industry by-products as food ingredients and additives", which received funding from the European Union's Seventh Framework program for research and innovation under the Marie Skłodowska-Curie grant agreement No. 609402-2020 researchers: Train to Move (T2M).

The first goal in this work was to get a deep knowledge in the current advances on CBS investigation, analyzing the uses that are already applied to it and its different characteristics for further functional food production and medical application purposes. For this purpose, a literature review was done over the current knowledge about CBS chemical and nutritional composition, as well as about the several uses that have been proposed up to date in order to valorize this by-product not only for food, livestock feed or industrial usages, but also for different medical applications.

In a second step, the characterization of both polyphenolic and aromatic profiles of CBS was addressed. The aim was to compare these chemical profiles with those of cocoa beans for

the consideration of different possible uses that could be given to CBS. Besides, authentication strategies for the CBS geographical origin and variety were sought by using the obtained chemical profiles. These works form **Part 1** of the Results and Discussion section. Within this first part of the results, **Chapter 1.1** addresses the characterization of CBS polyphenolic profile, while **Chapters 1.2 and 1.2.1** deal with the characterization of the volatile organic compounds (aromas) contained in CBS.

After the verification of CBS potential as a functional food ingredient and its cocoa-similar aroma profile, the next focus was given to the reintroduction of CBS into the value chain for the production innovative functional foods aimed at improving the well-being of consumers while promoting sustainability. **Part 2** of the results reports the production at pilot scale and the analysis of different CBS-based functional foods such as beverages (**Chapters 2.1 and 2.2**) and biscuits (**Chapters 2.3 and 2.4**). The aim of this part was to analyze the influence of CBS addition in the technological factors linked to the food production and the final product, as well as to research into the potential of these foods to promote health. *In vitro* biofunctionalities of the model foods were assessed and the bioaccessibility and intestinal permeability of these biofunctionalities and of some compounds of interest were studied in order to understand the potential effects of the CBS-based foods within the human organism.

Finally, the last task in this project, which is addressed in **Part 3** (**Chapter 3.1**), was to evaluate the antimicrobial potential of the cocoa bean shell against different food-borne bacteria and fungi strains based on the previous knowledge about CBS already acquired during the work. Here, CBS was not considered as a food ingredient anymore, but as a potential food additive or pharmaceutical ingredient.

RESULTS AND DISCUSSION

PART 1 – COCOA BEAN SHELL CHARACTERIZATION

In this first part of the Result and Discussion section, several CBS samples from different geographical origins and cocoa varieties were characterized for their polyphenolic and volatile organic compounds (VOC) profiles. Both characterization works followed a similar experimental design: in a first step, polyphenols (and methylxanthines) or VOCs were studied by a complete characterization method (liquid or gas chromatography, respectively) that allowed to identify single compounds. Polyphenolic and VOC fingerprints were established for CBS, several cocoa-marker compounds were identified for both polyphenols and aromas, and the results were compared to the values found for cocoa beans. Concurrently, CBS samples were also analyzed for their polyphenolic and aromatic profile by using screening and rapid methods allowing to detect different compound groups within the polyphenolic or VOC profiles (spectrophotometric assays and E-nose, respectively). The results obtained with the screening methods were compared to those obtained with the chromatographic techniques in order to assess their validity and to establish, in the case of polyphenols, some structure-activity relationships.

In a second step, the obtained profiles were used to establish some authentication techniques for the CBS geographical origin and/or variety. Principal component analyses of the obtained results were used for this purpose. The PCA differentiation results obtained with the chromatographic data were compared to those obtained with the screening methods. Indeed, the purpose of these comparisons was to establish the possibility of using more rapid, simpler, economic, and easier to handle techniques to interpret results more easily in order to obtain similar differentiation and product authentication strategies.

Part 1 is composed by three different chapters, each one issued from one scientific article. **Chapter 1.1** comprises the characterization of the CBS polyphenolic profile, while **Chapter 1.2** and **Chapter 1.2.1** address the characterization of the VOC profile of CBS. Chapter 1.2.1 was conceived as a data article that complements the research article forming Chapter 1.2.

CHAPTER 1.1

Determination of Polyphenolic Profile by RP-HPLC-PDA Analysis and Spectrophotometric Assays for the Chemometric Classification of Cocoa Bean Shells

Chapter 1.1 focuses on the determination of the polyphenolic fingerprint of CBS. In order to do this, a large CBS sample set was analyzed so as to obtain their polyphenolic composition and content by means of two different techniques: RP-HPLC-PDA, which was considered as a complete characterization method, and spectrophotometric assays, which were considered as screening methods. The results obtained by both techniques for the CBS polyphenolic content served for the proposition of an authentication strategy for CBS differentiation depending on its geographical content. Besides, it was found that a differentiation based on the CBS variety (Forastero, Trinitario, Criollo, and Nacional) was also possible by using the methylxanthine (caffeine and theobromine) ratio in the different CBS samples. This research is of great importance in the functional food development frame that will be presented next in the present thesis, since it confirms the potential of the by-product for being used as a functional ingredient.

This chapter was issued and adapted from a scientific article:

Determination of Polyphenolic Profile by RP-HPLC-PDA Analysis and Spectrophotometric Assays for the Chemometric Classification of Cocoa Bean Shells

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1. Introduction

The cocoa bean shell (CBS) is one of the main by-products of the chocolate manufacturing industry, usually discarded after cocoa bean husking. In contrast to other cocoa by-products such as the pod and the mucilage, CBS is usually discarded in a later step of the cocoa manufacture process, and it has been reported that more than 700 thousand tons of CBS waste are produced worldwide in a yearly basis [218]. CBS normally gets to the different chocolate manufacture countries along with the bean, and is separated from it after the roasting process [5]. This gives added costs in the cocoa manufacturing process, mainly linked to the added-weight that CBS represents during cocoa bean transportation and the environmental and economic cost of its disposal or underutilization. For this reason, different revalorization strategies linked to its polyphenolic content have appeared in the latest years for this by-product.

CBS represents about 10% - 17% of the total cocoa bean weight and has been reported to be rich in proteins, dietary fibers, minerals, vitamins, and, most importantly, methylxanthines and polyphenols [218]. Besides, it is well known that cocoa beans and their derivative products are rich in polyphenols, concretely flavanols [81]. These compounds are secondary metabolites of plants with several benefits for human health when consumed as food [219]. They display antioxidant properties and have been demonstrated to act as chemopreventers against several diseases such as cancer, diabetes, oxidative stress, cardiovascular diseases, and inflammatory bowel diseases, among others [82,84,169,170]. For this reason, and in the frame of a circular economy for a zero-waste cocoa industry, CBS has been lately proposed as a low-cost ingredient for functional foods [220], mainly baked goods [221] and beverages [13,222]. Besides, its aromatic profile was previously reported to be very similar to that of cocoa powder [66] and it could therefore be an interesting ingredient to produce cocoa-based products with a lower fat content and with higher amounts of dietary fibers [218].

However, as a vegetable product, CBS chemical composition can significantly differ based on several factors such as their geographical provenience or plant genotype and the globalization of the cocoa industry could lead to issues concerning cocoa products traceability. Concretely, geographic origin has been reported to be one of the main authenticity issues for food products in Europe [223]. Food policies require authenticity of products and demand that the information given to the consumer matches composition and origin of the product [223,224]. Besides, increasing attention has been given to sustainability in cocoa production, for which geographical traceability becomes mandatory. Improving this

aspect could ensure transparency along the supply chain and ensures cocoa and CBS sustainability, quality, and safety [225]. In the latest years, chemometric techniques implying multivariate analyses have arisen to assure food quality and authenticity, as well as to classify their geographical origin or genotype based in their chemical composition. Some of these techniques have been applied to cocoa beans [226]. However, being a novel ingredient, studies concerning CBS traceability are limited in the literature. On the other hand, the polyphenolic and methylxanthine composition of cocoa products, either qualitative and quantitative, is very variable and depends on many factors such as climatic conditions during the plant growth, area of cultivation, different species, and post-harvest treatments, among others [227]. Henceforth, since food authentication depends on measuring features that can discriminate foods of different origins [223], establishing fingerprints based on the concentration of key compounds in CBS could help in the estimation of the by-product traceability and quality in cases in which the purpose is to re-valorize it as a functional food ingredient.

As CBS attracts the interest of food researchers and industries, studies concerning its polyphenolic content have appeared in the literature in the latest years. Nevertheless, most of these studies imply one or few similar CBS samples or aim at few of the already known and representative compounds in CBS. The aim of the present study was to cover this gap by providing broader information about the polyphenolic profile and methylxanthine content in CBS, implying a large number of samples that could allow for extensive characterization of the cocoa by-product. Additionally, spectrophotometric assays were performed, and the feasibility of these methods as polyphenolic content determination screening methods was assessed. In a second step, the results obtained with both methods were employed for a chemometric classification of the CBS samples according to their geographical origin and cocoa variety.

2. Materials and Methods

2.1. Chemicals and Standars

Folin&Ciocalteu’s phenol reagent, sodium carbonate (≥99.5%), 2,2-diphenyl-1-picrylhydrazyl (95%; DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%; trolox), vanillin (99%), (+)-catechin hydrate (>98%), methanol (≥99.9%), hydrochloric acid (37%), aluminum chloride (99%), sodium nitrite (≥99%), formic acid (≥98%), quercetin-3-O-glucoside (≥90%; Q-3-G), theobromine (≥98.5%), caffeine (≥98.5%), *p*-coumaric acid

(≥98.0%) and quercetin (≥98.5%) were provided by Sigma-Aldrich (Milan, Italy). Gallic acid, ethanol (≥99.9%), sodium hydroxide (1 M), (-)-epicatechin (≥99%), procyanidin B1 (≥98.5%; PCB1) and protocatechuic acid (>97%) were supplied by Fluka (Milan, Italy).

Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

Table 1. Origin of fermented and dried cocoa beans used to yield the cocoa bean shells.

Sample code	Cultivar	Country	Region	Continent	Macroarea
BRA	*Trinitario*	Brazil	Cabruca	America	South America
CAM1	*Forastero*	Cameroon	N.D.	Africa	West Africa
CAM2	*Trinitario*	Cameroon	N.D.	Africa	West Africa
COL1	*Forastero*	Colombia	N.D.	America	South America
COL2	*Trinitario*	Colombia	Santander	America	South America
CON1	*Forastero*	Congo	N.D.	Africa	West Africa
CON2	*Forastero*	Congo	N.D.	Africa	West Africa
DOR1	*Trinitario*	Dominican Republic	N.D.	America	Central America
DOR2	*Forastero*	Dominican Republic	N.D.	America	Central America
DOR3	*Trinitario*	Dominican Republic	N.D.	America	Central America
DOR4	*Trinitario*	Dominican Republic	N.D.	America	Central America
ECU1	*Forastero*	Ecuador	Arriba	America	South America
ECU2	*Trinitario*	Ecuador	N.D.	America	South America
ECU3	*Forastero*	Ecuador	N.D.	America	South America
ECU4	*Nacional*	Ecuador	N.D.	America	South America
ECU5	*Nacional*	Ecuador	N.D.	America	South America
ECU6	*Forastero*	Ecuador	Palo Santo	America	South America
ECU7	*Criollo*	Ecuador	N.D.	America	South America
GHA	*Forastero*	Ghana	N.D.	Africa	West Africa
IVC	*Forastero*	Ivory Coast	N.D.	Africa	West Africa
JAM	*Trinitario*	Jamaica	N.D.	America	Central America
MAD	*Forastero*	Madagascar	N.D.	Africa	East Africa
MEX	*Trinitario*	Mexico	Chontalpa	America	Central America
PER1	*Forastero*	Peru	N.D.	America	South America
PER2	*Trinitario*	Peru	N.D.	America	South America
SAT1	*Forastero*	São Tomé	N.D.	Africa	West Africa
SAT2	*Forastero*	São Tomé	N.D.	Africa	West Africa
SAT3	*Forastero*	São Tomé	N.D.	Africa	West Africa
SLE	*Forastero*	Sierra Leone	N.D.	Africa	West Africa
TAN	*Forastero*	Tanzania	N.D.	Africa	East Africa
TOG1	*Forastero*	Togo	N.D.	Africa	West Africa
TOG2	*Forastero*	Togo	N.D.	Africa	West Africa
UGA1	*Forastero*	Uganda	N.D.	Africa	East Africa
UGA2	*Forastero*	Uganda	N.D.	Africa	East Africa
VEN1	*Trinitario*	Venezuela	Sur del Lago	America	South America
VEN2	*Trinitario*	Venezuela	Caucagua	America	South America
VEN3	*Trinitario*	Venezuela	Merida_1	America	South America
VEN4	*Trinitario*	Venezuela	Cuyagua	America	South America
VEN5	*Criollo*	Venezuela	Canoabo	America	South America
VEN6	*Trinitario*	Venezuela	Ocumare_1	America	South America
VEN7	*Criollo*	Venezuela	Merida_2	America	South America
VEN8	*Criollo*	Venezuela	Merida_3	America	South America
VEN9	*Criollo*	Venezuela	Carenero	America	South America
VEN10	*Criollo*	Venezuela	Ocumare_2	America	South America

2.2. Samples

A set of 88 CBS samples (44 different samples, 2 batches of each provided by the same supplier) were yielded from fermented and dried cocoa beans (*Theobroma cacao* L.) harvested during the season 2014/2015. The cocoa beans, purchased from several local cocoa manufacturers, came from 19 different countries in both continents America (n = 28) and Africa (n = 16) and belonged to four cocoa varieties: Forastero (n = 21), Trinitario (n = 15), Criollo (n = 6), and the Nacional variety of Ecuador (n = 2) as presented in Table 1. Due to confidentiality issues of the suppliers, information about the fermentation and drying conditions of the samples is not available. However, in order to standardize samples to a maximum, a similar roasting process was performed for all the cocoa beans at 130°C (isothermal) during 20 minutes on a ventilated Memmert UFE 550 oven (ENCO, Spinea, Italy), prior to the unshelling process. After manual separation from the beans, CBS samples were ground into a 250μm-particle size powder by using an ultra-centrifugal mill Retsch ZM 200 (RetschGmbh, Haan, Germany) and stored under vacuum at -20°C in no-light environment until extraction and analysis.

2.3. Polyphenolic Extracts

Extraction of polyphenols and methylxanthines was performed by mixing 0.50 g of cocoa bean shell powder with 10 mL of ethanol-water mixture (50:50, *v*/*v*) for 2h at 25°C under constant rotatory oscillation (3 oscillation/s) using a VRL 711 orbital shaker (AsalS.r.l., Milan, Italy), according to the optimized extraction conditions for CBS described by Barbosa-Pereira et al. [57]. The mix was then centrifuged on a centrifuge Heraeus Megafuge 11R (THERMO electron, Chateau-Gontier, France) at 4200 ×g for 10 minutes at 4ºC before collecting and filtering the supernatant through a 0.45 μm PTFE filter. Extractions were performed in duplicate for each CBS sample.

2.4. RP-HPLC-PDA Analysis

Detection and quantification of polyphenolic molecules and methylxanthines contained in the CBS extracts were performed by reversed-phase high-pressure liquid chromatography with a photodiode array detector (RP-HPLC-PDA) Thermo-Finnigan Spectra System (Thermo-Finnigan, Waltham, MA, USA). The instrument was composed by a P2000 binary gradient pump, SCM 1000 degasser, AS 3000 automatic injector, and Finnigan Surveyor

PDA Plus detector. Data were acquired, analyzed and processed by using the ChromQuest software 5.0 (Thermo-Finnigan, Waltham, MA, USA).

A reverse-phase Kinetex Phenyl-Hexyl column (150 × 4.6 mm, particle size of 5 μm) (Phenomenex, Castel Maggiore, Italy) was used at 35 °C in order to obtain the compound separation.

Water with 0.1% v/v formic acid and methanol served as mobile phases (solvent A and B, respectively). The sample injection volume was 10 μL and the gradient elution method described by [13] was applied with a flow rate of 1 mL/min during 45 minutes as follows: 0–2 min, 90% A and 10% B; 2–18 min, linear gradient from 10% to 50% B; 18–40 min, linear gradient from 50% to 80% B; 40–42 min, linear gradient from 80% to 90% B; and minutes 42–45, linear gradient until 90% A and 10% B.

Compound detection was performed with a continuous wavelength scanning between 200 and 400 nm. Quantification data for each compound were acquired at its maximum and more representative wavelengths, indicated in Table 2.

Compound identification was based on standards injection and on a previous work performed in our laboratory in which mass spectrometry was used coupled to HPLC as molecular identification method (yet unpublished).

For quantification, different external calibration curves that ranged from 0.2 to 100 mg/L of standard were analyzed under the same conditions, obtaining in all the cases correlation coefficients higher than R^2 = 0.9980. Results were expressed as g/kg of CBS for methylxanthines (theobromine and caffeine) and as mg/kg of CBS for polyphenols.

2.5. Spectrophotometric Assays

2.5.1. Total Phenolic, Flavonoid, and Tannin Contents

Three spectrophotometric screening methods were employed for rapid polyphenolic quantification depending on the different characteristics of these molecules. The total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) were assessed by the Folin–Ciocalteu, aluminum chloride, and vanillin methods, respectively. Methods were adapted as described in [57] to be performed in 96-well microplates, and a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy) was used.

As mentioned, TPC measurements were based on the Folin-Ciocalteu method. In this assay, the polyphenols are oxidized in a basic environment by means of the Folin-Ciocalteu

reagent, which consists of a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$. The Folin-Ciocalteu reagent oxidizes the phenols and reduces itself to a mixture of tungsten blue, W_8O_{23}, and molybdenum blue, Mo_8O_{23} (Figure 1). The reduction of the reagent, which is initially yellow in color and becomes blue, is followed by spectroscopy in the visible spectrum. The blue reduced mixture has a wide absorption band at λ max between 720 and 750 nm [228]. Generally, gallic acid (3,4,5 trihydroxybenzoic acid, GA) is used as a standard for quantification. However, although this method has been largely used for the total phenolic content determination in vegetal samples, it contains some drawbacks and the interpretation of the results should be always done with precaution. Indeed, this method is not specific for polyphenols but it could also detect other phenols such as those contained in proteins and reducing sugars. TPC results give therefore values that are usually overestimated [228].

For the measurement, 20 µL of sample was introduced into the wells of a 96-well microplate, to which 100 µL of 10% Folin-Ciocalteu reagent prepared in water (*v*/*v*) was subsequently added. After 3 minutes of incubation, 75 µL of 7.5% sodium carbonate solution was added to the wells. The solution was then incubated in the dark for 1 hour at 25 ° C and the absorbance was measured at 740 nm.

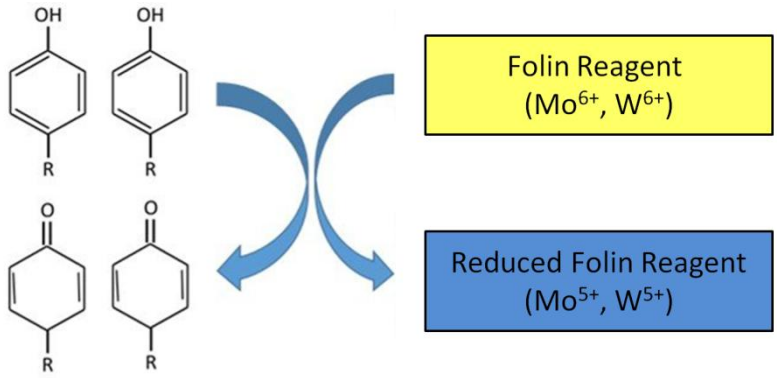

Figure 1. Reduction of the Folin-Ciocalteu reagent caused by the oxidation of the phenolics in a sample (adapted from [229]).

The total flavonoid content was determined with the colorimetric aluminum chloride assay described by Herald et al. [230]. The principle of this method is based on the determination of the complexes that are formed between aluminum chloride and flavonoids that possess ortho-dihydroxy-, peri-hydroxy-oxo-, and ortho-hydroxy-oxo- groups (Figure 2 a, b, and c, respectively [231]). The formation of the complexes is accompanied by a shift of the absorption band in the spectrum.

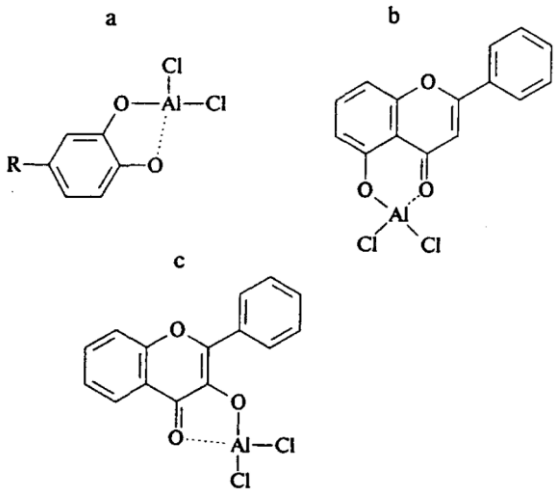

Figure 2. Formation of the aluminum chloride complex with the ortho-dihydroxy- (**a**), peri-hydroxy-oxo- (**b**), and ortho-hydroxy-oxo- (**c**) groups of flavonoids [231].

For the TFC determination, 25 µL of sample was introduced in the wells of the microplate, to which 110 µL of an aqueous solution of $NaNO_2$ (4.5 g /L) was added. After 5 minutes, 15 µL of an aqueous solution of $AlCl_3$ (100 g /L) was added. After another 6 minutes from this addition, 50 µL of 1 M NaOH and 50 µL of ultrapure water were introduced. The plate was then shaken for 30 seconds and the absorbance was measured at 510 nm.

The vanillin method, used for the TTC determination is specific for flavan-3-ols and proanthocyanins and is based on the condensation of the vanillin reagent with the proanthocyanins (condensed tannins) in an acidic solution (Figure 3 [219]). The protonated vanillin is a weak electrophilic radical, which reacts with the rings of these compounds in position 6 or 8. The intermediate product of this reaction is easily dehydrated to obtain a product with an intense red color [232]. The vanillin reaction is influenced by the acidic nature and the substrate concentration, the reaction time, the temperature, the vanillin concentration and the water content. Although the preparation of the vanillin reagent in methanol makes it more sensitive to polymeric tannins than to monomeric tannins (flavan-3-ols), it could also react with other molecules such as dihydrochalcones and anthocyanins. An overestimation of the TTC parameter could therefore occur in some cases in which concentrated extracts are analyzed [219].

For this assay, a 4% (*w*/*v*) vanillin solution was prepared in a mixture of methanol and 37% hydrochloric acid, in a 2:1 (*v*/*v*) ratio. 20 µL of sample was introduced into each well of the microplate and 200 µL of vanillin reagent was then added. The microplate was incubated for 15 minutes and then the absorbance was measured at 500 nm.

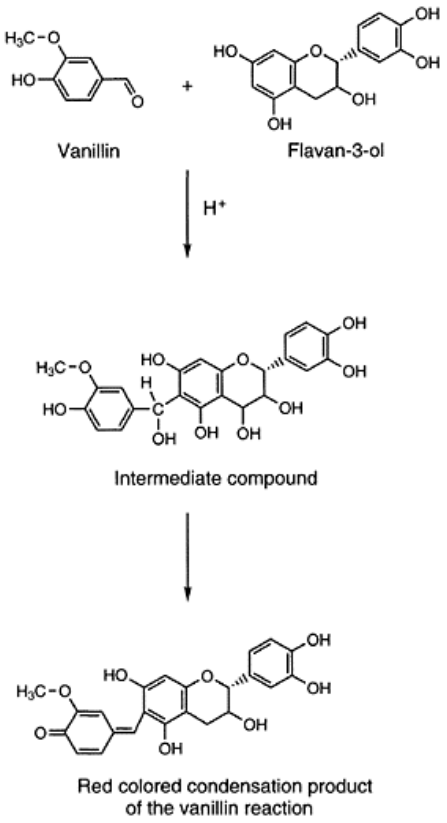

Figure 3. Reaction between vanillin and flavan-3-ol [219].

All the analyses were performed in triplicate and final concentrations were expressed according to different calibration curves. For the TPC, the obtained values were expressed as mg of gallic acid equivalents per gram of CBS (mg GAE/g CBS), according to a calibration curve drawn from 20 mg/L to 100 mg/L. For both TFC and TTC, catechin was used to build the standard curve (5–500 mg/L) and concentrations were expressed as mg of catechin equivalents per gram of CBS (mg GAE/g CBS).

2.5.2. Antioxidant Capacity

The antioxidant activity was determined with the DPPH radical method described by Gadow et al. [233]. DPPH (2,2'-Diphenyl-1-picrylhydrazyl, PM 394.33) is a nitrogenous organic radical, with a maximum absorption peak at 515 nm (Figure 4). When the DPPH reacts with an antioxidant compound capable of yielding a hydrogen atom to the radical compound, a discoloration of the solution occurs due to the disappearance (stabilization) of the radical. This discoloration can then be monitored by spectrophotometry.

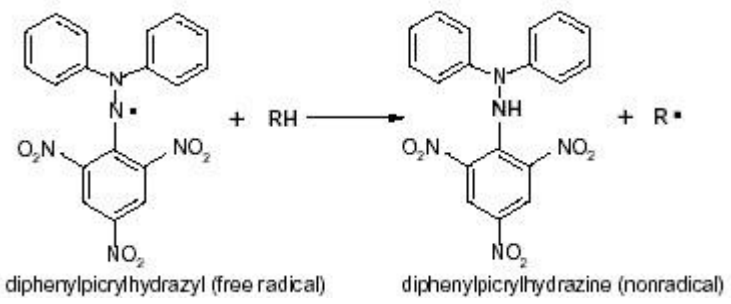

Figure 4. Stabilization of the DPPH radical by means of an antioxidant.

In practice, the antioxidant capacity of the CBS polyphenolic extracts was determined by adapting this method to 96-well microplates. To do so, 20 µL of sample was introduced in each well, to which they were added 180 µL of a DPPH radical solution, prepared with an ethanol:water solution (80:20 *v*/*v*). The microplate was then incubated in the dark for 30 minutes and the absorbance of the DPPH at 515 nm was measured with a BioTek Synergy HT microplate reader (BioTek Instruments, Milan, Italy).

The antioxidant capacity of the CBS extracts was calculated as the DPPH radical inhibition percentage as follows: IP (%) $= \frac{(A_0 - A_{30})}{A_0} \times 100$, where A_0 is the initial absorbance and A_{30} is the absorbance after 30 minutes. Analyses were performed in triplicate and the concentrations values were based on a Trolox standard curve (12.5–300 µM) and expressed as micromoles of Trolox equivalents per gram of CBS (µmol TE/g CBS), which is a water soluble vitamin E analog [234].

2.6. Statistical and Chemometric Analyses

Chemometric analysis was performed based on the data obtained for the 88 CBS samples (2 batches of 44 different CBS samples, Table 1). The 88 CBS samples were extracted and analyzed in duplicates for the HPLC methodology and in triplicates for the spectrophotometric assays. The package *made4* found in R (https://www.r-project.org) and the *dudi.pca* function were used in order to discriminate the groups of samples by geographic area of origin and different cultivars based on their polyphenolic compounds. A principal component analysis (PCA) was applied based on the normalized data ($\log_{10}$). To do so, an analysis of similarity with 999 permutations between the different concentration values obtained by both HPLC and spectrophotometric assays was carried out with the anosim function of the *vegan* package of R with the aim of finding significant differences depending on the sample geographic or cocoa cultivar. Non-parametrical tests such as Kruskal-Wallis and Wilcoxon tests were used for pairwise comparison in order to identify the specific

compounds or compound groups with marked significant abundance differences based on the different variables of sample origin and cultivar. Data for the specific compounds or compound groups driving the separations were represented by means of Box-plot graphics, which show the concentration range comprised between the first and third quartiles, with error bands that highlight the lowest and the highest values. The p-values were adjusted for the various tests and statistical significance was considered when the false discovery rate (FDR) < 0.05.

3. Results and Discussion

The present work was subdivided into two main parts. On the first step, the polyphenolic profile of the CBS samples was determined, following two different approaches. HPLC analysis was used as a complete characterization method, while different spectrophotometric assays (total phenolic content, total flavonoid content, total tannin content, and antioxidant capacity) were used as screening methods. On the second step of this work, principal component analyses (PCA) were applied to the obtained results for a chemometric differentiation of the CBS samples according to their geographical origin or variety based on their polyphenol and methylxanthine contents.

3.1. Polyphenolic and Methylxanthine Profile of the CBS Characterized by RP-HPLC-PDA

The polyphenolic compounds and methylxanthines identified and quantified by RP-HPLC-PDA are shown in Table 2. This table shows, for each polyphenolic compound or methylxanthine, the peak number within the total chromatogram, retention time and maximum wavelength at which the compound was quantified. The range of the different concentrations obtained for all of the different CBSs extracts in our sample set for each compound is shown and expressed as milligram per kilogram of CBS. A total of 27 compounds comprising polyphenols and methylxanthines were detected and quantified. These compounds were grouped in different categories for which the total quantities (sum of the compound concentrations) are shown in Table 3 for each CBS sample. Table 4 shows an extended vision of Table 3, with the concentration for each individual compound within the different group categories.

Table 2. Identification and concentration range of polyphenolic compounds and methylxanthines in CBS extracts yielded from roasted cocoa beans from different origins and cultivars.

Peak	R_t (min)	λ_{max}	Compound	Concentration range (mg kg^{-1} of CBS)
1	4.73	293	Protocatechuic acid	11.89 – 241.01
2	6.4	272	Theobromine	764.88 – 9028.28
3	6.8	279	*N*-Coumaroyl-L-aspartate_1	1.41 – 24.55
4	8.24	320	*N*-Caffeoyl-L-aspartate	5.86 – 652.58
5	8.63	278	Catechin	12.72 – 180.37
6	9.5	279	Catechin-3-O-glucoside	6.40 – 101.49
7	9.7	280	Procyanidin B-type trimer_1	0.90 – 130.89
8	10.6	306	*N*-Coumaroyl-L-aspartate_2	4.46 – 184.30
9	11.08	278	Procyanidin B-type trimer_2	0.00 – 163.15
10	11.1	285	Procyanidin B-type	6.84 – 672.55
11	11.5	278	Epicatechin	4.47 – 748.79
12	11.87	286	*N*-Coumaroyl-L-glutamate	1.10 – 12.58
13	12.2	272	Caffeine	150.81 – 3370.77
14	12.78	279	Procyanidin B-type trimer_3	1.51 – 97.04
15	13	278	Procyanidin B-type trimer_4	7.62 – 264.48
16	13.22	281	Procyanidin B-type trimer_5	6.52 – 280.29
17	13.86	280	Procyanidin A-type hexoside _1	8.51 – 560.65
18	14.6	279	Procyanidin B-type trimer_6	0.00 – 28.68
19	14.9	280	Procyanidin A-type pentoside_1	1.27 – 198.93
20	15.82	279	Procyanidin B-type trimer_7	2.49 – 104.37
21	16.03	279	Procyanidin A-type trimer arabinoside	2.44 – 114.09
22	17.2	278	Procyanidin A-type hexoside _2	2.49 – 73.34
23	17.5	283	*N*-Coumaroyl-L-tyrosine	1.99 – 48.70
24	17.7	278	Procyanidin A-type pentoside_2	0.00 – 40.02
25	18.2	355	Quercetin-3-O-glucoside	3.47 – 47.59
26	19.4	355	Quercetin-3-O-arabinoside	3.76 – 86.53
27	21.83	365	Quercetin	1.07 – 22.37

The compounds with the most notable concentrations throughout the CBS sample set were, in the first place, both methylxanthines: theobromine and caffeine, followed by flavanol monomers and procyanidins, similarly to what has been previously reported for cocoa beans [235]. Theobromine and caffeine are two well-known compounds present in cocoa and cocoa-derivative products which have been reported to be in part responsible for the high acceptance of cocoa among consumers. This fact is related to the capacity of these molecules to act in the central nervous system, influencing alertness and mood in a positive way. Besides the neuro-stimulating properties, caffeine and theobromine have been also reported to possess several other beneficial properties for human health, such as anticarcinogenic, diuretic, and anti-obesity effects, among others [101]. Theobromine concentrations in CBS ranged from 7.65 (CAM1) to 9.03 (DOR1) g/kg of CBS, while caffeine values were comprised between 0.15 (GHA) and 3.37 (VEN7) g/kg CBS. These values are in accordance with the ranges of 3.9 – 18.3 g of theobromine per kg of CBS and 0.4 – 4.2 g of caffeine per kg of CBS, which were reported in other studies gathered in the work of Rojo-Poveda [218]. In cocoa beans or cocoa

powder from cocoa nibs, the concentrations of both methylxanthines have been reported to be 1 – 24.28 g of theobromine per kg of cocoa powder and 0.5 – 7.28 g of caffeine per kg of cocoa beans, depending on the variety, origin, and fermentation, drying or roasting conditions [94,226,236-239]. Thus, contrary to some other chemical compounds such as polyphenols, which are generally more abundant in cocoa beans than in CBS, theobromine and caffeine concentrations found in the cocoa by-product can be considered to be almost equivalent to those found in cocoa powder, although in a lower extent in the case of caffeine. Methylxanthines are believed to be synthesized in the cocoa cotyledons. However, during the fermentation process, there is a considerable migration of caffeine and theobromine towards the shell [218]. Therefore, concentrations of these compounds will vary mostly depending on the fermentation state of cocoa beans. Studies focusing on CBS generally use fermented samples which have been obtained from cocoa manufacturers in an advance step of the cocoa manufacturing process, whereas studies focusing on cocoa beans use both non-fermented (directly obtained from the plantations) and fermented and dried samples. This could be the reason why methylxanthine concentration ranges vary more in cocoa bean sample when compared to CBS samples.

The total amount of polyphenols quantified by RP-HPLC-PDA ranges from 119.96 mg/kg CBS (GHA) to 3403.22 mg/kg CBS (SAT2). Throughout the whole sample set, a total of 25 individual phenolic compounds were detected, identified, and quantified in the CBS samples. These individual compounds were further classified into nine different categories (Table 4): phenolic acids, flavan-3-ols, catechin-3-O-glycosides, procyanidins B-type (PCB), procyanidins B-type trimers (PCB trimers), procyanidins A-type (PCA) glycosides, flavonols, flavonol-3-O-glycosides, and N-phenylpropenoyl-L-aminoacids. The range of concentration among the CBS samples for each compound is shown in Table 2 while the sums of the concentrations of all the individual compounds forming each category for each CBS sample are shown in Table 3.

Table 3. Sums (∑) for the total contents of the different polyphenolic categories, methylxanthines and spectrophotometric assays values determined in CBS powders from different geographical origins and cultivars. Concentrations are expressed as mg/kg CBS, unless indicated differently. An extended version of this table with the individual compound concentrations can be consulted in Table 4.

Sample code	∑ Phenolic acids	∑ Flavan-3-ols	∑ Catechin-3-O-glycosides	∑ PCB	∑ PCB trimers	∑ PCA glycosides	∑ Flavonols	∑ Flavonol-3-O-glycosides	∑ N-phenyl propenoyl-L-aminoacids	**∑ Total polyphenols**	**∑ Total Methylxantines (g/kg CBS)**	**TPC (mg GAE/g CBS)**	**TFC (mg CE/g CBS)**	**TTC (mg CE/g CBS)**	**RSA (µmol TE/g CBS)**
BRA	11.89	58.48	30.91	9.81	195.06	103.02	3.49	39.20	40.96	492.81	4.09	18.06	4.85	2.81	60.38
CAM1	111.79	116.44	31.51	68.13	181.49	225.04	3.42	30.63	105.35	873.80	7.27	18.46	6.07	4.83	78.24
CAM2	27.77	56.56	16.75	16.07	46.07	31.84	1.09	12.18	29.31	237.64	3.15	12.17	5.00	3.43	54.34
COL1	121.68	344.29	25.51	209.71	362.70	338.56	6.62	56.13	220.46	1685.65	7.32	28.02	12.35	7.67	118.99
COL2	130.38	858.91	41.04	289.49	649.51	497.35	9.36	89.06	543.26	3108.35	10.75	34.66	16.67	8.09	143.72
CON1	138.31	85.96	16.21	274.48	149.56	127.82	3.29	47.34	197.03	1040.00	8.37	16.10	5.81	3.01	64.24
CON2	141.24	179.95	66.65	140.65	237.59	270.78	5.48	40.66	161.13	1244.12	8.44	17.91	7.26	4.08	76.36
DOR1	176.42	587.80	70.54	583.92	601.29	424.66	10.26	94.28	584.36	3133.51	12.28	33.99	17.72	8.00	145.67
DOR2	136.27	245.49	39.09	274.53	305.40	296.11	3.08	71.72	459.36	1831.05	9.32	20.98	6.89	4.96	78.67
DOR3	130.95	227.01	13.75	78.29	252.48	183.96	1.41	19.31	68.06	975.23	2.89	17.75	5.64	7.10	78.46
DOR4	140.63	287.58	48.08	235.90	275.95	309.76	6.97	61.68	395.46	1762.00	11.07	18.42	6.55	3.46	74.13
ECU1	98.57	249.62	24.79	309.99	216.07	301.69	2.61	35.23	155.49	1394.07	6.83	18.84	6.83	4.75	71.24
ECU2	122.20	213.86	12.15	167.26	162.40	121.70	3.09	23.81	92.71	919.17	7.20	12.31	4.14	3.03	52.56
ECU3	206.06	479.52	48.41	233.42	476.00	495.96	9.94	111.69	666.71	2727.72	10.65	35.82	18.50	7.89	150.25
ECU4	145.43	413.75	22.20	198.00	248.28	306.14	6.92	56.55	213.08	1610.35	9.08	19.94	7.22	5.84	84.05
ECU5	112.64	286.93	27.22	96.36	214.41	293.67	3.04	37.41	159.64	1231.32	7.95	14.70	4.66	4.06	59.92
ECU6	88.40	461.77	15.62	276.73	297.94	371.45	6.08	35.61	264.49	1818.09	8.07	18.77	8.08	4.49	77.73
ECU7	108.36	436.71	37.32	52.01	283.86	292.36	2.84	65.39	277.06	1555.90	10.76	14.56	6.40	3.78	62.44
GHA	20.89	17.19	6.77	6.84	26.28	16.34	1.29	7.23	17.13	119.96	0.92	9.09	3.06	2.27	38.98
IVC	54.10	77.29	20.62	45.85	73.91	68.23	1.07	13.40	50.04	404.50	4.15	10.00	3.17	2.50	44.26
JAM	186.53	245.34	57.87	57.49	455.22	286.80	6.85	76.36	450.02	1822.48	11.92	19.92	8.56	3.65	81.54
MAD	87.20	887.71	31.54	478.32	518.45	412.03	6.90	50.10	370.99	2843.24	10.55	34.02	12.53	7.73	139.19
MEX	241.01	523.32	88.79	261.13	537.93	657.51	22.37	92.85	810.01	3234.91	9.75	38.92	18.29	9.27	172.32
PER1	120.10	412.87	26.66	137.69	272.16	417.17	7.50	54.61	310.63	1759.39	8.29	26.39	13.38	6.68	115.60
PER2	86.82	384.68	33.03	341.40	340.01	351.00	6.31	51.92	403.61	1998.77	9.68	42.97	17.70	11.00	181.98
SAT1	120.30	351.85	41.51	640.40	483.68	387.07	4.67	38.39	203.27	2271.15	8.89	26.30	10.85	5.26	103.32

Sample code	∑ Phenolic acids	∑ Flavan-3-ols	∑ Catechin-3-O-glycosides	∑ PCB	∑ PCB trimers	∑ PCA glycosides	∑ Flavonols	∑ Flavonol-3-O-glycosides	∑ N-phenyl propenoyl-L-aminoacids	**∑ Total polyphenols**	**∑ Total Methylxantines (g/kg CBS)**	**TPC (mg GAE/g CBS)**	**TFC (mg CE/g CBS)**	**TTC (mg CE/g CBS)**	**RSA (µmol TE/g CBS)**
SAT2	197.73	751.33	101.49	190.76	653.73	833.22	13.91	121.98	539.09	3403.22	10.07	33.88	16.69	10.91	154.87
SAT3	164.18	656.23	79.34	246.44	565.53	681.77	7.98	62.35	405.59	2869.41	9.95	37.94	19.67	10.40	165.19
SLE	14.43	225.39	29.39	135.56	199.40	128.52	1.95	27.52	60.64	822.81	4.73	21.00	6.72	5.47	73.48
TAN	88.40	172.76	17.90	253.36	262.97	199.35	2.88	31.99	93.19	1122.82	7.96	13.08	3.08	3.64	54.59
TOG1	13.56	43.48	27.47	136.45	154.39	72.79	1.10	24.32	60.85	534.42	4.78	12.71	2.97	1.95	44.55
TOG2	15.84	57.63	28.10	145.65	123.36	85.13	1.43	24.36	44.93	526.44	4.06	13.50	3.04	3.02	49.04
UGA1	56.96	44.97	6.40	128.58	76.14	45.00	2.04	16.29	61.27	437.65	4.47	8.96	2.49	2.06	36.95
UGA2	107.95	67.24	13.41	38.30	97.40	48.32	2.38	15.75	58.40	449.16	7.76	6.10	1.42	1.14	27.67
VEN1	118.99	314.23	16.79	407.82	330.00	259.13	3.19	27.99	104.47	1582.61	7.25	22.09	7.73	6.85	98.23
VEN2	73.43	194.72	14.17	672.55	617.10	178.83	5.93	41.54	216.66	1135.76	8.80	27.46	8.68	4.94	99.41
VEN3	107.87	412.40	52.35	77.63	377.03	467.31	3.41	75.60	318.65	2014.94	11.15	20.02	8.93	5.07	87.02
VEN4	46.42	171.18	19.63	83.42	125.55	98.29	2.56	21.54	133.30	1892.25	4.43	19.94	8.93	4.47	82.02
VEN5	120.54	163.93	25.05	55.12	220.20	127.34	3.33	32.95	188.48	701.88	8.28	15.48	5.88	3.53	66.38
VEN6	124.26	256.07	29.70	181.77	374.65	251.86	6.11	48.94	281.23	936.94	9.85	21.75	9.05	4.49	85.75
VEN7	103.68	318.56	34.77	106.02	342.45	267.07	1.63	26.63	321.96	1554.60	11.11	14.90	5.57	2.44	55.62
VEN8	136.29	346.70	50.05	53.90	285.84	328.77	2.94	58.00	321.96	1522.77	11.44	18.53	6.16	3.29	67.84
VEN9	85.51	214.74	24.70	67.85	207.76	230.16	3.46	23.56	178.35	1584.45	9.57	12.51	2.82	2.17	46.56
VEN10	12.05	306.47	31.95	104.07	220.17	209.09	2.43	35.88	213.64	1036.08	7.18	19.39	5.02	5.37	64.89

Protocatechuic acid was the only phenolic acid detected in CBS in the present study, although other scientific studies have reported the presence of other phenolic acids in CBS such as gallic acid (up to 147 mg/kg) [64], homovanillic acid, vanillic acid glycoside isomers, and cinnamic acid derivatives [240]. In our work, protocatechuic acid represented on average the 7.0% of the total quantified phenolic compounds, and its concentrations ranged from 11.89 mg/kg CBS (BRA) to 241.01 mg/kg CBS (MEX). Protocatechuic is a known phenolic acid contained in cocoa beans and it has been found in concentrations comprised between 2.20 and 22.2 mg/kg of cocoa bean [235], which is considerably lower than the concentrations found in CBS. Nevertheless, Cádiz-Gurrea et al. [240] claimed that CBS samples are characterized by a higher content in phenolic acids when compared to cocoa beans, while the latter have in general higher flavonoid contents.

Flavan-3-ols was one of the most abundant polyphenolic groups in the CBS samples, with concentrations that ranged from 17.19 (GHA) to 887.71 (MAD) mg/kg CBS and amounted on average the 20.0 % of the total analyzed compounds, which is in agreement with the 20.5% of abundance found for the same compound group in cocoa beans by Rodríguez-Carrasco et al. [241]. Indeed, this group was formed by catechin and epicatechin, which are two of the most representative polyphenolic compounds in cocoa beans and CBS [81,218]. In our samples, catechin was present in concentrations that varied from 12.72 (GHA) to 180.37 (ECU7) mg/kg CBS. Other researchers that performed different types of optimized extractions with CBS such as the application of high-voltage electric discharge [56,64] and pressurized liquid extraction [62] found concentrations up to 290, 284, and 178 mg of catechin per kg of CBS, respectively, which is in accordance with the amounts quantified in this work. When it comes to cocoa beans, values of catechin ranging from 40.1 to 1297 mg/kg are reported in the literature [226,235,237], which is not far from what is observed for CBS. However, as stated before, these concentrations vary and decrease with the fermentation process [81], which happens to be more variable when in it refers to cocoa bean studies. Epicatechin was in general the most abundant individual polyphenolic compound found in our samples, and its concentration varied from 4.47 (GHA) to 748.79 (MAD) mg/kg CBS. Epicatechin has been found in concentrations ranging from 210 up to 3500 mg/kg CBS in the previously mentioned works in which assisted extractions were performed [56,57,62,64], which is still in line with the values found in the present study. Regarding the concentrations of epicatechin in cocoa beans that are reported in the literature, these are slightly higher than those found in the cocoa by-products, with values that vary from 500 to 4787 mg of epicatechin per kg of cocoa bean [226,235,237,238], although some studies have reported

Table 4. Content of the single identified polyphenolic compounds and methylxantines in CBS powders from different geographical origins and cultivars

POLYPHENOLS	Concentration (mg kg-1 of CBS)										
	BRA	CAM1	CAM2	COL1	COL2	CON1	CON2	DOR1	DOR2	DOR3	DOR4
Phenolic acids											
Protocatechuic acid	11.89 ± 0.81	111.79 ± 3.34	27.77 ± 1.92	121.68 ± 5.08	130.38 ± 8.22	138.31 ± 7.51	141.24 ± 8.89	176.42 ± 8.57	136.27 ± 9.88	130.95 ± 3.50	140.63 ± 6.14
Σ	**11.89**	**111.79**	**27.77**	**121.68**	**130.38**	**138.31**	**141.24**	**176.42**	**136.27**	**130.95**	**140.63**
Flavan-3-ol											
Catechin	14.77 ± 1.03	20.44 ± 1.78	13.52 ± 0.55	65.57 ± 5.80	128.29 ± 6.73	14.22 ± 0.05	25.82 ± 2.11	94.66 ± 2.41	76.15 ± 6.29	45.72 ± 2.18	58.94 ± 4.51
Epicatechin	43.71 ± 4.45	96.01 ± 1.30	43.03 ± 5.10	278.72 ± 20.80	730.62 ± 10.03	71.75 ± 0.55	154.13 ± 8.96	493.14 ± 44.55	169.34 ± 8.71	181.29 ± 22.55	228.64 ± 17.45
Σ	**58.48**	**116.44**	**56.56**	**344.29**	**858.91**	**85.96**	**179.95**	**587.80**	**245.49**	**227.01**	**287.58**
Catechin-3-O-glycosides											
Catechin-3-O-glucoside	30.91 ± 3.05	31.51 ± 1.15	16.75 ± 0.66	25.51 ± 1.12	41.04 ± 3.66	16.21 ± 0.60	66.65 ± 3.55	70.54 ± 4.47	39.09 ± 3.26	13.75 ± 0.90	48.08 ± 1.74
Σ	**30.91**	**31.51**	**16.75**	**25.51**	**41.04**	**16.21**	**66.65**	**70.54**	**39.09**	**13.75**	**48.08**
Procyanidins B-type (PCB)											
PCB	9.81 ± 0.85	68.13 ± 7.67	16.07 ± 0.44	209.71 ± 21.04	289.49 ± 29.45	274.48 ± 30.49	140.65 ± 16.47	583.92 ± 20.67	274.53 ± 20.91	78.29 ± 10.74	235.90 ± 25.72
Σ	**9.81**	**68.13**	**16.07**	**209.71**	**289.49**	**274.48**	**140.65**	**583.92**	**274.53**	**78.29**	**235.90**
Procyanidins B-type (PCB) trimers											
PCB trimer 1	47.68 ± 4.21	11.21 ± 0.15	0.90 ± 0.09	34.10 ± 1.64	73.57 ± 5.43	19.86 ± 0.16	13.34 ± 0.59	76.33 ± 3.15	53.61 ± 3.63	12.79 ± 0.94	48.42 ± 5.71
PCB trimer 2	4.80 ± 0.29	3.99 ± 0.03	3.11 ± 0.18	2.95 ± 0.22	22.88 ± 1.19	4.58 ± 0.10	8.91 ± 0.74	36.76 ± 2.96	34.43 ± 2.95	2.04 ± 0.17	19.07 ± 1.58
PCB trimer 3	9.96 ± 1.15	13.60 ± 1.62	1.56 ± 0.18	8.34 ± 0.47	3.43 ± 0.27	18.50 ± 0.38	20.13 ± 1.55	14.17 ± 1.23	16.65 ± 1.69	20.66 ± 0.52	11.75 ± 1.57
PCB trimer 4	31.73 ± 2.98	89.70 ± 2.47	22.75 ± 0.70	139.53 ± 13.65	199.53 ± 20.62	38.49 ± 1.74	81.18 ± 6.80	125.84 ± 12.24	64.99 ± 3.95	114.24 ± 9.18	84.83 ± 3.82
PCB trimer 5	78.69 ± 8.56	31.55 ± 2.93	14.18 ± 1.72	128.42 ± 11.08	280.29 ± 26.47	43.85 ± 2.23	82.09 ± 5.56	234.83 ± 3.02	87.17 ± 6.24	53.01 ± 3.43	57.43 ± 6.09
PCB trimer 6	1.67 ± 0.15	7.96 ± 0.88	1.09 ± 0.15	9.85 ± 1.07	12.26 ± 1.21	1.45 ± 0.03	6.71 ± 0.60	18.46 ± 2.28	7.06 ± 0.56	28.68 ± 0.87	12.57 ± 0.80
PCB trimer 7	20.53 ± 2.15	23.49 ± 1.26	2.49 ± 0.10	39.51 ± 4.52	57.54 ± 5.06	22.83 ± 0.90	25.23 ± 1.47	94.89 ± 6.70	41.49 ± 3.25	21.05 ± 0.20	41.87 ± 3.26
Σ	**195.06**	**181.49**	**46.07**	**362.70**	**649.51**	**149.56**	**237.59**	**601.29**	**305.40**	**252.48**	**275.95**
Procyanidins A-type (PCA) glycosides											
PCA hexoside 1	40.81 ± 2.70	142.54 ± 4.46	15.74 ± 1.49	218.41 ± 3.03	284.11 ± 23.98	70.06 ± 2.98	165.13 ± 13.32	178.19 ± 13.57	155.46 ± 13.58	140.82 ± 8.81	198.19 ± 10.13
PCA pentoside 1	20.09 ± 1.51	17.86 ± 0.77	3.16 ± 0.36	30.42 ± 1.39	65.01 ± 8.15	21.31 ± 0.85	15.13 ± 0.39	66.43 ± 4.08	61.00 ± 5.94	19.28 ± 1.86	36.14 ± 2.52
PCA trimer arabinoside	13.05 ± 1.64	20.03 ± 0.86	2.44 ± 0.31	34.15 ± 2.14	69.03 ± 4.72	17.65 ± 2.07	35.39 ± 2.43	82.82 ± 8.55	35.65 ± 3.53	14.03 ± 1.54	34.31 ± 1.09
PCA hexoside 2	19.62 ± 1.02	29.98 ± 0.75	6.94 ± 0.37	32.80 ± 3.30	53.91 ± 1.81	14.02 ± 1.32	37.47 ± 2.58	69.46 ± 3.32	34.28 ± 1.55	8.16 ± 0.36	27.66 ± 3.41
PCA pentoside 2	9.46 ± 0.26	14.62 ± 0.49	3.56 ± 0.02	22.78 ± 2.76	25.28 ± 3.49	4.78 ± 0.38	17.64 ± 2.26	27.76 ± 1.22	9.73 ± 0.69	1.67 ± 0.14	13.46 ± 0.76
Σ	**103.02**	**225.04**	**31.84**	**338.56**	**497.35**	**127.82**	**270.78**	**424.66**	**296.11**	**183.96**	**309.76**
Flavonols											
Quercetin	3.49 ± 0.32	3.42 ± 0.18	1.09 ± 0.13	6.62 ± 0.47	9.36 ± 0.98	3.29 ± 0.42	5.48 ± 0.30	10.26 ± 0.73	3.08 ± 0.17	1.41 ± 0.14	6.97 ± 0.28
Σ	**3.49**	**3.42**	**1.09**	**6.62**	**9.36**	**3.29**	**5.48**	**10.26**	**3.08**	**1.41**	**6.97**
Flavonol-3-O-glycosides											
Quercetin-3-O-glucoside	6.41 ± 0.38	8.41 ± 0.05	5.08 ± 0.25	22.82 ± 2.06	37.00 ± 3.30	17.74 ± 0.54	12.89 ± 0.75	34.82 ± 3.04	25.90 ± 2.36	5.43 ± 0.17	22.39 ± 1.16
Quercetin-3-O- arabinoside	32.79 ± 2.82	22.22 ± 0.09	7.10 ± 0.46	33.31 ± 3.75	52.06 ± 4.01	29.60 ± 1.72	27.77 ± 3.52	59.46 ± 1.79	45.82 ± 2.91	13.88 ± 0.66	39.29 ± 2.50
Σ	**39.20**	**30.63**	**12.18**	**56.13**	**89.06**	**47.34**	**40.66**	**94.28**	**71.72**	**19.31**	**61.68**
N-phenylpropenoyl-L-aminoacids											
N-Coumaroyl-L-aspartate 1	3.92 ± 0.17	10.46 ± 0.20	7.54 ± 0.54	6.18 ± 0.41	9.41 ± 0.52	5.63 ± 0.34	9.19 ± 0.58	6.41 ± 0.42	9.31 ± 0.67	9.88 ± 0.46	6.11 ± 0.50
N-Caffeoyl-L-aspartate	24.31 ± 1.35	61.72 ± 1.79	13.73 ± 1.17	152.72 ± 7.33	408.59 ± 7.09	122.64 ± 9.10	102.41 ± 8.63	370.78 ± 10.14	294.74 ± 25.54	37.43 ± 1.62	211.47 ± 19.96
N-Coumaroyl-L-aspartate 2	5.65 ± 0.36	20.63 ± 0.86	4.70 ± 0.01	45.89 ± 3.75	103.52 ± 9.05	51.65 ± 4.11	33.05 ± 2.10	148.67 ± 10.33	124.13 ± 7.38	14.51 ± 0.77	148.40 ± 13.96
N-Coumaroyl-L-glutamate	3.98 ± 0.30	3.96 ± 0.13	1.11 ± 0.09	3.42 ± 0.24	6.90 ± 0.59	6.77 ± 0.08	3.71 ± 0.18	9.80 ± 1.08	9.16 ± 0.57	3.07 ± 0.13	5.45 ± 0.58
N-coumaroyl-L-tyrosine	3.10 ± 0.30	8.56 ± 0.39	2.23 ± 0.09	12.25 ± 1.05	14.85 ± 0.55	10.34 ± 0.34	12.76 ± 1.71	48.70 ± 1.61	22.01 ± 2.09	3.17 ± 0.10	24.03 ± 1.86
Σ	**40.96**	**105.35**	**29.31**	**220.46**	**543.26**	**197.03**	**161.13**	**584.36**	**459.36**	**68.06**	**395.46**
Total polyphenols	**492.81**	**873.80**	**237.64**	**1685.65**	**3108.35**	**1040.00**	**1244.12**	**3133.51**	**1831.05**	**975.23**	**1762.00**

METHYLXANTHINES	Concentration (g kg-1 of CBS)										
Theobromine	3.40 ± 0.19	6.54 ± 0.08	2.99 ± 0.27	6.13 ± 0.42	8.17 ± 0.12	7.18 ± 0.39	7.50 ± 0.17	9.03 ± 0.06	7.44 ± 0.32	2.38 ± 0.23	8.45 ± 0.13
Caffeine	0.69 ± 0.04	0.74 ± 0.01	0.16 ± 0.00	1.20 ± 0.07	2.57 ± 0.05	1.18 ± 0.10	0.94 ± 0.03	3.25 ± 0.08	1.89 ± 0.18	0.51 ± 0.04	2.62 ± 0.10
Total Methylxantines	**4.09**	**7.27**	**3.15**	**7.32**	**10.75**	**8.37**	**8.44**	**12.28**	**9.32**	**2.89**	**11.07**

Data are presented as the mean (n=4) ± standard deviation

Table 4 (continuation). Content of the single identified polyphenolic compounds and methylxantines in CBS powders from different geographical origins and cultivars.

POLYPHENOLS	Concentration (mg kg-1 of CBS)										
	ECU1	ECU2	ECU3	ECU4	ECU5	ECU6	ECU7	GHA	IVC	JAM	MAD
Phenolic acids											
Protocatechuic acid	98.57 ± 8.87	122.20 ± 8.98	206.06 ± 19.25	145.43 ± 2.28	112.64 ± 7.60	88.40 ± 4.59	108.36 ± 2.69	20.89 ± 0.80	54.10 ± 3.72	186.53 ± 18.32	87.20 ± 3.99
Σ	**98.57**	**122.20**	**206.06**	**145.43**	**112.64**	**88.40**	**108.36**	**20.89**	**54.10**	**186.53**	**87.20**
Flavan-3-ol											
Catechin	46.43 ± 4.15	46.19 ± 1.68	113.86 ± 10.88	45.49 ± 3.39	47.64 ± 1.05	88.08 ± 5.41	180.37 ± 1.99	12.72 ± 0.48	27.50 ± 2.22	84.59 ± 2.67	138.92 ± 9.69
Epicatechin	203.19 ± 19.95	167.67 ± 7.46	365.66 ± 6.62	368.25 ± 13.11	239.29 ± 0.04	373.69 ± 5.94	256.34 ± 12.71	4.47 ± 0.29	49.79 ± 6.53	160.75 ± 15.63	748.79 ± 62.26
Σ	**249.62**	**213.86**	**479.52**	**413.75**	**286.93**	**461.77**	**436.71**	**17.19**	**77.29**	**245.34**	**887.71**
Catechin-3-O-glycosides											
Catechin-3-O-glucoside	24.79 ± 2.28	12.15 ± 0.73	48.41 ± 1.04	22.20 ± 1.11	27.22 ± 0.14	15.62 ± 0.25	37.32 ± 2.78	6.77 ± 0.07	20.62 ± 1.27	57.87 ± 2.43	31.54 ± 0.61
Σ	**24.79**	**12.15**	**48.41**	**22.20**	**27.22**	**15.62**	**37.32**	**6.77**	**20.62**	**57.87**	**31.54**
Procyanidins B-type (PCB)											
PCB	309.99 ± 23.06	167.26 ± 14.17	233.42 ± 12.15	198.00 ± 10.49	96.36 ± 8.27	276.73 ± 27.53	52.01 ± 1.90	6.84 ± 0.39	45.85 ± 4.60	57.49 ± 6.41	478.32 ± 19.05
Σ	**309.99**	**167.26**	**233.42**	**198.00**	**96.36**	**276.73**	**52.01**	**6.84**	**45.85**	**57.49**	**478.32**
Procyanidins B-type (PCB) trimers											
PCB trimer 1	31.15 ± 1.07	18.33 ± 2.08	81.52 ± 4.76	39.67 ± 0.67	49.71 ± 5.40	33.13 ± 1.89	64.93 ± 2.53	2.54 ± 0.16	15.60 ± 0.97	130.89 ± 9.01	51.46 ± 0.95
PCB trimer 2	8.14 ± 0.48	3.34 ± 0.29	15.71 ± 0.55	2.65 ± 0.12	0.00 ± 0.00	23.84 ± 1.67	41.02 ± 2.61	0.47 ± 0.05	2.31 ± 0.21	163.15 ± 11.79	20.34 ± 1.51
PCB trimer 3	10.86 ± 0.87	5.65 ± 0.59	23.20 ± 0.74	13.52 ± 1.26	2.60 ± 0.08	11.91 ± 0.94	9.58 ± 0.50	3.56 ± 0.34	3.45 ± 0.43	8.39 ± 0.93	20.03 ± 1.63
PCB trimer 4	81.94 ± 7.04	73.45 ± 4.52	121.99 ± 10.23	124.25 ± 4.61	121.83 ± 12.91	112.43 ± 12.12	94.88 ± 3.45	7.62 ± 0.72	25.46 ± 2.67	66.96 ± 7.90	169.37 ± 13.19
PCB trimer 5	49.41 ± 5.96	50.12 ± 7.02	140.04 ± 6.28	51.11 ± 1.61	9.19 ± 0.93	73.12 ± 6.78	24.18 ± 2.28	6.52 ± 0.81	9.14 ± 0.81	46.24 ± 4.21	182.56 ± 14.19
PCB trimer 6	9.00 ± 1.01	0.00 ± 0.00	9.75 ± 0.87	12.34 ± 0.47	11.89 ± 0.71	4.34 ± 0.53	2.65 ± 0.02	2.14 ± 0.19	2.22 ± 0.28	7.26 ± 0.38	9.92 ± 0.25
PCB trimer 7	25.58 ± 0.92	11.50 ± 0.98	83.79 ± 1.18	4.74 ± 0.47	19.20 ± 0.81	39.18 ± 1.41	46.62 ± 4.10	3.43 ± 0.29	15.73 ± 1.00	32.33 ± 1.46	64.78 ± 4.00
Σ	**216.07**	**162.40**	**476.00**	**248.28**	**214.41**	**297.94**	**283.86**	**26.28**	**73.91**	**455.22**	**518.45**
Procyanidins A-type (PCA) glycosides											
PCA hexoside 1	222.21 ± 30.77	86.54 ± 3.52	334.13 ± 20.47	171.83 ± 4.58	183.30 ± 6.90	136.19 ± 8.06	159.10 ± 11.68	8.51 ± 0.40	43.16 ± 0.81	171.37 ± 15.54	307.80 ± 8.06
PCA pentoside 1	28.96 ± 2.26	12.66 ± 0.90	46.87 ± 5.70	98.74 ± 10.93	31.98 ± 0.03	198.93 ± 16.27	28.05 ± 1.56	1.27 ± 0.07	2.61 ± 0.34	38.70 ± 2.68	30.07 ± 0.53
PCA trimer arabinoside	22.83 ± 2.93	14.11 ± 0.72	64.87 ± 2.88	7.50 ± 0.19	46.30 ± 3.43	13.28 ± 1.09	42.80 ± 2.50	3.42 ± 0.37	14.08 ± 1.52	38.89 ± 2.52	48.40 ± 3.54
PCA hexoside 2	20.47 ± 2.01	8.39 ± 0.79	39.73 ± 2.62	22.53 ± 2.10	25.46 ± 3.36	17.29 ± 2.05	40.08 ± 0.84	2.49 ± 0.13	5.88 ± 0.69	24.69 ± 3.02	18.42 ± 0.96
PCA pentoside 2	7.23 ± 0.43	0.00 ± 0.00	10.36 ± 0.96	5.54 ± 0.32	6.63 ± 0.77	5.76 ± 0.33	22.33 ± 0.89	0.65 ± 0.03	2.50 ± 0.29	13.15 ± 1.42	7.34 ± 0.16
Σ	**301.69**	**121.70**	**495.96**	**306.14**	**293.67**	**371.45**	**292.36**	**16.34**	**68.23**	**286.80**	**412.03**
Flavonols											
Quercetin	2.61 ± 0.11	3.09 ± 0.28	9.94 ± 0.87	6.92 ± 0.45	3.04 ± 0.32	6.08 ± 0.59	2.84 ± 0.23	1.29 ± 0.04	1.07 ± 0.13	6.85 ± 0.89	6.90 ± 0.14
Σ	**2.61**	**3.09**	**9.94**	**6.92**	**3.04**	**6.08**	**2.84**	**1.29**	**1.07**	**6.85**	**6.90**
Flavonol-3-O-glycosides											
Quercetin-3-O-glucoside	14.11 ± 0.87	8.76 ± 0.43	47.59 ± 1.96	21.49 ± 0.04	14.40 ± 1.52	15.79 ± 1.48	21.18 ± 0.70	3.47 ± 0.37	5.30 ± 0.34	23.14 ± 0.56	22.92 ± 1.42
Quercetin-3-O- arabinoside	21.12 ± 1.87	15.04 ± 0.37	64.10 ± 1.48	35.06 ± 0.68	23.01 ± 0.10	19.81 ± 1.25	44.20 ± 0.41	3.76 ± 0.19	8.10 ± 0.72	53.22 ± 0.83	27.19 ± 0.82
Σ	**35.23**	**23.81**	**111.69**	**56.55**	**37.41**	**35.61**	**65.39**	**7.23**	**13.40**	**76.36**	**50.10**
N-phenylpropenoyl-L-aminoacids											
N-Coumaroyl-L-aspartate 1	12.80 ± 0.85	8.59 ± 0.52	14.12 ± 0.72	8.19 ± 0.71	13.35 ± 0.14	5.40 ± 0.31	22.15 ± 1.36	3.71 ± 0.35	6.39 ± 0.64	24.55 ± 2.02	5.20 ± 0.14
N-Caffeoyl-L-aspartate	101.55 ± 7.08	50.24 ± 2.77	430.78 ± 31.82	124.26 ± 5.66	56.92 ± 1.60	179.51 ± 7.50	138.22 ± 5.02	5.86 ± 0.40	22.36 ± 1.74	256.06 ± 16.73	268.66 ± 1.66
N-Coumaroyl-L-aspartate 2	24.56 ± 2.63	25.60 ± 2.52	184.30 ± 11.79	65.88 ± 2.97	65.78 ± 0.47	63.70 ± 6.34	93.88 ± 1.76	4.46 ± 0.47	10.62 ± 1.24	132.56 ± 3.00	74.54 ± 3.20
N-Coumaroyl-L-glutamate	4.82 ± 0.44	2.55 ± 0.32	8.54 ± 0.91	3.40 ± 0.03	5.83 ± 0.26	7.18 ± 0.66	5.35 ± 0.61	1.10 ± 0.14	4.17 ± 0.37	7.83 ± 0.35	5.69 ± 0.66
N-coumaroyl-L-tyrosine	11.76 ± 0.98	5.73 ± 0.79	28.97 ± 1.71	11.35 ± 1.55	17.76 ± 0.55	8.70 ± 1.05	17.46 ± 0.15	1.99 ± 0.19	6.51 ± 0.78	29.02 ± 1.49	16.91 ± 0.99
Σ	**155.49**	**92.71**	**666.71**	**213.08**	**159.64**	**264.49**	**277.06**	**17.13**	**50.04**	**450.02**	**370.99**
Total polyphenols	**1394.07**	**919.17**	**2727.72**	**1610.35**	**1231.32**	**1818.09**	**1555.90**	**119.96**	**404.50**	**1822.48**	**2843.24**

METHYLXANTHINES	Concentration (g kg-1 of CBS)										
Theobromine	5.88 ± 0.46	6.05 ± 0.35	8.57 ± 0.29	7.43 ± 0.15	6.30 ± 0.12	6.86 ± 0.34	8.01 ± 0.72	0.76 ± 0.07	3.48 ± 0.28	8.92 ± 0.16	8.25 ± 0.02
Caffeine	0.95 ± 0.04	1.15 ± 0.03	2.08 ± 0.07	1.65 ± 0.11	1.66 ± 0.07	1.21 ± 0.07	2.75 ± 0.27	0.15 ± 0.00	0.67 ± 0.04	3.00 ± 0.02	2.29 ± 0.01
Total Methylxantines	**6.83**	**7.20**	**10.65**	**9.08**	**7.95**	**8.07**	**10.76**	**0.92**	**4.15**	**11.92**	**10.55**

Data are presented as the mean (n=4) ± standard deviation

Table 4 (continuation). Content of the single identified polyphenolic compounds and methylxantines in CBS powders from different geographical origins and cultivars.

POLYPHENOLS	Concentration (mg kg-1 of CBS)										
	MEX	PER1	PER2	SAT1	SAT2	SAT3	SLE	TAN	TOG1	TOG2	UGA1
Phenolic acids											
Protocatechuic acid	241.01 ± 6.22	120.10 ± 6.90	86.82 ± 3.11	120.30 ± 3.76	197.73 ± 10.63	164.18 ± 15.92	14.43 ± 0.66	88.40 ± 6.81	13.56 ± 1.07	15.84 ± 1.11	56.96 ± 2.65
Σ	**241.01**	**120.10**	**86.82**	**120.30**	**197.73**	**164.18**	**14.43**	**88.40**	**13.56**	**15.84**	**56.96**
Flavan-3-ol											
Catechin	93.44 ± 4.88	61.23 ± 4.03	84.76 ± 2.60	26.44 ± 0.83	119.50 ± 8.70	114.34 ± 7.38	34.65 ± 3.34	35.29 ± 2.30	18.67 ± 1.09	20.25 ± 1.24	13.52 ± 1.14
Epicatechin	429.87 ± 12.30	351.64 ± 29.91	299.92 ± 41.54	325.41 ± 28.65	631.82 ± 18.37	541.89 ± 45.38	190.74 ± 13.87	137.48 ± 17.45	24.81 ± 3.01	37.38 ± 1.59	31.45 ± 2.47
Σ	**523.32**	**412.87**	**384.68**	**351.85**	**751.33**	**656.23**	**225.39**	**172.76**	**43.48**	**57.63**	**44.97**
Catechin-3-O-glycosides											
Catechin-3-O-glucoside	88.79 ± 4.00	26.66 ± 1.58	33.03 ± 2.49	41.51 ± 1.40	101.49 ± 2.82	79.34 ± 3.16	29.39 ± 1.68	17.90 ± 0.74	27.47 ± 1.53	28.10 ± 2.11	6.40 ± 0.27
Σ	**88.79**	**26.66**	**33.03**	**41.51**	**101.49**	**79.34**	**29.39**	**17.90**	**27.47**	**28.10**	**6.40**
Procyanidins B-type (PCB)											
PCB	261.13 ± 34.96	137.69 ± 2.13	341.40 ± 26.01	640.40 ± 40.81	190.76 ± 20.36	246.44 ± 29.55	135.56 ± 12.40	253.36 ± 17.84	136.45 ± 11.85	145.65 ± 14.37	128.58 ± 5.01
Σ	**261.13**	**137.69**	**341.40**	**640.40**	**190.76**	**246.44**	**135.56**	**253.36**	**136.45**	**145.65**	**128.58**
Procyanidins B-type (PCB) trimers											
PCB trimer 1	106.32 ± 2.91	41.59 ± 0.99	51.23 ± 5.03	23.95 ± 2.80	95.53 ± 6.12	65.35 ± 6.87	2.72 ± 0.07	24.03 ± 1.87	12.04 ± 0.65	11.43 ± 1.17	10.35 ± 1.02
PCB trimer 2	18.86 ± 1.26	1.50 ± 0.10	11.93 ± 1.10	34.91 ± 3.35	3.49 ± 0.27	5.87 ± 0.41	4.69 ± 0.44	7.65 ± 0.66	17.27 ± 0.85	13.03 ± 0.61	0.00 ± 0.00
PCB trimer 3	12.12 ± 0.96	10.26 ± 1.06	9.07 ± 0.66	40.23 ± 4.90	10.79 ± 1.00	10.76 ± 0.76	1.51 ± 0.10	9.45 ± 1.02	4.03 ± 0.37	11.58 ± 1.16	3.07 ± 0.36
PCB trimer 4	128.22 ± 17.23	123.89 ± 7.64	158.98 ± 10.53	143.47 ± 15.17	264.48 ± 31.21	242.38 ± 29.58	73.07 ± 1.28	130.12 ± 15.69	14.91 ± 0.71	16.64 ± 1.79	31.48 ± 4.37
PCB trimer 5	163.38 ± 13.98	72.56 ± 5.59	55.74 ± 5.68	222.80 ± 10.69	176.18 ± 17.00	131.55 ± 2.79	71.07 ± 9.44	50.78 ± 5.68	73.82 ± 6.54	44.51 ± 3.36	20.89 ± 1.58
PCB trimer 6	4.67 ± 0.29	6.22 ± 0.64	15.92 ± 2.08	2.28 ± 0.19	14.44 ± 1.24	20.81 ± 1.90	8.08 ± 0.39	19.66 ± 1.29	7.36 ± 0.35	4.42 ± 0.56	3.27 ± 0.12
PCB trimer 7	104.37 ± 6.39	16.15 ± 1.18	37.14 ± 2.90	16.05 ± 0.92	88.83 ± 5.34	88.81 ± 10.52	38.25 ± 4.86	21.28 ± 2.56	24.95 ± 2.84	21.74 ± 1.87	7.08 ± 0.63
Σ	**537.93**	**272.16**	**340.01**	**483.68**	**653.73**	**565.53**	**199.40**	**262.97**	**154.39**	**123.36**	**76.14**
Procyanidins A-type (PCA) glycosides											
PCA hexoside 1	416.69 ± 36.57	301.62 ± 19.35	236.30 ± 5.56	198.81 ± 18.78	560.65 ± 17.39	478.55 ± 60.02	85.91 ± 9.99	126.04 ± 15.90	20.27 ± 2.22	21.48 ± 0.76	29.99 ± 3.41
PCA pentoside 1	49.45 ± 6.68	30.64 ± 2.09	45.80 ± 3.67	103.11 ± 8.25	74.87 ± 6.70	43.21 ± 5.64	3.10 ± 0.14	23.84 ± 1.53	10.25 ± 0.67	20.62 ± 1.69	3.02 ± 0.17
PCA trimer arabinoside	114.09 ± 3.82	24.92 ± 1.85	31.75 ± 2.91	42.84 ± 2.08	84.34 ± 4.20	65.44 ± 2.63	12.70 ± 0.99	23.60 ± 1.10	21.99 ± 0.42	22.02 ± 0.95	5.84 ± 0.39
PCA hexoside 2	46.33 ± 4.33	45.82 ± 3.26	16.32 ± 1.93	34.88 ± 1.55	73.34 ± 7.21	70.98 ± 8.74	19.63 ± 1.72	11.66 ± 1.13	12.85 ± 0.92	18.61 ± 1.82	4.54 ± 0.27
PCA pentoside 2	30.95 ± 3.70	14.17 ± 0.71	20.82 ± 1.91	7.42 ± 0.31	40.02 ± 3.73	23.58 ± 2.57	7.18 ± 0.69	14.20 ± 1.14	7.43 ± 1.02	2.39 ± 0.27	1.61 ± 0.15
Σ	**657.51**	**417.17**	**351.00**	**387.07**	**833.22**	**681.77**	**128.52**	**199.35**	**72.79**	**85.13**	**45.00**
Flavonols											
Quercetin	22.37 ± 0.64	7.50 ± 0.29	6.31 ± 0.50	4.67 ± 0.35	13.91 ± 0.54	7.98 ± 0.93	1.95 ± 0.06	2.88 ± 0.25	1.10 ± 0.06	1.43 ± 0.10	2.04 ± 0.05
Σ	**22.37**	**7.50**	**6.31**	**4.67**	**13.91**	**7.98**	**1.95**	**2.88**	**1.10**	**1.43**	**2.04**
Flavonol-3-O-glycosides											
Quercetin-3-O-glucoside	23.13 ± 1.50	17.97 ± 1.18	19.76 ± 0.22	13.13 ± 0.55	35.45 ± 3.18	20.75 ± 1.42	6.91 ± 0.17	9.34 ± 0.85	4.76 ± 0.50	4.06 ± 0.44	5.86 ± 0.66
Quercetin-3-O- arabinoside	69.72 ± 3.81	36.64 ± 0.82	32.16 ± 0.65	25.27 ± 2.35	86.53 ± 5.25	41.60 ± 1.41	20.61 ± 1.14	22.65 ± 1.46	19.56 ± 1.40	20.29 ± 2.19	10.43 ± 0.12
Σ	**92.85**	**54.61**	**51.92**	**38.39**	**121.98**	**62.35**	**27.52**	**31.99**	**24.32**	**24.36**	**16.29**
N-phenylpropenoyl-L-aminoacids											
N-Coumaroyl-L-aspartate 1	14.12 ± 1.24	11.13 ± 0.69	7.11 ± 0.71	9.41 ± 0.77	11.66 ± 1.10	14.79 ± 0.52	4.59 ± 0.43	6.88 ± 0.64	3.10 ± 0.21	2.76 ± 0.21	4.36 ± 0.36
N-Caffeoyl-L-aspartate	652.58 ± 40.18	195.38 ± 15.63	248.27 ± 5.56	141.25 ± 13.13	358.23 ± 29.35	244.26 ± 2.50	36.54 ± 1.24	50.29 ± 4.32	33.86 ± 2.26	23.16 ± 2.12	34.05 ± 1.84
N-Coumaroyl-L-aspartate 2	93.97 ± 3.14	85.24 ± 3.29	121.75 ± 8.39	28.89 ± 1.59	118.16 ± 7.68	93.55 ± 10.54	9.39 ± 0.16	23.54 ± 0.86	7.22 ± 0.10	5.42 ± 0.33	14.87 ± 0.83
N-Coumaroyl-L-glutamate	9.40 ± 0.18	6.17 ± 0.55	8.42 ± 0.42	7.42 ± 0.78	12.58 ± 0.44	10.61 ± 0.98	3.25 ± 0.08	5.14 ± 0.21	9.34 ± 0.69	9.08 ± 0.88	3.12 ± 0.24
N-coumaroyl-L-tyrosine	39.93 ± 1.57	12.71 ± 1.76	18.06 ± 1.04	16.31 ± 0.99	38.46 ± 2.97	42.37 ± 1.29	6.88 ± 0.61	7.34 ± 0.37	7.32 ± 0.58	4.52 ± 0.44	4.88 ± 0.23
Σ	**810.01**	**310.63**	**403.61**	**203.27**	**539.09**	**405.59**	**60.64**	**93.19**	**60.85**	**44.93**	**61.27**
Total polyphenols	**3234.91**	**1759.39**	**1998.77**	**2271.15**	**3403.22**	**2869.41**	**822.81**	**1122.82**	**534.42**	**526.44**	**437.65**

METHYLXANTHINES	Concentration (g kg-1 of CBS)										
Theobromine	8.49 ± 0.26	6.76 ± 0.43	8.09 ± 0.74	7.43 ± 0.32	8.71 ± 0.20	8.47 ± 0.26	4.36 ± 0.32	6.86 ± 0.54	4.38 ± 0.31	3.71 ± 0.12	3.97 ± 0.36
Caffeine	1.26 ± 0.08	1.52 ± 0.14	1.59 ± 0.08	1.46 ± 0.05	1.36 ± 0.07	1.49 ± 0.04	0.38 ± 0.03	1.10 ± 0.06	0.40 ± 0.04	0.35 ± 0.02	0.50 ± 0.03
Total Methylxantines	**9.75**	**8.29**	**9.68**	**8.89**	**10.07**	**9.95**	**4.73**	**7.96**	**4.78**	**4.06**	**4.47**

Data are presented as the mean (n=4) ± standard deviation

Table 4 (continuation). Content of the single identified polyphenolic compounds and methylxantines in CBS powders from different geographical origins and cultivars.

POLYPHENOLS	Concentration (mg kg-1 of CBS)										
	UGA2	VEN1	VEN2	VEN3	VEN4	VEN5	VEN6	VEN7	VEN8	VEN9	VEN10
Phenolic acids											
Protocatechuic acid	107.95 ± 7.55	118.99 ± 4.56	73.43 ± 2.41	107.87 ± 3.62	46.42 ± 3.05	120.54 ± 8.76	124.26 ± 8.81	103.68 ± 3.83	136.29 ± 4.26	85.51 ± 5.29	12.05 ± 0.02
Σ	**107.95**	**118.99**	**73.43**	**107.87**	**46.42**	**120.54**	**124.26**	**103.68**	**136.29**	**85.51**	**12.05**
Flavan-3-ol											
Catechin	51.34 ± 4.67	20.52 ± 1.98	35.49 ± 1.66	72.59 ± 3.97	52.85 ± 1.53	56.38 ± 3.32	66.48 ± 3.14	132.57 ± 4.77	117.69 ± 5.69	127.19 ± 5.09	77.24 ± 1.84
Epicatechin	15.90 ± 1.34	293.71 ± 25.61	159.23 ± 19.16	339.81 ± 31.23	118.34 ± 4.72	107.56 ± 11.70	189.59 ± 15.88	185.99 ± 1.39	229.01 ± 4.14	87.54 ± 4.61	229.23 ± 0.05
Σ	**67.24**	**314.23**	**194.72**	**412.40**	**171.18**	**163.93**	**256.07**	**318.56**	**346.70**	**214.74**	**306.47**
Catechin-3-O-glycosides											
Catechin-3-O-glucoside	13.41 ± 1.32	16.79 ± 0.69	14.17 ± 0.98	52.35 ± 4.73	19.63 ± 1.08	25.05 ± 1.20	29.70 ± 2.22	34.77 ± 0.53	50.05 ± 2.87	24.70 ± 1.67	31.95 ± 0.56
Σ	**13.41**	**16.79**	**14.17**	**52.35**	**19.63**	**25.05**	**29.70**	**34.77**	**50.05**	**24.70**	**31.95**
Procyanidins B-type (PCB)											
PCB	38.30 ± 4.82	407.82 ± 53.09	672.55 ± 51.82	77.63 ± 3.71	83.42 ± 2.75	55.12 ± 3.42	181.77 ± 9.98	106.02 ± 8.84	53.90 ± 2.37	67.85 ± 5.48	104.07 ± 12.15
Σ	**38.30**	**407.82**	**672.55**	**77.63**	**83.42**	**55.12**	**181.77**	**106.02**	**53.90**	**67.85**	**104.07**
Procyanidins B-type (PCB) trimers											
PCB trimer 1	13.85 ± 1.12	19.64 ± 1.39	70.47 ± 5.00	73.91 ± 2.20	15.89 ± 0.97	59.51 ± 4.65	26.97 ± 1.47	65.15 ± 0.18	92.25 ± 7.01	49.24 ± 4.91	16.53 ± 0.49
PCB trimer 2	6.72 ± 0.54	20.82 ± 1.71	73.22 ± 7.69	26.28 ± 0.95	13.45 ± 1.56	35.54 ± 2.93	22.79 ± 1.19	109.57 ± 2.79	48.42 ± 0.11	36.28 ± 2.55	1.50 ± 0.06
PCB trimer 3	13.50 ± 1.00	15.51 ± 1.30	97.04 ± 10.55	53.24 ± 3.37	16.60 ± 1.58	30.49 ± 1.91	17.96 ± 1.68	19.74 ± 2.15	10.29 ± 1.09	8.55 ± 0.75	14.59 ± 1.71
PCB trimer 4	18.09 ± 2.39	104.98 ± 9.66	101.48 ± 5.34	103.89 ± 9.58	29.64 ± 1.01	38.42 ± 1.25	99.12 ± 6.22	47.89 ± 5.02	62.30 ± 4.20	34.44 ± 0.09	122.69 ± 2.84
PCB trimer 5	13.39 ± 1.09	129.78 ± 9.02	259.51 ± 33.07	79.20 ± 3.47	42.28 ± 1.52	35.32 ± 4.15	167.55 ± 14.71	61.51 ± 5.29	21.95 ± 2.89	39.94 ± 3.99	26.83 ± 1.68
PCB trimer 6	3.72 ± 0.21	6.98 ± 0.76	1.57 ± 0.17	3.88 ± 0.35	0.82 ± 0.10	4.53 ± 0.25	2.37 ± 0.20	2.88 ± 0.12	3.63 ± 0.03	3.28 ± 0.45	8.58 ± 0.05
PCB trimer 7	28.13 ± 2.84	32.28 ± 1.53	13.81 ± 0.64	36.62 ± 4.49	6.87 ± 0.33	16.40 ± 1.41	37.89 ± 5.28	35.72 ± 0.34	47.00 ± 3.78	36.02 ± 4.20	29.46 ± 0.38
Σ	**97.40**	**330.00**	**617.10**	**377.03**	**125.55**	**220.20**	**374.65**	**342.45**	**285.84**	**207.76**	**220.17**
Procyanidins A-type (PCA) glycosides											
PCA hexoside 1	34.02 ± 4.25	171.55 ± 8.35	33.97 ± 0.95	315.20 ± 14.31	55.70 ± 2.99	62.43 ± 1.94	131.47 ± 15.00	181.20 ± 0.34	210.71 ± 20.94	158.48 ± 13.86	160.41 ± 11.07
PCA pentoside 1	3.01 ± 0.42	33.68 ± 3.58	98.31 ± 4.24	65.39 ± 4.11	12.61 ± 1.72	15.06 ± 0.59	45.50 ± 3.94	29.03 ± 2.19	37.51 ± 0.56	22.26 ± 1.80	10.95 ± 1.20
PCA trimer arabinoside	7.64 ± 0.26	32.13 ± 2.84	23.79 ± 3.27	34.44 ± 4.00	15.93 ± 1.36	24.61 ± 1.80	36.65 ± 2.83	25.80 ± 0.23	36.17 ± 1.04	26.83 ± 0.12	17.69 ± 1.73
PCA hexoside 2	2.74 ± 0.29	13.47 ± 1.49	20.47 ± 1.65	29.81 ± 2.45	9.21 ± 0.62	19.70 ± 1.54	28.91 ± 2.34	14.92 ± 0.38	28.56 ± 2.46	12.27 ± 1.56	11.48 ± 0.09
PCA pentoside 2	0.91 ± 0.05	8.29 ± 0.68	2.28 ± 0.19	22.48 ± 3.04	4.85 ± 0.25	5.54 ± 0.71	9.33 ± 0.14	16.12 ± 1.97	15.82 ± 1.22	10.32 ± 0.56	8.57 ± 0.19
Σ	**48.32**	**259.13**	**178.83**	**467.31**	**98.29**	**127.34**	**251.86**	**267.07**	**328.77**	**230.16**	**209.09**
Flavonols											
Quercetin	2.38 ± 0.18	3.19 ± 0.27	5.93 ± 0.78	3.41 ± 0.27	2.56 ± 0.09	3.33 ± 0.32	6.11 ± 0.53	1.63 ± 0.02	2.94 ± 0.04	3.46 ± 0.43	2.43 ± 0.08
Σ	**2.38**	**3.19**	**5.93**	**3.41**	**2.56**	**3.33**	**6.11**	**1.63**	**2.94**	**3.46**	**2.43**
Flavonol-3-O-glycosides											
Quercetin-3-O-glucoside	5.11 ± 0.42	10.44 ± 0.77	18.18 ± 0.61	24.61 ± 1.58	7.09 ± 0.55	10.89 ± 0.56	20.23 ± 1.83	11.73 ± 0.99	20.99 ± 1.07	6.51 ± 0.19	10.55 ± 0.26
Quercetin-3-O- arabinoside	10.64 ± 0.31	17.55 ± 0.76	23.36 ± 1.67	50.99 ± 3.26	14.45 ± 0.36	22.07 ± 0.66	28.71 ± 0.75	14.90 ± 0.38	37.01 ± 0.79	17.05 ± 1.33	25.32 ± 1.32
Σ	**15.75**	**27.99**	**41.54**	**75.60**	**21.54**	**32.95**	**48.94**	**26.63**	**58.00**	**23.56**	**35.88**
N-phenylpropenoyl-L-aminoacids											
N-Coumaroyl-L-aspartate 1	12.52 ± 1.19	5.38 ± 0.24	1.41 ± 0.13	21.32 ± 0.87	6.54 ± 0.27	19.33 ± 1.63	8.96 ± 0.64	8.69 ± 0.03	13.35 ± 0.44	5.81 ± 0.32	4.19 ± 0.30
N-Caffeoyl-L-aspartate	17.77 ± 1.45	59.97 ± 3.01	168.62 ± 16.11	193.80 ± 10.43	94.35 ± 9.41	97.61 ± 5.21	191.28 ± 16.84	200.15 ± 4.10	188.57 ± 4.29	89.79 ± 1.85	149.23 ± 5.89
N-Coumaroyl-L-aspartate 2	21.33 ± 1.32	23.14 ± 2.48	29.30 ± 1.61	75.08 ± 3.88	24.17 ± 1.17	55.32 ± 1.98	64.63 ± 3.40	91.94 ± 3.20	87.43 ± 3.00	61.90 ± 3.48	48.63 ± 2.84
N-Coumaroyl-L-glutamate	2.17 ± 0.18	5.70 ± 0.26	9.84 ± 0.62	5.23 ± 0.60	3.77 ± 0.48	3.40 ± 0.36	4.30 ± 0.45	2.41 ± 0.28	5.78 ± 0.73	6.91 ± 0.96	3.21 ± 0.32
N-coumaroyl-L-tyrosine	4.61 ± 0.45	10.29 ± 1.13	7.49 ± 0.54	23.23 ± 2.15	4.47 ± 0.22	12.82 ± 1.26	12.07 ± 0.87	18.78 ± 0.17	26.83 ± 0.54	13.95 ± 0.47	8.38 ± 1.00
Σ	**58.40**	**104.47**	**216.66**	**318.65**	**133.30**	**188.48**	**281.23**	**321.96**	**321.96**	**178.35**	**213.64**
Total polyphenols	**449.16**	**1582.61**	**2014.94**	**1892.25**	**701.88**	**936.94**	**1554.60**	**1522.77**	**1584.45**	**1036.08**	**1135.76**

METHYLXANTHINES	Concentration (g kg-1 of CBS)										
Theobromine	6.77 ± 0.28	5.97 ± 0.37	6.55 ± 0.35	8.19 ± 0.61	3.22 ± 0.10	6.04 ± 0.50	7.24 ± 0.69	7.74 ± 0.03	8.41 ± 0.19	6.93 ± 0.28	5.31 ± 0.17
Caffeine	0.99 ± 0.01	1.27 ± 0.08	2.25 ± 0.07	2.97 ± 0.14	1.21 ± 0.04	2.24 ± 0.22	2.61 ± 0.18	3.37 ± 0.03	3.03 ± 0.15	2.64 ± 0.10	1.88 ± 0.12
Total Methylxantines	**7.76**	**7.25**	**8.80**	**11.15**	**4.43**	**8.28**	**9.85**	**11.11**	**11.44**	**9.57**	**7.18**

Data are presented as the mean (n=4) ± standard deviation

concentrations up to 8510 mg of epicatechin per kg of cocoa bean [239]. However, it is worth mention that in this latter work, fermented but not roasted cocoa beans were studied, so a further decrease in epicatechin concentration due to the high roasting temperature did not take place.

Catechin-3-O-glucoside represented in average 2.3% of the total amount of quantified phenolic compounds, with a concentration range of 6.40 (UGA1) – 101.49 (SAT2) mg/kg CBS. Catechin-3-O-glucoside has been previously detected in aqueous extractions of CBS, prepared as beverages [13,222].

Procyanidin B-type (dimer) was, after epicatechin, the most abundant individual polyphenolic compound in CBS. It represented 12.9% of the total amount of quantified polyphenols and was present at concentrations of 6.84 (GHA) – 672.55 (VEN2) mg/kg CBS. Seven different procyanidin B-type trimers were detected and quantified in almost all the CBS samples. Only PCB trimer 6 was absent in ECU2 and PCB trimer 2 was absent for UGA1. The sum of the seven PCB trimers was comprised between 26.28 (GHA) and 653.73 (SAT2) mg/kg CBS and accounted on average for 19.8% of the total quantified polyphenols. On the other hand, five glycosides of procyanidins A-type (PCA) were detected and quantified, among which PCA hexoside 1 was the most abundant. The sums of the PCA glycosides ranged from 16.34 (GHA) to 833.22 (SAT2) mg/kg CBS and this compound group accounted for 18.3% of the total amount of polyphenols. Procyanidins are, together with their monomers (catechin and epicatechin), cocoa polyphenol markers [81]. In cocoa powder, procyanidin dimers have been detected in concentrations of 1820 – 2710 mg/kg while trimers have been detected in concentrations of 500 – 1200 mg/kg [237]. As observed before for flavanol monomers, procyanidin concentrations in CBS could be considered to be an order of magnitude below those found in cocoa nibs. This fact was also observed by Cádiz-Gurrea et al. [240], who stated that higher flavonoid contents were present in bean samples, while a higher content of phenolic acids was observed in CBS samples. However, it was found that flavanol monomers and procyanidins (including glycosides) in CBS accounted for 73.2% of the total quantified phenolic compounds, which is in line with the 60% stated for cocoa beans by Barnaba et al. [235].

Concerning flavonols, quercetin and two quercetin glycosides (quercetin-3-O-glucoside, also named isoquercitrin, and quercetin-3-O-arabinoside) were detected and quantified throughout the CBS sample set. Quercetin was found in concentrations going from 1.07 (IVC) to 22.37 (MEX) mg/kg CBS, while isoquercitrin concentrations ranged from 3.47 (GHA) to 47.59 (ECU3) mg/kg CBS, and quercetin-3-O-arabinoside was present in concentrations

comprised between 3.76 (GHA) and 86.53 (SAT2) mg/kg CBS. These compounds have been claimed to increased their contents in the outer plant of the cocoa fruit in order to protect the plant when it is submitted to intense sunlight and irrigation [227], which could be related with their different concentrations in the different CBS samples. Quercetin accounted only for 0.3% of the total quantified polyphenols while its glycosides were 3.1%. In CBS, flavonols were, therefore, present in much lower concentrations compared to flavanols, as it was already noticed for cocoa beans [81]. Nevertheless, the three flavonol compounds quantified in this study are considered to be cocoa markers [242]. In previous studies involving CBS, both quercetin and isoquercitrin have been detected, together with other quercetin glycosides such as quercetin-3-O-galactoside or quercetin-3-O-rhamnoside [13,100,222]. In cocoa powder, these compounds have been found in slightly higher concentrations when compared to the cocoa by-product studied here. Quercetin-3-O-glucoside (isoquercitrin) was found in concentrations of 117 – 323 mg/kg of cocoa powder while quercetin-3-O arabinoside was detected in concentrations of 180 – 486 mg/kg of cocoa powder [237].

N-phenylpropenoyl-L-aminoacids are hydroxycinnamate derivatives unique to cocoa products and are, therefore, cocoa markers [243]. The presence of these compounds has been related to the astringent properties of cocoa [240], which are also very perceptible in CBS [13]. In this study, five amino acid derivatives were identified and quantified: N-coumaroyl-L-aspartate (isomers 1 and 2), N-caffeoyl-L-aspartate, N-coumaroyl-L-glutamate, and N-coumaroyl-L-tyrosine. Among the CBS samples, the sums of their concentrations were comprised between 17.13 (GHA) and 810.01 (MEX) mg/kg CBS, and they accounted on average for a 16.4% of the total quantified polyphenolic compounds. The most abundant compound in this category was N-caffeoyl-L-aspartate, which showed concentrations up to 652.58 mg/kg CBS (MEX). This amino acid derivative was also found to be the most abundant in cocoa beans [237,243]. In fermented cocoa beans, the sum of N-phenylpropenoyl-L-aminoacids was found to be 1470 mg/kg, an order of magnitude higher than that found in CBS [244].

3.2. Polyphenolic Quantification by Spectrophotometric Analyses

Spectrophotometric analysis of the phenolic content in CBS were performed in this study as screening methods, with the aim of comparing their results to those obtained by RP-HPLC-PDA Analysis. Concretely, the total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and the radical scavenging activity (RSA) were assessed through the Folin-Ciocalteu, aluminum chloride, vanillin, and DPPH radical scavenging methods,

respectively. The results obtained for these for parameters for each CBS sample are shown in Table 3.

TPC values among the different samples ranged from 6.10 (UGA2) to 42.97 (PER2) mg GAE/g CBS. It has been claimed that for cocoa beans, Criollo variety usually presents lower polyphenolic contents that account for 2/3 of what is found in Forastero variety beans [81]. Trinitario variety is a consequence of the botanical cross of the two previously mentioned varieties and should therefore have TPC values among those two. Nacional variety is close in aroma to the Criollo and Trinitario varieties and is therefore considered as the third 'fine aroma' variety. Nevertheless, from a botanical point of view it actually belongs to the Forastero group [227], although it is a native variety to Ecuador, and it should therefore contain TPC values close to the ones found for Forastero CBS. However, when looking into our sample set results, those differences and variety-dependence of the TPC values are not observed, and values seem to vary independently of the CBS variety. A clear example of this can be observed in the fact that the sample with the lowest TPC value (UGA2) is a Forastero CBS while the one with the highest value (PER2) belongs to the Trinitario variety. To the contrary, a tendency depending on the CBS origin continent can be observed in which America samples tend to show higher TPC values than African ones. This general line can be observed with the fact that the lowest TPC values were found for samples coming from Uganda and Ghana (UGA1, UGA2, and GHA) while the highest ones were found for CBS samples coming for Mexico or Peru (PER2 and MEX). This trend can be explained by the different climatic conditions in both continents and the different cultivation traditions that lead to different chemical transformations because of the different fermentation and drying processes employed by the local populations. In our work, cocoa bean roasting and husking processes for yielding the different CBS were standardized as it could have been done in the cocoa manufacturing industry. However, fermentation and drying processes that normally take place at the cocoa farm are hard or impossible to standardize when it comes to samples with different origins. Indeed, it has been reported that regulations applied in EU countries where cocoa is manufactured are not applied in the beginning of the supply chain (including fermentation and drying of cocoa beans) [225], which explains the common absence of exchanged information concerning the first treatments of cocoa raw material and, therefore, the impossibility to standardized these first steps on the manufacturing process. Polyphenol content in cocoa beans and CBS gets reduced during fermentation since cocoa polyphenols can diffuse into fermentation sweating during the process. It has been previously claimed that non-standardized fermentations could led to 6-fold variations in the final product polyphenol

concentration [81]. Previous studies have reported values of CBS TPC varying for 2.72 to 55.16 mg GAE/g CBS [13,57-60,240], which is in line with the ones obtained in the present work and confirms that variation in these parameters is mainly linked to the different origins, cultivation techniques, and fermentation and drying processes. When it comes to cocoa beans, previous studies have found TPC values of 30.49 – 94.33 mg GAE/g [94,226,239]. TPC values in cocoa beans get to double those found for CBS, which is in line with what observed for individual polyphenolic compounds in the previous section.

A comparison was made between the obtained TPC values (considered as a screening method) and the sum of the polyphenolic compound concentration obtained by HPLC in order to compare both methods. In a general line, it was observed that TPC values were an order of magnitude higher than the values obtained when adding all the individual compound concentrations. One of the reasons explaining this difference could be the fact that there is probably a large amount of CBS polyphenols that remained still undetermined and were not quantified in our study, although the most abundant and known compounds were taken into account. Spectrophotometric analyses are rapid and easy methods that can be performed by less experimented personnel than HPLC requires. However, these methods have demonstrated to present several interferences that usually result in overestimated values. These are valid methods for screening and comparison between similar samples. However, chromatographic techniques are recommended to accompany these methods and establish a structure-activity relationship [98]. Nevertheless, a correlation coefficient of r = 0.8671 was found among the obtained TPC values and the HPLC-determined sum of concentrations, which confirms that spectrophotometric assays remain useful as approximate methods for semi-quantitative phenolic amount determination and comparison among the different CBS samples. This correlation was much higher than the r = 0.32 found for the sum of individual phenols with TPC reported for cocoa beans [94].

TFC accounted for a 22.51 – 52.12% of the TPC. These numbers are in line with the 25.6% that the sum of flavan-3-ol, catechin-3-O-glycoside, flavonols, and flavonol-3-O-glucosides represented in the total polyphenolic compound concentration determined by RP-HPLC-PDA. On the other hand, TFC values correlated with the flavonoid sum with a correlation coefficient of r = 0.8264. TFC values were comprised between 1.42 (UGA2) and 19.67 (SAT3) mg CE/g CBS. These values are slightly lower than previously found TFC values for CBS such as those in the work of Barbosa-Pereira et al. [57], in which TFC values of 5.39 – 43.94 mg CE/g CBS were reported. Nevertheless, in that work, a pulsed electric field assisted extraction was performed, which could have influenced a bigger CBS flavonoid

extraction over the rest of phenolic compounds. Although TFC values seemed to be overestimated as observed before for TPC, these values were highly correlated with TPC results (r = 0.9607) as it has been reported in previous works concerning CBS [13,57,245,246].

TTC results accounted for a 15.33 – 40.0% of the TPC values, ranged from 1.14 (UGA2) to 11.00 (PER2) mg CE/g CBS and were correlated with this parameter with r = 0.9251. The sum of all the procyanidins detected and quantified by HPLC, which are considered as condensed tannins [81], accounted for a 51.0% of the total polyphenols quantified by HPLC, which is higher than the percentage range of TTC with respect to TPC, but still in line with those numbers. TTC values correlated with the values of HPLC-determined procyanidins with r = 0.7716. These compounds are believed to increase their concentration during the cocoa bean fermentation process, when polyphenols diffuse from the storage cells and undergo oxidation to condensed tannins [81]. TTC values will therefore highly depend on cocoa fermentation conditions and time. In previous works concerning CBS, TTC has been reported to be comprised between 7.24 and 25.79 mg CE/g CBS when performing a pulsed electric field assisted extraction [57]. As happened for TFC, this kind of extraction performed in the mentioned work could have favored a major tannin extraction from CBS.

Polyphenols, and concretely cocoa and CBS polyphenols, have been reported to possess antioxidative properties that, together with other biofunctionalities can help to treat or prevent several chronic diseases such as cancer, diabetes, and cardiovascular diseases, among others [84,169,170,218]. In the present study, the antioxidant characteristics of the CBS samples were measured as their radical scavenging activity (RSA) and the results gave values comprised between 27.69 (UGA2) and 181.98 (PER2) µmol TE/g CBS. It is worth mentioning than the lowest and highest RSA values were obtained for the same samples that gave the lowest and highest values of TPC, TFC and TTC. Indeed, it was found that RSA values were highly correlated with those three parameters with the following correlation coefficients: $r_{(RSA\text{-}TPC)}$ = 0.9871, $r_{(RSA\text{-}TFC)}$ = 0.9771, and $r_{(RSA\text{-}TTC)}$ = 0.9480. These values are higher than those reported by Gültekin-Özgüven et al. [245] ($r_{(RSA\text{-}TPC)}$ = 0.7010 and $r_{(RSA\text{-}TFC)}$ = 0.7725), and those reported by Barbosa-Pereira et al. [57] ($r_{(RSA\text{-}TPC)}$ = 0.9647, $r_{(RSA\text{-}TFC)}$ = 0.9740, and $r_{(RSA\text{-}TTC)}$ = 0.7290). Besides, the correlations of RSA with the HPLC-determined counterparts of the spectrophotometric parameters were $r_{(RSA\text{-Total sum of polyphenols})}$ = 0.8721, $r_{(RSA\text{-Sum of flavonoids})}$ = 0.8165, and $r_{(RSA\text{-Sum of procyanidins})}$ = 0.8446. Concerning the potential of the different HPLC-determined groups to act as antioxidant compounds, it was found that the highest correlation with RSA were found for PCA glycosides and PCB trimers ($r_{(RSA\text{-}}$

$_{PCAglycosides)}$ = 0.8221 and $r_{(RSA\text{-}PCBtrimers)}$ = 0.8100, respectively). Other works in which CBS antioxidant activity was studied reported values of 36 – 321.97 µmol TE/ g CBS when employing assisted extractions [57,62]. These values are again higher than the ones reported in the present study, but they are logical if we take into account the also higher values of TPC, TFC, TTC that were found in those works, as previously mentioned. Concerning cocoa beans, they have been reported to show an antioxidant activity of 173 µmol TE/ g roasted cocoa beans [245], which is in the range of the values found for CBS in the present work. It can therefore be concluded that CBS, which is considered a by-product, shows similar antioxidant capacity to that of cocoa nibs.

3.3. Classification of CBS Based on Their Polyphenolic Fingerprints and Spectrophotometric measurements

In this section of the work, the HPLC-determined polyphenolic profiles of CBS as well as the spectrophotometric analyses results were employed for a chemometric analysis with the aim of obtaining the classification of the different samples according to their geographic origins and varieties. Principal component analyses (PCAs) were employed for the different sample group classification and differentiation.

3.3.1. Classification of CBS Samples According to their Geographical Origin

PCA analyses of the entire sample set data were performed to evaluate sample separation according to their geographic origin and variety. Polyphenolic amounts in cocoa have been reported to be dependent on the cocoa cultivar [81]. However, in our study, this was not observed. Taking into account the CBS varieties of all the American and African CBS samples together, PCA separation among the four different variety groups (Forastero, Trinitario, Nacional, and Criollo) was not significant. All the four groups clustered together in both HPLC-determined polyphenolic profile and spectrophotometric analyses results diagrams. The no-significance for both HPLC and spectrophotometric analyses data following a classification by CBS variety was expected since no tendencies with respect to the CBS variety were a priori found when observing the results obtained in sections 3.1 and 3.2. This can occur due to the fact that our matrix of samples is not completely balance. As mentioned before, a completely balanced sample matrix was not possible to achieve due to the lack of information concerning the fermentation and drying steps.

On the other hand, a clear separation among the American and African samples was found when performing the PCA based on the continent of origin of the CBS (Figure 5).

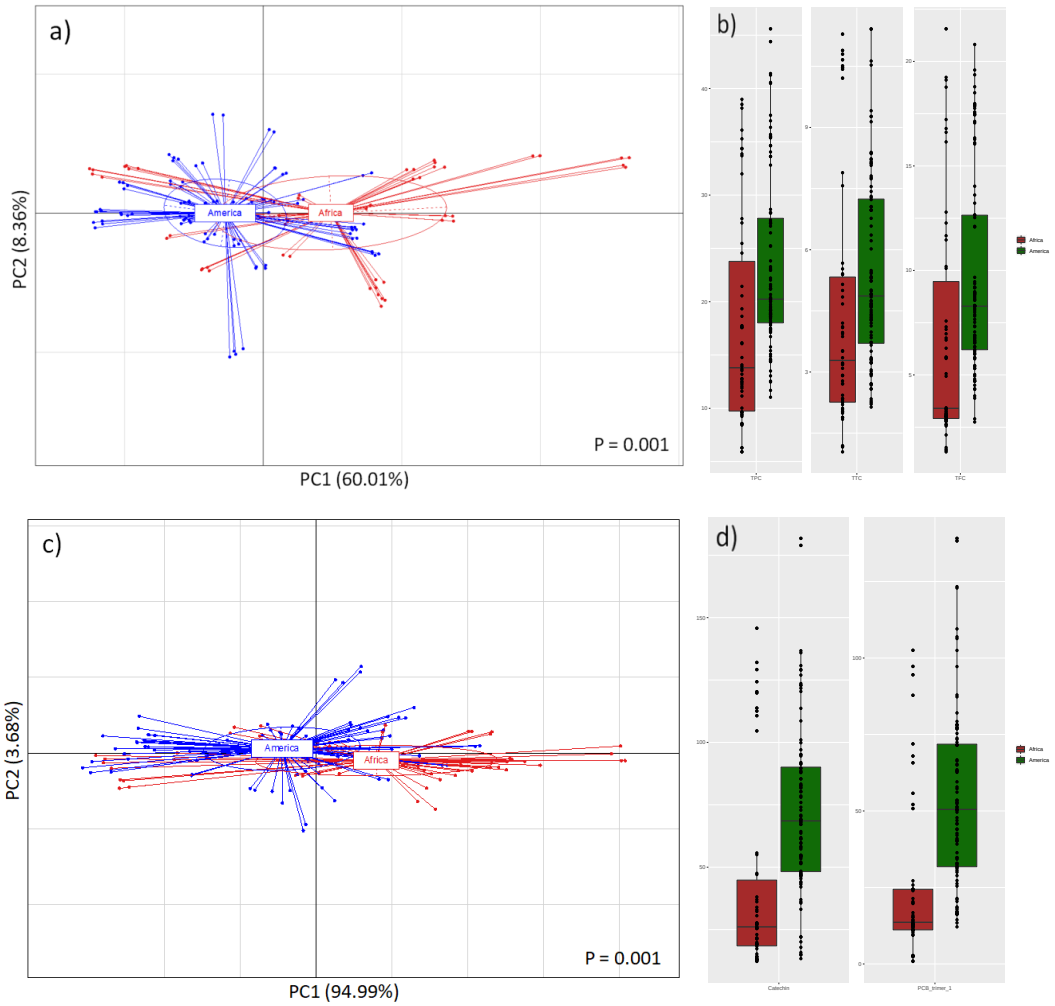

Figure 5. PCA based on the HPLC-determined polyphenolic profile (**a**) and spectrophotometric analyses results (**c**) as function of the continent of origin of the CBS. The variance explained by the first and second components of PCA is shown in parenthesis for PC1 and PC2, respectively, in each graph. Boxplots showing abundance of two key polyphenolic compounds (catechin and PCB trimer 1) (**b**) and TPC, TTC and TFC (**d**) that can be used as possible markers for the separation based on the continent of origin are shown next to their correspondent PCA graph. For interpretation of the legends, see Table 1.

These PCAs explained 68.42% and 98.67% of the variance for the HPLC and spectrophotometric analyses plots, respectively. The separation was significant for both techniques (p = 0.001) and occurred in a similar way. The American samples appeared clustered at the left side of the PCA diagram while the African samples were placed at the right side of the PCA diagram for both techniques (HPLC and spectrophotometric assays, Figure 5a and 5c, respectively). Thus, the screening methods allowed for a similar separation of samples than the more sophisticated characterization method (HPLC). This could be enormously interesting from the cocoa industry point of view in terms of quick, easy, and cheap origin authentication of CBS samples. Origin classification based on the carbohydrate composition of fermented and unfermented cocoa beans has been previously sought. However, this differentiation could not be clearly obtained and indicators to discern cocoa

beans depending on a specific origin could not be proposed [247]. In another study involving different cocoa metabolites, a differentiation among African an Asian cocoa beans was attempted, although American samples seemed to cluster together with both groups [225]. Regarding the cocoa by-product, attempts to chemometrically classify CBS samples have already been reported. Mandrile et al. [112] worked on the authentication of CBS by infrared spectroscopy and optical emission spectroscopy, observing that the combination of those techniques allowed to individualize some patterns related to the geographical origin. Barbosa-Pereira et al. [29,66] found that it was possible to separate the Criollo variety from the rest of varieties according to the CBS volatile profile. On the contrary, in this work, the variety separation was not possible according to the polyphenolic profile, whereas the geographic separation was observed. As mentioned in previous sections, CBS polyphenolic profile seemed to be more dependent on the different factors linked to the cultivation areas (cultivation traditions, different climate conditions and different ecophysiological conditions) than the CBS varieties, a fact that was previously reported also for cocoa beans [239].

A deeper analysis into the continent classification allowed to observe that 23 out of the 25 HPLC-determined polyphenolic compounds had a significantly different content in CBS samples among both continent groups (FDR < 0.001 (12 compounds), FDR < 0.01 (9 compounds), and FDR < 0.05 (1 compound)), as shown in Table 5. Among these compounds driving the CBS separation by the sample continent of origin, two compounds were selected for the representation of their abundance boxplots (Figure 5b): catechin (FDR = 3.4×10^{-8}) and PCB timer 1 (FDR = 3.3×10^{-11}). For the spectrophotometric analysis PCA, boxplots for the three parameters (TPC, TTC, and TFC) were represented (Figure 5d). In the mentioned boxplots, higher values for the different marker compounds and parameters were observed for the American content in all cases. A similar tendency was found in a previous work involving cocoa beans [235], in which catechin was found to be more abundant in American cocoa beans than in African cocoa beans, and was also selected as a marker for the geographical separation of cocoa bean samples.

Table 5. Pairwise comparison of the phenolic compounds quantified in CBSs by RP-HPLC-PDA that could be used as potential geographical origin markers for the American and African continents.

Protocatechuic_acid	N-Coumaroyl-L-aspartate_2	PCA_pentoside_1
Africa	Africa	Africa
America 0.0023	America 2.60E-09	America 2.10E-07
N-Coumaroyl-L-aspartate_1	PCB_trimer_2	PCB_trimer_7
Africa	Africa	Africa
America 0.008	America 1.80E-06	America 0.0048
N-Caffeoyl-L-aspartate	PCB	PCA_trimer_arabinoside
Africa	Africa	Africa
America 3.10E-08	America 0.046	America 0.0046
Catechin	Epicatechin	PCA_hexoside_2
Africa	Africa	Africa
America 3.40E-08	America 4.10E-06	America 0.004
PCA_hexoside_1	N-Coumaroyl-L-glutamate	N-coumaroyl-L-tyrosine
Africa	Africa	Africa
America 9.70E-05	America 0.41	America 0.00025
Catechin-3-O-glucoside	PCB_trimer_3	PCA_pentoside_2
Africa	Africa	Africa
America 0.19	America 0.0098	America 0.005
Quercetin	PCB_trimer_4	Quercetin-3-O-glucoside
Africa	Africa	Africa
America 1.00E-05	America 0.0061	America 1.60E-08
PCB_trimer_1	PCB_trimer_5	Quercetin-3-O-arabinoside
Africa	Africa	Africa
America 3.30E-11	America 0.0069	America 1.90E-05
PCB_trimer_6		
Africa		
America 0.71		

FDR (false discovery rate)<0.001; FDR<0.01 and FDR<0.05 are highlighted in green, yellow, and red, respectively. Compounds highlighted in bold were used to build the box plots that are displayed in the main

Figure 6 shows the PCA plots obtained for the African CBS samples, classified as function of the different countries of origin. These PCAs explained 77.85% of the total variance for the HPLC technique (Figure 6a) and 98.90% for the spectrophotometric analyses (Figure 6c). Both PCAs gave significant separation of the samples (p = 0.001) depending on the country of origin, while the PCAs of the same samples and analytical techniques

depending on the CBS variety gave no-significant separations and the two variety groups of the African CBS samples (Forastero and Trinitario) clustered together.

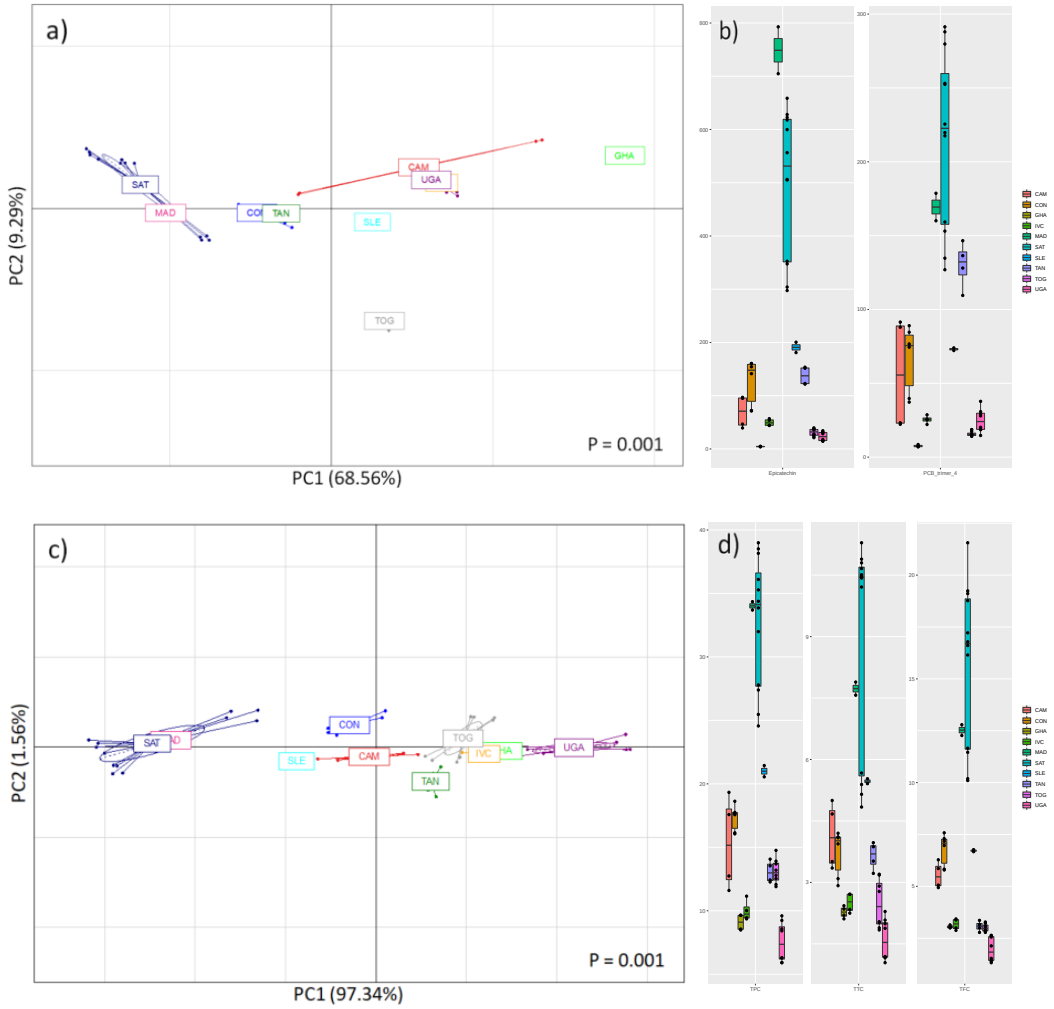

Figure 6. PCA based on the HPLC-determined polyphenolic profile (**a**) and spectrophotometric analyses results (**c**) as function of the country of origin among the African CBS. The variance explained by the first and second components of PCA is shown in parenthesis for PC1 and PC2, respectively, in each graph. Boxplots showing abundance of two key polyphenolic compounds (epicatechin and PCB trimer 4) (**b**) and TPC, TTC and TFC (**d**) that can be used as possible markers for the separation based in the country of origin are shown next to their correspondent PCA graph. For interpretation of the legends, see Table 1.

In both PCA plots shown in Figure 6a and 6c, the most notably characteristic was found to be the clusterization of both Madagascar and São Tomé CBS, which appeared together at the left side of both plots, and well differentiated from the other countries. Again, the results obtained for the screening method (spectrophotometric assays) were similar to those obtained with the HPLC results, allowing both for the separation of the two island samples from the rest of the African countries samples. Two main compounds that drove the separation in the HPLC-results PCA plot were Epicatechin and PCB trimer 4 (Figure 6b). The pairwise comparisons among the different countries for these compounds and the rest of HPLC-

determined phenolic compounds can be consulted in Table 6. Epicatechin and PCB trimer 4 were found at their highest concentrations in Madagascar and São Tomé CBS. These two countries are located in islands. This fact can cause that, despite the distance between these two locations, they share specific climate conditions that make CBS polyphenol content have developed in a similar extent. An island climate is in general characterized by high temperatures and humidity. These two conditions have been reported to give cocoa beans with higher concentrations of catechins, low molecular weight flavan-3-ol oligomers, and total extractable procyanidins [226]. In light of the obtained results, these characteristics can also be extended to CBS. Concerning the TPC, TTC, and TFC boxplots (Figure 6d), similar tendencies are found. Madagascar and São Tomé CBS give the highest concentrations for all the three parameters, which was the cause of their differentiation from the rest of samples, although this caused also the clusterization of both countries. As reported in section 3.2, UGA samples were those with the lowest values in TPC, TTC, and TFC. This fact can also be observed in the plots of Figure 6d and was also the driving cause for the differentiation of the Uganda samples at the right side of the spectrophotometric assay PCA plot.

Table 6. Pairwise comparison of the phenolic compounds quantified in CBSs by RP-HPLC-PDA that could be used as potential geographical origin markers for different countries within the African continent.

Protocatechuic_acid

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02381	-							
GHA	0.05357	0.02381	-						
IVC	1	0.02381	0.05357	-					
MAD	1	0.11285	0.17143	0.17143	-				
SAT	0.00549	0.60038	0.00549	0.00549	0.04945	-			
SLE	0.17143	0.11285	0.17143	0.17143	0.41667	0.04945	-		
TAN	1	0.02381	0.05357	0.05357	1	0.00549	0.17143	-	
TOG	0.01299	0.00549	0.01299	0.01299	0.07692	0.00036	1	0.01299	-
UGA	0.80861	0.00549	0.01299	0.11285	1	0.00036	0.07692	1	0.00233

N-Coumaroyl-L-aspartate_2

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02381	-							
GHA	0.16216	0.02381	-						
IVC	1	0.02381	0.05357	-					
MAD	0.16216	0.10714	0.16216	0.16216	-				
SAT	0.00549	0.33551	0.00549	0.00549	0.5887	-			
SLE	1	0.10714	0.16216	0.31579	0.375	0.04945	-		
TAN	0.05357	0.02381	0.05357	0.05357	0.16216	0.00549	0.16216	-	
TOG	1	0.00549	0.01299	0.01299	0.07143	0.00036	0.07143	0.01299	-
UGA	0.50554	0.00549	0.01299	0.01299	0.07143	0.00036	0.07143	0.03828	0.00233

PCA_pentoside_1

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.5102	-							
GHA	0.05357	0.02679	-						
IVC	0.09184	0.02679	0.05357	-					
MAD	0.15385	0.10557	0.15385	0.15385	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.05205	-			
SLE	0.55814	0.10557	0.15385	0.15385	0.36585	0.05205	-		
TAN	0.05357	0.05042	0.05357	0.05357	0.15385	0.00549	0.15385	-	
TOG	0.24091	0.58578	0.01399	0.01399	0.07407	0.00036	0.07407	0.02597	-
UGA	0.15341	0.00549	0.01399	0.10557	0.07407	0.00036	0.71111	0.01399	0.00175

N-Coumaroyl-L-aspartate_1

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.56044	-							
GHA	0.06767	0.03297	-						
IVC	0.06767	0.44048	0.06767	-					
MAD	0.17859	0.13393	0.17859	0.17859	-				
SAT	0.11913	0.02133	0.00824	0.00824	0.06593	-			
SLE	0.17859	0.13393	0.17859	0.17859	0.42857	0.06593	-		
TAN	0.17859	0.56044	0.06767	0.56044	0.17859	0.00824	0.17859	-	
TOG	0.0202	0.00824	0.0303	0.0202	0.09524	0.00071	0.09524	0.0202	-
UGA	1	1	0.0303	1	1	0.17859	0.8	1	0.0035

PCB_trimer_2

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.039	-							
GHA	0.071	0.039	-						
IVC	0.071	0.039	0.071	-					
MAD	0.188	0.14	0.188	0.188	-				
SAT	0.146	0.836	0.012	0.012	0.634	-			
SLE	0.188	0.367	0.188	0.188	0.417	0.634	-		
TAN	0.071	0.836	0.071	0.071	0.188	0.461	0.188	-	
TOG	0.023	0.012	0.023	0.023	0.091	0.315	0.091	0.023	-
UGA	1	0.091	1	1	0.091	0.239	1	0.071	0.012

PCB_trimer_7

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.214	-							
GHA	1	0.071	-						
IVC	1	0.071	0.125	-					
MAD	0.214	0.169	0.214	0.214	-				
SAT	0.154	0.422	0.045	0.125	0.651	-			
SLE	0.214	0.169	0.214	0.214	0.469	0.651	-		
TAN	0.791	0.125	0.125	0.125	0.214	0.487	0.214	-	
TOG	0.576	0.47	0.045	0.045	0.125	0.357	0.125	0.487	-
UGA	0.332	0.935	0.045	1	0.125	0.08	0.125	1	0.898

N-Caffeoyl-L-aspartate

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02521	-							
GHA	0.05844	0.02521	-						
IVC	1	0.02521	0.05844	-					
MAD	0.16667	0.12363	0.16667	0.16667	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.65067	-			
SLE	1	0.12363	0.16667	0.16667	0.40541	0.05495	-		
TAN	1	0.02521	0.05844	0.05844	0.16667	0.00549	0.16667	-	
TOG	1	0.00549	0.01399	0.16667	0.08333	0.00036	0.14286	0.01399	-
UGA	1	0.00549	0.01399	1	0.08333	0.00036	0.14286	0.01399	0.662

PCB

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02857	-							
GHA	0.05844	0.02857	-						
IVC	1	0.02857	0.05844	-					
MAD	0.17647	0.12363	0.17647	0.17647	-				
SAT	0.00989	0.08	0.00989	0.00989	0.5887	-			
SLE	0.17647	0.48214	0.17647	0.17647	0.39474	0.05818	-		
TAN	0.05844	0.32143	0.05844	0.05844	0.17647	0.9744	0.17647	-	
TOG	0.01653	0.47722	0.01653	0.01653	0.08	0.00036	0.58537	0.01653	-
UGA	0.27532	0.05619	0.01653	0.84567	0.08	0.00036	0.32432	0.01653	0.01748

PCA_trimer_arabinoside

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.16667	-							
GHA	1	0.02381	-						
IVC	1	0.02381	0.0559	-					
MAD	0.16667	0.11084	0.16667	0.16667	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.60306	-			
SLE	1	0.11084	0.16667	0.6	0.40541	0.05205	-		
TAN	0.0559	0.54945	0.0559	0.0559	0.16667	0.00549	0.16667	-	
TOG	0.01136	0.40854	0.01136	0.01136	0.07407	0.00036	0.07407	0.01136	-
UGA	1	0.00549	0.01136	0.01136	0.07407	0.00036	0.07407	0.01136	0.00175

Catechin

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.1875	-							
GHA	0.12245	0.04762	-						
IVC	0.08036	0.23377	0.08036	-					
MAD	0.1875	0.13975	0.1875	0.1875	-				
SAT	0.01648	0.03047	0.01648	0.1875	0.08036	-			
SLE	0.1875	0.13975	0.1875	0.1875	0.44118	0.66825	-		
TAN	0.08036	0.04762	0.08036	0.08036	0.1875	0.4739	0.90909	-	
TOG	0.67464	0.44356	0.0303	0.0303	0.10526	0.00071	0.10526	0.0303	-
UGA	0.90909	0.93492	0.10909	1	0.10526	0.0558	1	1	1

Epicatechin

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.14286	-							
GHA	0.04945	0.02256	-						
IVC	0.7013	0.02256	0.04945	-					
MAD	0.14286	0.10045	0.14286	0.14286	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.0471	-			
SLE	0.14286	0.10045	0.14286	0.14286	0.34884	0.0471	-		
TAN	0.04945	0.7619	0.04945	0.04945	0.14286	0.00549	0.14286	-	
TOG	0.02139	0.00549	0.01136	0.01136	0.06667	0.00036	0.06667	0.01136	-
UGA	0.01136	0.00549	0.01136	0.01136	0.06667	0.00036	0.06667	0.01136	0.14286

PCA_hexoside_2

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.16667	-							
GHA	0.0559	0.02679	-						
IVC	0.09184	0.02679	0.0559	-					
MAD	1	0.72321	0.16667	0.16667	-				
SAT	0.00618	0.06	0.00618	0.00618	0.05495	-			
SLE	1	0.72321	0.16667	0.16667	0.73171	0.05495	-		
TAN	1	0.02679	0.0559	0.0559	0.16667	0.00618	0.16667	-	
TOG	1	0.12608	0.01399	0.01399	0.47368	0.00036	0.21622	0.08081	-
UGA	0.01399	0.00618	0.0559	0.01399	0.07692	0.00036	0.07692	0.01399	0.00233

PCA_hexoside_1

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.16667	-							
GHA	0.0559	0.02381	-						
IVC	1	0.02381	0.0559	-					
MAD	0.16667	0.1148	0.16667	0.16667	-				
SAT	0.00495	0.00162	0.00495	0.00495	0.63398	-			
SLE	1	0.72321	0.16667	0.16667	0.40541	0.05205	-		
TAN	1	0.56391	0.0559	0.0559	0.16667	0.00495	0.16667	-	
TOG	1	0.00495	0.01136	0.01136	0.07407	0.00036	0.07407	0.01136	-
UGA	1	0.00495	0.01136	0.01136	0.07407	0.00036	0.07407	0.01136	0.00175

N-Coumaroyl-L-glutamate

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.40659	-							
GHA	0.25	0.02521	-						
IVC	0.17647	0.95681	0.0559	-					
MAD	0.17647	0.68878	0.17647	0.17647	-				
SAT	0.00618	0.00618	0.00618	0.00618	0.05205	-			
SLE	1	0.11905	0.17647	0.17647	0.39474	0.05205	-		
TAN	0.0559	0.52265	0.0559	0.0559	0.32432	0.00618	0.17647	-	
TOG	0.01136	0.00618	0.01136	0.01136	0.07692	0.48022	0.07692	0.01136	-
UGA	1	0.00666	0.01136	0.01136	0.07692	0.00071	0.22857	0.01136	0.0035

N-coumaroyl-L-tyrosine

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02521	-							
GHA	0.10714	0.02521	-						
IVC	1	0.02521	0.06429	-					
MAD	0.1875	0.12363	0.1875	0.1875	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.33333	-			
SLE	1	0.12363	0.1875	0.92308	0.40541	0.05495	-		
TAN	1	0.02521	0.06429	0.1875	0.1875	0.00549	0.33333	-	
TOG	1	0.00549	0.01399	0.80861	0.08696	0.00036	1	0.29201	-
UGA	1	0.00549	0.01399	0.01399	0.08696	0.00036	0.08696	0.01399	0.31037

Catechin-3-O-glucoside

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.45306	-							
GHA	0.06122	0.02857	-						
IVC	1	0.57915	0.06122	-					
MAD	0.63158	0.72321	0.19355	0.19355	-				
SAT	0.00618	0.12524	0.00618	0.00618	0.05818	-			
SLE	1	0.72321	0.19355	0.19355	0.45306	0.05818	-		
TAN	1	0.57915	0.06122	0.06122	0.19355	0.00618	0.19355	-	
TOG	1	0.45306	0.01299	0.01299	0.16	0.00036	0.375	0.01299	-
UGA	0.01299	0.00618	1	0.01299	0.08696	0.00036	0.08696	0.01299	0.00233

PCB_trimer_3

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.054	-							
GHA	1	0.054	-						
IVC	1	0.054	0.662	-					
MAD	0.222	0.804	0.222	0.222	-				
SAT	0.533	0.436	0.012	0.012	0.727	-			
SLE	0.414	0.161	0.222	0.222	0.484	0.092	-		
TAN	1	0.054	0.092	0.092	0.222	0.092	0.222	-	
TOG	1	0.012	0.092	0.092	0.111	0.112	0.111	1	-
UGA	0.809	0.012	0.809	0.732	0.111	0.382	0.111	1	1

PCA_pentoside_2

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.18182	-							
GHA	0.06767	0.03061	-						
IVC	0.06767	0.03061	0.06767	-					
MAD	1	0.68878	0.18182	0.18182	-				
SAT	0.11395	0.15574	0.00824	0.00824	0.18182	-			
SLE	1	0.68878	0.18182	0.18182	1	0.18182	-		
TAN	0.44082	0.54945	0.06767	0.06767	0.18182	0.4739	0.18182	-	
TOG	0.28342	0.09091	0.01515	0.54545	0.48649	0.00824	0.6	0.01515	-
UGA	0.01515	0.00824	0.01515	0.01515	0.09091	0.00071	0.09091	0.01515	0.0035

Quercetin

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.08	-							
GHA	1	0.02857	-						
IVC	0.40602	0.02857	0.06767	-					
MAD	0.17143	0.1148	0.17143	0.17143	-				
SAT	0.00549	0.1148	0.00549	0.00549	0.76078	-			
SLE	1	0.1148	0.17143	0.17143	0.40541	0.06181	-		
TAN	1	0.08	0.06767	0.06767	0.17143	0.00549	0.17143	-	
TOG	0.76818	0.00549	1	0.26768	0.08	0.00036	0.08	0.01399	-
UGA	1	0.00549	0.01399	0.01399	0.08	0.00036	0.08	0.01399	0.00233

PCB_trimer_4

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.95455	-							
GHA	0.05143	0.02381	-						
IVC	0.73469	0.02381	0.05143	-					
MAD	0.16216	0.10714	0.16216	0.16216	-				
SAT	0.00706	0.00162	0.00706	0.00706	0.61813	-			
SLE	1	0.70557	0.16216	0.16216	0.38462	0.04945	-		
TAN	0.05143	0.02381	0.05143	0.05143	0.16216	0.04685	0.16216	-	
TOG	0.01299	0.00706	0.01299	0.01299	0.06897	0.00036	0.06897	0.01299	-
UGA	0.25359	0.00749	0.01299	0.95455	0.06897	0.00036	0.06897	0.01299	0.02098

Quercetin-3-O-glucoside

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02679	-							
GHA	0.05357	0.02679	-						
IVC	0.71761	0.02679	0.05357	-					
MAD	0.15385	0.10369	0.15385	0.15385	-				
SAT	0.00618	0.06897	0.00618	0.00618	0.70644	-			
SLE	1	0.10369	0.15385	0.15385	0.375	0.04945	-		
TAN	0.15385	0.02679	0.05357	0.05357	0.15385	0.00618	0.15385	-	
TOG	0.03828	0.00618	0.03828	0.03828	0.06897	0.00036	0.06897	0.01653	-
UGA	0.50554	0.00618	0.01653	0.95455	0.06897	0.00036	0.06897	0.01653	0.01748

PCB_trimer_1

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02857	-							
GHA	1	0.02857	-						
IVC	0.05844	0.52265	0.05844	-					
MAD	0.15789	0.10909	0.15789	0.15789	-				
SAT	0.00706	0.00162	0.00706	0.00706	0.5887	-			
SLE	1	0.10909	0.30769	0.15789	0.375	0.05818	-		
TAN	0.05844	0.02857	0.05844	0.05844	0.15789	0.14526	0.15789	-	
TOG	0.10909	0.00706	0.01515	0.01515	0.07692	0.00036	0.07692	0.01515	-
UGA	0.10909	0.11795	0.01515	0.04545	0.07692	0.00036	0.07692	0.01515	0.67539

PCB_trimer_5

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02381	-							
GHA	0.05143	0.02381	-						
IVC	0.05143	0.02381	0.05143	-					
MAD	0.16216	0.11084	0.16216	0.16216	-				
SAT	0.00549	0.00162	0.00549	0.00549	0.80919	-			
SLE	0.16216	0.85714	0.16216	0.16216	0.38462	0.05143	-		
TAN	0.05143	0.52265	0.05143	0.05143	0.16216	0.00549	0.16216	-	
TOG	0.01212	0.27052	0.01212	0.01212	0.07143	0.00036	0.74419	0.61039	-
UGA	0.51818	0.00549	0.01212	0.01212	0.07143	0.00036	0.07143	0.01212	0.00175

Quercetin-3-O-arabinoside

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	0.02381	-							
GHA	0.0559	0.02381	-						
IVC	0.92691	0.02381	0.0559	-					
MAD	0.16667	0.70557	0.16667	0.16667	-				
SAT	0.00618	0.16667	0.00618	0.00618	0.40575	-			
SLE	1	0.1148	0.16667	0.16667	0.39474	0.05205	-		
TAN	0.16667	0.02381	0.0559	0.0559	0.16667	0.01413	0.32432	-	
TOG	0.86858	0.00599	0.01399	0.01399	0.07692	0.00036	0.6	0.08081	-
UGA	1	0.00599	0.01399	0.01399	0.07692	0.00036	0.07692	0.01399	0.00233

PCB_trimer_6

	CAM	CON	GHA	IVC	MAD	SAT	SLE	TAN	TOG
CON	1	-							
GHA	1	0.612	-						
IVC	1	0.612	1	-					
MAD	0.207	0.161	0.207	0.207	-				
SAT	0.154	0.161	0.066	0.125	0.651	-			
SLE	0.651	0.161	0.207	0.207	0.469	0.651	-		
TAN	0.107	0.054	0.107	0.107	0.207	0.207	0.207	-	
TOG	0.909	0.661	0.026	0.026	0.125	0.345	0.19	0.026	-
UGA	1	0.47	0.026	0.026	0.125	0.345	0.125	0.026	0.026

FDR (false discovery rate)<0.001; FDR<0.01 and FDR<0.05 are highlighted in green, yellow, and red, respectively. Compounds highlighted in bold were used to build the box plots that are displayed in the main manuscript.

Figure 7 shows the PCA plots obtained for the CBS samples from the American countries. These PCAs explained 63.83% of the total variance for the HPLC technique (Figure 7a) and 97.96% for the spectrophotometric analyses (Figure 7c). As it was found for the African countries, both PCA plots for the separation of the American samples depending on the country of origin were found to be significant (p = 0.001), while the PCAs of the same

samples and analytical techniques depending on the CBS variety gave no-significant separations.

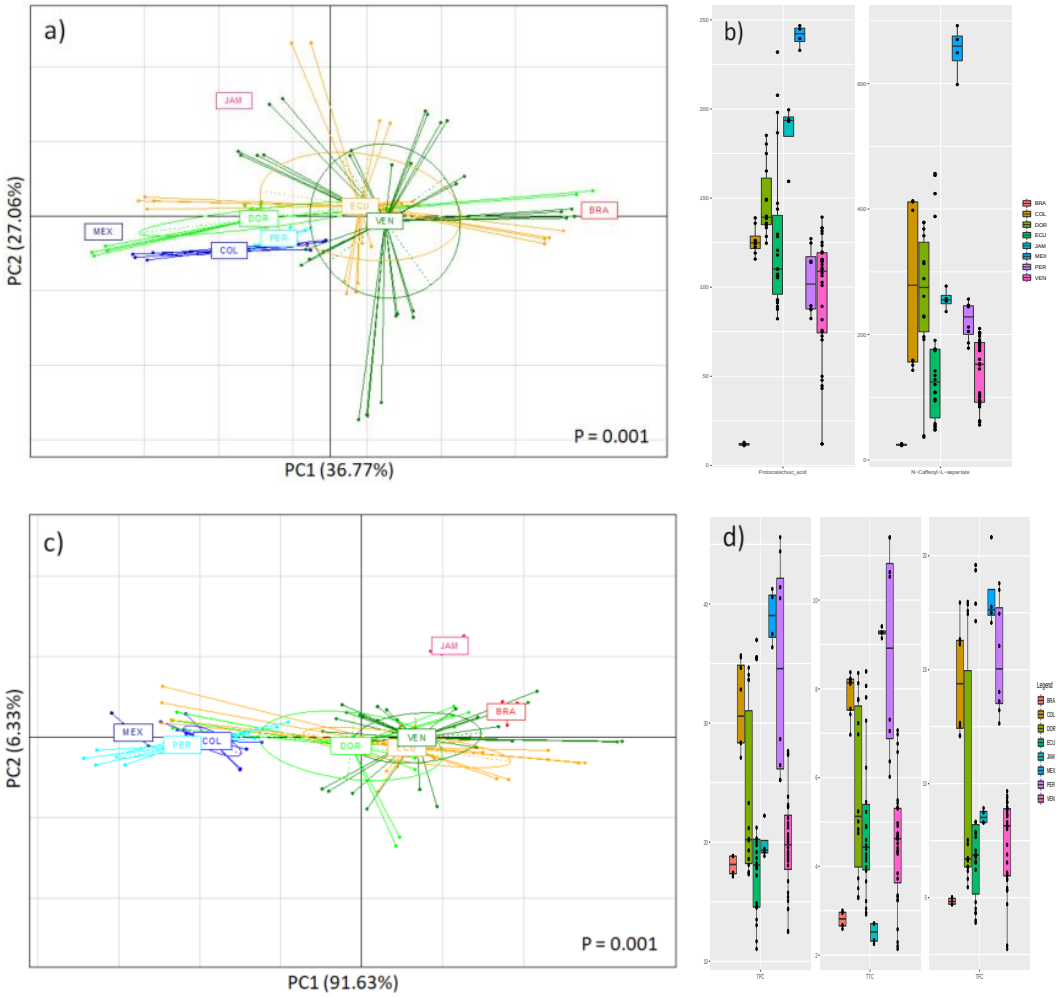

Figure 7. PCA based on the HPLC-determined polyphenolic profile (**a**) and spectrophotometric analyses results (**c**) as function of the country of origin among the American CBS. The variance explained by the first and second components of PCA is shown in parenthesis for PC1 and PC2, respectively, in each graph. Boxplots showing abundance of two key polyphenolic compounds (protocatechuic acid and N-caffeoyl-L-aspartate) (**b**) and TPC, TTC and TFC (**d**) that can be used as possible markers for the separation based in the country of origin are shown next to their correspondent PCA graph. For interpretation of the legends, see Table 1.

HPLC results allowed for a separation of the Mexican, Jamaican and Brazilian samples (at the left, upper center, and right sides, respectively) from the rest of the samples, which clustered together in the central part of the plot. All the HPLC-determined polyphenolic compounds (except for PCA pentoside 1) contributed significantly to the differentiation of samples among the American countries (Table 7).

Table 7. Pairwise comparison of the phenolic compounds quantified in CBSs by RP-HPLC-PDA that could be used as potential geographical origin markers for different countries within the American continent.

Protocatechuic_acid

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00665	-					
DOR	0.00203	0.00665	-				
ECU	0.00062	0.26582	0.01404	-			
JAM	0.03333	0.00665	0.0122	0.02765	-		
MEX	0.03333	0.00665	0.00203	0.00062	0.03333	-	
PER	0.00665	0.02765	0.00024	0.1995	0.00665	0.00665	-
VEN	0.00165	0.00665	2.10E-07	0.07636	0.00024	0.00024	0.77807

N-Coumaroyl-L-aspartate_1

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00943	-					
DOR	0.00305	0.97338	-				
ECU	0.00187	0.17333	0.04875	-			
JAM	0.04875	0.00943	0.00305	0.0025	-		
MEX	0.04875	0.00943	0.00305	0.18223	0.04875	-	
PER	0.00943	0.19012	0.07803	0.19012	0.00943	0.00943	-
VEN	0.03766	0.92114	0.97338	0.19012	0.00095	0.19012	0.55508

N-Caffeoyl-L-aspartate

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.0087	-					
DOR	0.00203	0.6903	-				
ECU	0.00062	0.05827	0.06292	-			
JAM	0.04706	1	0.85943	0.06404	-		
MEX	0.04706	0.0087	0.00203	0.00062	0.04706	-	
PER	0.0087	1	0.22838	0.01986	0.09256	0.0087	-
VEN	0.00032	0.05079	0.00089	0.63787	0.00032	0.00032	0.00059

Catechin

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01886	-					
DOR	0.0061	0.20529	-				
ECU	0.00187	0.15817	0.72262	-			
JAM	0.0834	1	0.37744	0.72262	-		
MEX	0.0834	1	0.15817	0.37744	0.0834	-	
PER	0.01886	0.37744	0.99113	0.72262	0.3527	0.01886	-
VEN	0.00095	0.0834	0.37744	0.79231	0.10236	0.10236	0.34485

PCA_hexoside_1

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00754	-					
DOR	0.00229	8.80E-05	-				
ECU	0.00075	0.03211	0.97258	-			
JAM	0.0381	0.00754	0.97258	0.97258	-		
MEX	0.0381	0.00754	0.00229	0.00075	0.0381	-	
PER	0.00754	0.27358	8.80E-05	0.02008	0.00754	0.00754	-
VEN	0.02008	0.0024	0.10165	0.05258	0.27726	0.00032	0.00108

Catechin-3-O-glucoside

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.93333	-					
DOR	0.06062	0.08262	-				
ECU	0.32061	0.24189	0.01049	-			
JAM	0.05333	0.01049	0.31024	0.00095	-		
MEX	0.05333	0.01049	0.00366	0.00095	0.05333	-	
PER	0.93333	0.77636	0.01486	0.2504	0.01049	0.01049	-
VEN	0.32745	0.25957	0.01049	0.44396	0.00095	0.00095	0.34186

Quercetin

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01131	-					
DOR	0.6695	0.37337	-				
ECU	1	0.05255	0.43574	-			
JAM	0.05714	0.46795	0.85943	0.24722	-		
MEX	0.05714	0.01131	0.00366	0.00094	0.05714	-	
PER	0.01131	0.34558	0.63934	0.09986	1	0.01131	-
VEN	0.72202	1.40E-05	0.09986	0.27939	0.00602	0.00032	5.00E-05

PCB_trimer_1

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	1	-					
DOR	0.61856	0.99113	-				
ECU	0.5183	0.34514	0.32418	-			
JAM	0.06154	0.01131	0.00305	0.00094	-		
MEX	0.06154	0.01131	0.00305	0.00094	0.06154	-	
PER	0.7596	1	0.32418	0.32418	0.01131	0.01131	-
VEN	0.92117	0.34514	0.88715	0.95717	0.00048	0.00048	0.91638

PCB_trimer_6

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01028	-					
DOR	0.00366	0.27795	-				
ECU	0.07819	0.02587	0.00576	-			
JAM	0.04706	0.01028	0.10488	0.86502	-		
MEX	0.04706	0.01028	0.00366	0.61052	0.04706	-	
PER	0.01028	1	0.27795	0.08913	0.9679	0.01028	-
VEN	0.08421	1.50E-06	2.10E-07	0.02673	0.01909	0.10488	0.00051

N-Coumaroyl-L-aspartate_2

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01028	-					
DOR	0.00261	0.01156	-				
ECU	0.00075	0.53208	0.06623	-			
JAM	0.05	0.01028	0.77637	0.06724	-		
MEX	0.05	0.83801	0.05471	0.12208	0.05	-	
PER	0.01028	0.19543	0.05471	0.05	0.01028	1	-
VEN	0.00032	0.11724	0.00037	0.38674	0.00032	0.00111	0.00025

PCB_trimer_2

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	1	-					
DOR	0.06062	0.16207	-				
ECU	0.50363	1	0.03645	-			
JAM	0.05333	0.01131	0.00366	0.00795	-		
MEX	0.05333	1	0.29614	0.22098	0.05333	-	
PER	1	0.16207	0.00293	0.19538	0.01131	0.01131	-
VEN	0.00795	0.02473	0.69453	0.00197	0.00048	0.1067	0.00048

PCB

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01257	-					
DOR	0.00458	0.51166	-				
ECU	0.00187	0.42694	0.14592	-			
JAM	0.05714	0.01257	0.00458	0.01257	-		
MEX	0.05714	0.6903	0.77637	0.42694	0.05714	-	
PER	0.01257	1	0.6903	0.6903	0.01257	1	-
VEN	0.00095	0.06671	0.03345	0.12914	0.02805	0.16992	0.14726

Epicatechin

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00808	-					
DOR	0.00261	0.00925	-				
ECU	0.00094	0.04	0.69001	-			
JAM	0.04	0.00808	0.02059	0.00749	-		
MEX	0.04	1	0.28326	0.00094	0.04	-	
PER	0.00808	0.61787	0.13997	0.34754	0.00808	0.00808	-
VEN	0.00048	0.00129	0.12347	0.00598	0.50432	0.00048	0.00129

N-Coumaroyl-L-glutamate

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.9333	-					
DOR	0.0693	0.1478	-				
ECU	0.2541	0.8241	0.0774	-			
JAM	0.0667	0.0323	0.8074	0.0693	-		
MEX	0.0667	0.0226	0.3258	0.0037	0.0667	-	
PER	0.0226	0.0827	0.7966	0.0827	0.7966	0.0226	-
VEN	0.4204	0.8601	0.0428	0.596	0.0455	0.0307	0.0226

PCB_trimer_3

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01616	-					
DOR	0.01165	8.80E-05	-				
ECU	0.53357	0.01616	0.03306	-			
JAM	0.14545	0.31677	0.00261	0.23941	-		
MEX	0.08421	0.0115	0.11072	0.4191	0.04444	-	
PER	0.5697	0.00653	0.00042	0.29905	0.14545	0.01616	-
VEN	0.0115	2.90E-06	0.03513	0.00024	0.00111	0.02275	0.00024

PCB_trimer_4

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00808	-					
DOR	0.00261	0.00053	-				
ECU	0.00075	1.30E-05	0.37312	-			
JAM	0.04211	0.00808	0.06497	0.00674	-		
MEX	0.04211	0.12727	0.07479	0.07338	0.04211	-	
PER	0.00808	0.06651	0.00285	0.00209	0.00808	0.30458	-
VEN	0.01753	7.30E-07	0.13302	0.01696	0.61007	0.00285	2.50E-06

PCB_trimer_5

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.0132	-					
DOR	1	0.0139	-				
ECU	0.1844	0.0015	0.0362	-			
JAM	0.0571	0.0132	0.0046	0.3603	-		
MEX	0.0571	1	0.3258	0.0015	0.0571	-	
PER	0.1273	0.0015	0.1969	0.3603	0.0132	0.0132	-
VEN	0.8427	0.0132	0.2646	0.4476	0.634	0.1209	0.9492

PCA_pentoside_1

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.057	-					
DOR	0.16	0.765	-				
ECU	0.224	0.801	0.627	-			
JAM	0.16	1	0.765	0.923	-		
MEX	0.16	1	0.984	0.627	0.229	-	
PER	0.057	0.765	0.308	0.976	1	0.255	-
VEN	0.627	0.627	0.303	0.627	0.765	0.529	0.765

PCB_trimer_7

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01187	-					
DOR	0.01187	0.87813	-				
ECU	0.46586	0.08768	0.04069	-			
JAM	0.05	0.01886	0.05705	0.87813	-		
MEX	0.05	0.01187	0.01187	0.00094	0.05	-	
PER	1	0.01187	0.00904	0.7123	0.9679	0.01187	-
VEN	0.6304	0.00092	0.00092	0.72581	0.84894	0.00092	0.84894

PCA_trimer_arabinoside

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.00943	-					
DOR	0.00985	0.76843	-				
ECU	0.10863	0.0251	0.07375	-			
JAM	0.04211	0.93333	0.49485	0.45875	-		
MEX	0.04211	0.00943	0.00432	0.00125	0.04211	-	
PER	0.00943	0.00746	0.03439	0.45875	0.00943	0.00943	-
VEN	0.00095	0.00432	0.0251	0.38929	0.00602	0.00095	0.8334

PCA_hexoside_2

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01616	-					
DOR	0.08818	0.6429	-				
ECU	0.71907	0.00946	0.08905	-			
JAM	0.08	0.01616	0.1958	0.65484	-		
MEX	0.08	1	0.36195	0.0025	0.08	-	
PER	1	0.1958	0.71907	0.44829	1	0.3527	-
VEN	1	3.30E-05	0.00681	0.38109	0.24848	0.00048	0.15059

N-coumaroyl-L-tyrosine

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.0087	-					
DOR	0.0087	0.00949	-				
ECU	0.00075	0.55401	0.01738	-			
JAM	0.04	0.0087	0.2606	0.00949	-		
MEX	0.04	0.0087	0.2606	0.00075	0.04	-	
PER	0.0087	0.30007	0.01114	0.2606	0.0087	0.0087	-
VEN	0.00032	0.2606	0.0087	0.72046	0.00032	0.00032	0.20455

PCA_pentoside_2

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01004	-					
DOR	0.18377	0.06688	-				
ECU	0.15324	0.00121	0.01004	-			
JAM	0.04444	0.01004	0.87843	0.02146	-		
MEX	0.04444	0.01616	0.01004	0.01004	0.04444	-	
PER	0.01004	0.01004	0.21011	0.00391	0.06688	0.01004	-
VEN	0.40767	0.00019	0.06677	0.27578	0.11771	0.00048	0.01004

Quercetin-3-O-glucoside

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.01257	-					
DOR	0.06062	0.49407	-				
ECU	0.00145	0.02897	0.08856	-			
JAM	0.05333	0.34431	0.54409	0.0791	-		
MEX	0.05333	0.34431	0.59511	0.12006	0.88571	-	
PER	0.01257	0.00145	0.01387	0.37358	0.01257	0.01257	-
VEN	0.00285	0.00096	0.00366	0.18373	0.01677	0.02844	0.16417

Quercetin-3-O-arabinoside

	BRA	COL	DOR	ECU	JAM	MEX	PER
COL	0.34431	-					
DOR	0.06466	0.63917	-				
ECU	0.43586	0.08272	0.1605	-			
JAM	0.05714	0.20733	0.29614	0.08272	-		
MEX	0.05714	0.01131	0.00366	0.00366	0.05714	-	
PER	0.68283	0.38291	0.02068	0.20733	0.01131	0.01131	-
VEN	0.08889	0.00366	0.00666	0.45917	0.00366	0.00095	0.01131

FDR (false discovery rate)<0.001; FDR<0.01 and FDR<0.05 are highlighted in green, yellow, and red, respectively.
Compounds highlighted in bold were used to build the box plots that are displayed in the main manuscript.

The boxplots obtained for the abundance differences of protocatechuic acid and N-caffeoyl-L-aspartate among American countries are shown in Figure 7b. These two compounds were found to be those with the highest significant differences among samples and can, therefore, be used as markers for the mentioned separations. Mexican samples showed to be those with the highest concentrations of both markers, appearing for this reason at the left side of the PCA plot. On the contrary, Brazilian samples were those with the lowest

marker concentrations, and appeared at the right side of the plot, separated from the rest of samples. Jamaican samples showed to possess high amounts of protocatechuic acid, but intermediate concentrations of N-caffeoyl-L-aspartate, while the rest of the samples showed contents of both compounds that varied proportionally. This, together with variations in concentrations of other compounds found in Table 7, could be the reason for the differentiation of Jamaican CBS samples. For the PCA of the spectrophotometric results, the CBS samples from Mexico, Jamaica, and Brazil were again differentiated from the others at the left, upper centre, and right sides, respectively. With this, an equivalence of the three screening methods together (TPC, TFC, and TTC) and the HPLC analysis was again demonstrated. In this case, the boxplots shown in Figure 7d suggest again that the Mexican samples were differentiated because of their high TPC, TFC, and TTC values while the Brazilian ones appeared differentiated at the right side of the correspondent PCA plot because of having the lowest spectrophotometric values. On the other hand, Jamaican samples were probably separated because of their unusual low values of TTC in comparison with their TPC and TFC values, while the rest of samples had values that varied proportionally among the three parameters.

3.3.2. Classification of CBS According to the Cultivar – Localized Case Study (Ecuador)

In this section, CBS data from one representative country of cocoa production (Ecuador), with one of the most representative numbers of samples among our sample set (n=7), and offering CBS samples belonging to the 4 varieties were used. The aim was to verify whether both HPLC and spectrophotometric assays were able to classify CBS samples among one same country depending on the cocoa variety. Although it was previously observed that variety differentiation was not possible when taking into account all the samples from our sample set or all the samples from one single continent, it was found that this differentiation was possible if samples from just one country were analyzed. The separation of CBS samples from Ecuador according to their varieties by both HPLC and spectrophotometric results PCA are shown in Figure 8a and 8c, respectively. The PCA plot built with the HPLC polyphenolic contents was significant (p = 0.002) and explained 70.59% of the total variance, while the PCA plot built from the spectrophotometric assays results was also significant, although in a smaller extent (p = 0.032), and explained 99.27% of the total variance. Both plots allowed for the separation of the four CBS varieties.

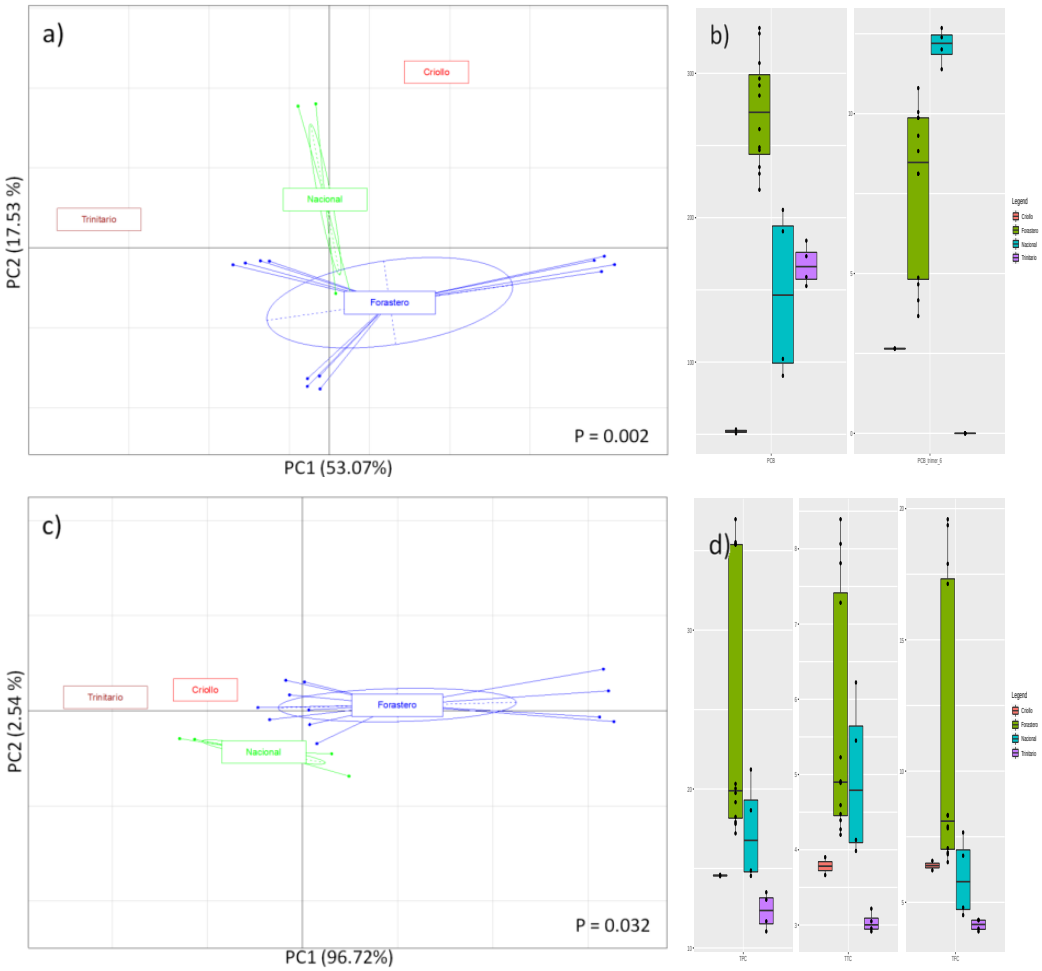

Figure 8. PCA based on the HPLC-determined polyphenolic profile (**a**) and spectrophotometric analyses results (**c**) as function of the CBS variety among the Ecuadorian CBS. The variance explained by the first and second components of PCA is shown in parenthesis for PC1 and PC2, respectively, in each graph. Boxplots showing abundance of two key polyphenolic compounds (PCB and PCB trimer 6) (**b**) and TPC, TTC and TFC (**d**) that can be used as possible markers for the separation based in the CBS variety are shown next to their correspondent PCA graph. For interpretation of the legends, see Table 1.

For the PCA built from the HPLC results, 18 out of the 25 determined compounds gave significant pairwise comparison among the four different CBS varieties (Table 8). Boxplots corresponding to PCB and PCB trimer 6 were chosen for representation for being the marker compounds that gave the highest number of significant differences among comparisons and for being compounds with high concentrations in the analyzed CBS (Figure 8b). On the one hand, PCB was found to be in high concentrations in the Ecuadorian Forastero CBSs, which allowed to differentiate this variety from the Criollo CBS that presented the lowest concentration of this compound. On the other hand, PCB trimer 6 allowed to separate the four varieties, being more present in the Nacional variety, followed by the Forastero, Criollo, and Trinitario CBS. Regarding the separation by spectrophotometric analysis results, the three studied parameters gave proportional results for the four CBS varieties (Figure 8d). These techniques allowed to separate the Forastero, Trinitario, and Criollo varieties, while the

Nacional variety clustered together with Forastero and Criollo in TPC, with Forastero in TTC, and with both Forastero and Criollo in TFC. The Nacional variety belongs to the Forastero genotype from a botanical point of view [227], which would explain the clusterization of these boxplots. Forastero CBSs seemed to be the ones with the highest polyphenol concentrations among the four Ecuadorian varieties, which confirms in this case the observations previously described for the variety of cocoa beans [81].

Table 8. Pairwise comparison of the phenolic compounds quantified in CBSs by RP-HPLC-PDA that could be used as potential markers for cultivars among Ecuadorian samples.

Protocatechuic_acid

	Criollo	Forastero	Nacional
Forastero	0.79	-	-
Nacional	0.57	0.57	-
Trinitario	0.57	0.57	0.89

N-Coumaroyl-L-aspartate_2

	Criollo	Forastero	Nacional
Forastero	0.66	-	-
Nacional	0.2	1	-
Trinitario	0.2	0.2	0.17

PCA_pentoside_1

	Criollo	Forastero	Nacional
Forastero	0.16	-	-
Nacional	0.16	1	-
Trinitario	0.16	0.0066	0.0857

N-Coumaroyl-L-aspartate_1

	Criollo	Forastero	Nacional
Forastero	0.13	-	-
Nacional	0.27	0.95	-
Trinitario	0.27	0.57	0.58

PCB_trimer_2

	Criollo	Forastero	Nacional
Forastero	0.044	-	-
Nacional	0.1203	0.013	-
Trinitario	0.1333	0.0066	0.0441

PCB_trimer_7

	Criollo	Forastero	Nacional
Forastero	0.6593	-	-
Nacional	0.2	0.0033	-
Trinitario	0.2	0.0033	1

N-Caffeoyl-L-aspartate

	Criollo	Forastero	Nacional
Forastero	0.5495	-	-
Nacional	0.16	0.1165	-
Trinitario	0.16	0.0066	0.0857

PCB

	Criollo	Forastero	Nacional
Forastero	0.044	-	-
Nacional	0.16	0.0033	-
Trinitario	0.16	0.0033	1

PCA_trimer_arabinoside

	Criollo	Forastero	Nacional
Forastero	0.82	-	-
Nacional	0.96	0.76	-
Trinitario	0.64	0.64	1

Catechin

	Criollo	Forastero	Nacional
Forastero	0.13	-	-
Nacional	0.16	0.16	-
Trinitario	0.16	0.16	0.49

Epicatechin

	Criollo	Forastero	Nacional
Forastero	0.8242	-	-
Nacional	1	1	-
Trinitario	0.2667	0.0066	0.0857

PCA_hexoside_2

	Criollo	Forastero	Nacional
Forastero	0.16	-	-
Nacional	0.16	0.5209	-
Trinitario	0.16	0.0066	0.0857

PCA_hexoside_1

	Criollo	Forastero	Nacional
Forastero	0.5495	-	-
Nacional	0.2	0.4549	-
Trinitario	0.2	0.0066	0.0857

N-Coumaroyl-L-glutamate

	Criollo	Forastero	Nacional
Forastero	0.5275	-	-
Nacional	0.8	0.1165	-
Trinitario	0.2	0.0066	0.0857

N-coumaroyl-L-tyrosine

	Criollo	Forastero	Nacional
Forastero	0.8	-	-
Nacional	0.8	0.8	-
Trinitario	0.2667	0.0066	0.0857

Catechin-3-O-glucoside

	Criollo	Forastero	Nacional
Forastero	0.6593	-	-
Nacional	0.2	0.9527	-
Trinitario	0.2	0.0066	0.0857

PCB_trimer_3

	Criollo	Forastero	Nacional
Forastero	0.0659	-	-
Nacional	1	0.5687	-
Trinitario	0.2667	0.0066	1

PCA_pentoside_2

	Criollo	Forastero	Nacional
Forastero	0.044	-	-
Nacional	0.133	0.124	-
Trinitario	0.083	0.025	0.044

Quercetin

	Criollo	Forastero	Nacional
Forastero	0.64	-	-
Nacional	0.64	0.95	-
Trinitario	0.64	0.64	0.64

PCB_trimer_4

	Criollo	Forastero	Nacional
Forastero	0.659	-	-
Nacional	0.2	0.204	-
Trinitario	0.2	0.026	0.086

Quercetin-3-O-glucoside

	Criollo	Forastero	Nacional
Forastero	0.6593	-	-
Nacional	0.6593	0.8615	-
Trinitario	0.2667	0.0066	0.0857

PCB_trimer_1

	Criollo	Forastero	Nacional
Forastero	0.5495	-	-
Nacional	0.2	0.4549	-
Trinitario	0.2	0.0066	0.0857

PCB_trimer_5

	Criollo	Forastero	Nacional
Forastero	0.066	-	-
Nacional	1	0.066	-
Trinitario	0.2	0.156	0.583

Quercetin-3-O-arabinoside

	Criollo	Forastero	Nacional
Forastero	0.5495	-	-
Nacional	0.2	0.5495	-
Trinitario	0.2	0.0066	0.0857

PCB_trimer_6

	Criollo	Forastero	Nacional
Forastero	0.033	-	-
Nacional	0.1333	0.0066	-
Trinitario	0.0663	0.0123	0.033

FDR (false discovery rate)<0.001; FDR<0.01 and FDR<0.05 are highlighted in green, yellow, and red, respectively. Compounds highlighted in bold were used to build the box plots that are displayed in the main manuscript.

3.4. Classification of CBS Based on Their Methylxanthine Ratio According to Variety

Methylxanthine content in cocoa beans, and concretely the ratio between theobromine and caffeine contents, have been previously reported to be dependent on the cocoa variety. Studies carried out by Davrieux et al. [248] reported a classification of cocoa samples into Forastero, Trinitario, and Criollo genotypes depending on the methylxanthine ratio determined by near infrared spectroscopy. After that, other works concerning the study of cocoa bean methylxanthine content through HPLC [226,236] and HPTLC [239] have reported similar classifications of cocoa varieties when building a graph in which theobromine/caffeine concentration ratio is represented in axe Y and the percentage of caffeine content in the sample is represented in axe X. A similar graph was built in the present study with the results of the methylxanthine content obtained for the different CBS samples and is shown in Figure 9.

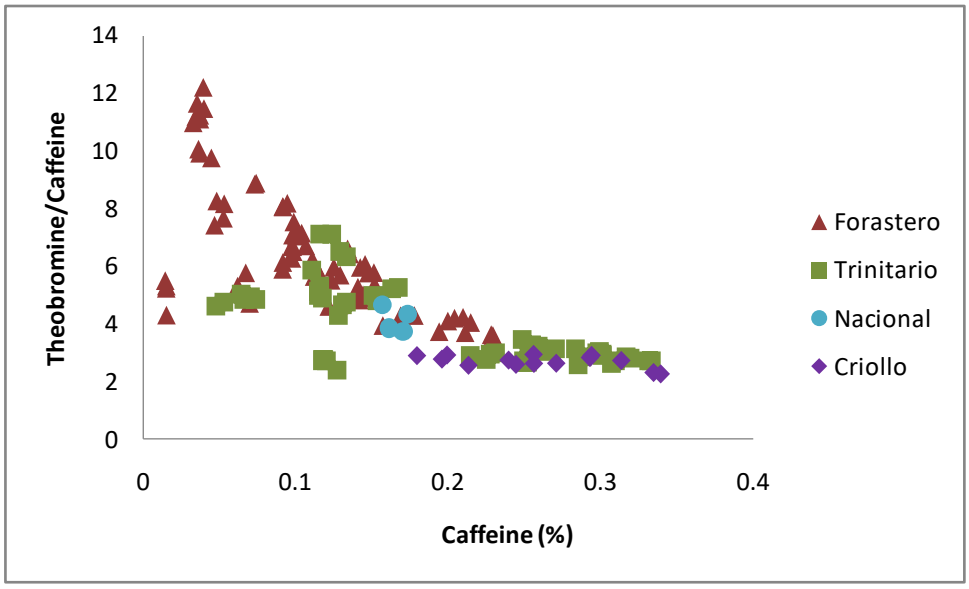

Figure 9. Relationship between methylxanthine content in CBS and CBS variety.

For the first time for the cocoa by-product, a variety distribution depending on the methylxanthine content is reported. This distribution is similar to the one observed before for cocoa beans and allow to classify the different CBS varieties. In a general line, Forastero samples seemed to have high concentrations of theobromine and low concentrations of caffeine, which situated these samples in the upper left corner of the graph. On the other hand, Criollo CBS presented the opposite characteristics; they had lower content of theobromine and higher content of caffeine, appearing in the lower right corner of the graph. Trinitario genotype is a hybrid from the two previously mentioned varieties [227], and samples belonging to this group appeared in an intermediate position of the graph. Finally, as it has been previously mentioned, Nacional variety belongs to the Forastero group from a botanical

point of view [227] and CBS samples belonging to Nacional variety were therefore found together with the Forastero group.

The average theobromine/caffeine ratios for the CBS samples were 6.45, 4.15, 3.88, and 2.69 for Forastero, Nacional, Trinitario, and Criollo varieties, respectively. These values are in accordance to those obtained by Davrieux et al. [248] for cocoa beans (7.3 for Forastero, 4.0 and 3.6 for Trinitario, and 1.5 for Criollo variety). While the HPLC-determined polyphenolic content did not allow for a separation of the complete sample set CBSs depending on their varieties, this finding could allow for a rapid variety classification of CBS depending on their HPLC-determined theobromine and caffeine content.

4. Conclusions

This study provides information about the polyphenolic profile of CBS, obtained by both RP-HPLC-PDA Analysis and Spectrophotometric Assays for a large set of samples. Different cocoa polyphenolic markers were individualized thanks to the HPLC analysis, such as catechin, epicatechin, procyanidins, quercetin glycosides, and N-phenylpropenoyl-L-aminoacids, among others. CBS was therefore demonstrated to have a similar polyphenolic profile to that of cocoa beans. Generally, CBS polyphenolic concentrations were lower than those found for cocoa beans. Some compounds such as monomeric flavanols and N-phenylpropenoyl-L-aminoacids were found in CBS in concentrations that were an order of magnitude below those of cocoa powder. Though, other polyphenolic compounds such as protocatechuic acid were found in higher concentrations in CBS than in cocoa powder. Individual polyphenolic compounds concentration showed high variability among the CBS samples set in the present study, which was related to the different geographic origin of samples (different cultivation traditions and climates) rather than their different genotypes.

Spectrophotometric analyses of the CBS polyphenolic content, concretely TPC, TFC, TFC, and RSA demonstrated to be powerful tools for polyphenol content comparison among samples. The spectrophotometric results showed to be highly correlated to the HPLC-determined results. However, these parameters highly overestimated polyphenol content in CBS and should therefore be used with precaution, and preferably always supported by chromatographic techniques that allow to establish structure-activity relationships. In this frame, it was also found that the obtained values of RSA (antioxidant capacity) were highly correlated with the HPLC-determined total polyphenolic content and, concretely, PCA glycosides and PCB trimers seemed to be the compound groups contributing the most to this parameter.

Chemometric analysis of the CBS polyphenolic contents through PCA did not allow for CBS sample differentiation according to genotype when taking into account our whole CBS sample set. Nevertheless, it was possible to differentiate CBS samples according to their continent of origin (America or Africa). This was possible thanks to some marker compounds such as catechin and PCB trimer 6 that were significantly more present in American samples than in African samples. The same separation was also obtained with the spectrophotometric results, with American CBSs presenting higher values of TPC, TFC, and TTC than African samples. This finding has a great utility for CBS origin traceability from an instrumental point of view since similar differentiation results can be obtained by using screening, rapid, and easy to handle methods such the spectrophotometric assays instead of more sophisticated methods such as chromatography techniques. On the other hand, marker compounds such as epicatechin and PCB trimer 4 allowed for a differentiation among African CBS according to the country of origin, whereas protocatechuic acid and N-caffeoyl-L-aspartate served for the classification of American CBS according to the country of origin. In these latest cases, similar differentiation results could again be obtained by using spectrophotometric techniques.

Finally, it was found that the classification of the whole CBS sample set according to their genotype, which could not be obtained with the polyphenolic profile, could be achieved by analyzing their theobromine/caffeine ratio. This ratio showed to be the highest for Forastero CBS, followed by Nacional and Trinitario, and with Criollo being the genotype with the lowest theobromine/caffeine ratio. This finding has been previously reported for cocoa beans and, for the first time, the equivalent has been successfully applied for CBS in the present study.

CBS has been recently proposed as a high-added value ingredient for functional foods and, therefore, its polyphenolic and methylxanthine contents are important parameters to this end. The present study demonstrates that CBS is an interesting ingredient to yield polyphenolic and methylxanthine rich products by being recycled inside the cocoa manufacturing industry in the frame of a circular economy and offers interesting tools for the selection of the by-product in this context.

CHAPTER 1.2

Assessment of Volatile Fingerprint by HS-SPME/GC-qMS and E-nose for the Classification of Cocoa Bean Shells Using Chemometrics

Chapter 1.2 is devoted to the determination of the aromatic (volatile organic compounds, VOCs) profile of CBS. To achieve this, a similar experimental design to that presented in the previous chapter (Chapter 1.1) was followed. A large CBS sample set was analyzed in order to obtain their VOC composition and content by means of two different techniques: HS-SPME/GC-qMS, which was considered as a complete characterization method, and electronic nose (E-nose), which was considered as a rapid screening method. In this case, the authentication strategy was also proposed basing on the obtained results for both methods. Besides, the results obtained in this chapter served to confirm the cocoa-similar aroma profile of CBS, which would allow to propose its use in the functional food preparation that will be presented later in Part 2 of the results.

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Assessment of Volatile Fingerprint by HS-SPME/GC-qMS and E-nose for the Classification of Cocoa Bean Shells Using Chemometrics

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1. Introduction

The cocoa bean (*Theobroma cacao* L.) is a ubiquitous edible product, consumed across the world, of great economic significance, and the key raw material in chocolate manufacturing [249]. According to the International Cocoa Organization, theworld production of cocoa beans reached 4.7 million tonnes in the 2016-2017 season, and the major producers were the West African countries, Ivory Coast and Ghana, and countries located in other tropical areas, like Central and South America (Brazil and Ecuador) or Southern Asia [250].

The world cocoa market typically separates cocoa beans into two main categories according to their flavour, namely bulk or basic cocoa and fine or flavour cocoa [251]. Bulk cocoa is mainly produced from the *Forastero* cultivar, which has ordinary flavour properties and makes up 95% of the world's total cocoa production. Fine grade cocoa is exclusively produced from *Criollo*, *Trinitario*, and *Nacional* cultivars, the latter is grown in Ecuador and is characterised by its remarkable flavour properties, due to the fruity and floral aroma attributes [249,252]. Even though almost all the cocoa cultivated worldwide is *Forastero*, differences can be found in the flavour profiles of cocoa-derived products produced with cocoa beans from different geographical origins [253-255]. Indeed, the quality and flavour of cocoa are not simply affected by genotype and geographical origin but also by other factors, such as growth conditions, post-harvest treatments, and industrial processing of beans [256]. In particular, fermentation and roasting are the key steps responsible for the characteristics and desirable organoleptic properties of cocoa, such as aroma and flavour, that are important quality attributes for consumer acceptability [251,252].

To date, several hundreds of volatile organic compounds (VOCs) have been reported to characterise the cocoa aroma, mainly represented by pyrazines, aldehydes, ketones, alcohols, esters, furans, acids, pyrroles, phenols, and terpenes [251]. Some of these molecules might be used as key indicators to certify the quality and enable the discrimination of the cocoa products with label of origin to ensure food authentication, a new market trend of great interest for law enforcement, food producers, importers and exporters, and consumers [223,253].

The solid-phase microextraction (SPME) coupled with gas chromatography (GC) mass spectrometry (MS) methodology has been widely used to identify and quantify VOCs and, more recently, in combination with multivariate analysis to consent the classification and discrimination of cocoa and cocoa-related products for the traceability of such products [253-255,257]. However, a limited number of studies have explored the potential applicability of

electronic nose technologies to assess cocoa quality and origin [258,259]. To the best of our knowledge, no studies are available in literature that explore and compare the potential applicability of both techniques for the classification of cocoa and related products.

Similar to other agro-food sectors, the cocoa industry also produces large amounts of by-products during manufacturing. The cocoa bean shell (CBS) is one of the main by-products generated after the roasting and husking of cocoa beans (about 12% of the total weight), and consequently more than 500 thousand tonnes are produced every year that represent a disposal problem for the cocoa sector. However, recent studies have established that the CBS might also be an economic source of fibre, minerals, polyphenols, and methylxanthines with potential health benefits and, therefore, with great potential to be used as an ingredient for functional foods, creating new food market perspectives [57,77,112,260]. Wang et al. [261] patented a process for chocolate flavour production, with a real chocolate aroma, from dried CBSs using an enzymatic technology. Nevertheless, despite this product exhibiting great potential as a food ingredient, to the best of our knowledge, no information is available in the literature describing the volatile composition of CBSs. As for cocoa beans, the study of the CBS volatile fingerprint is very important to define the quality and flavour of the product. Moreover, selecting the CBS with a specific flavour profile according to the food application could be an approach to valorise this by-product as a food ingredient. Hence, the aim of this study was to describe, for the first time, the volatile fingerprint of the CBS by HS-SPME/GC-qMS and determine the volatile compounds responsible for differences among several CBSs yielded from cocoa beans collected in different geographical origins and cultivars to allow for the traceability of this material. Moreover, we also explored the applicability of E-nose as a rapid methodology for the classification of the CBS and evaluated the correlation between E-nose and GC-qMS data sets.

2. Materials and Methods

2.1. Chemicals and Standards

Methanol (≥99.9%), sodium chloride (≥99%), sodium hydroxide standard solution (0.1001mol L^{-1}), and n-alkanes (n-C7–n-C30) mix standard (Supelco, Italy) for retention index determination were obtained from Sigma-Aldrich (Milano, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

The internal standard (ISTD), 5-nonanol (≥95% GC), for analyte response normalisation was provided by Sigma-Aldrich (Milano, Italy). A standard stock solution of 5-nonanol was

prepared in ultrapure water at a 10 mgL^{-1} concentration for the semi-quantification and stored in a sealed vial at -20 ºC.

2.2. CBS Samples

CBS samples were obtained as described in section 2.2 of Chapter 1.1. Samples were stored under a vacuum at −20 °C before sample preparation and headspace analysis. The humidity content of the CBS samples ranged between 5.46% and 9.22% as reported by [29].

2.3. HS-SPME/GC-qMS analysis

The VOCs from the CBS samples were identified and analysed using a headspace solid phase micro extraction (HS-SPME) coupled with gas chromatography/quadrupole mass spectrometry (GC/qMS).

2.3.1. HS-SPME/ GC-qMS conditions

For the extraction of VOCs, 0.1 g of CBS powder was accurately weighed in a 20 mL headspace vial. Then, 2 mL of sodium chloride (40% *w*/*v*) and 10 µL of internal standard (IS) 5-nonanol (10 µg/mL) were added to the sample, and the vial was immediately hermetically capped with a PTFE-silicon septum. The extraction was performed in a COMBI PAL System Autosampler for SPME (CTC Analytics AG, Zwingen, Switzerland) equipped with an HS-SPME unit. The sample was equilibrated at 60 ºC with stirring at 250 rpm for 10 min to reach equilibrium. Next, a well-conditioned SPME fibre coated with divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) (d_f 50/30 µm, 1 cm) (Supelco, Bellefonte, PA, USA) was exposed to the headspace of the sample for another 30 min with continuous heating and agitation. After extraction, the fibre was inserted into the injection port of the GC system in splitless mode and desorbed at 260 °C for 2 min. Three identical samples were prepared for each analysis.

GC-qMS analyses were performed on a Shimadzu GC-2010 gas chromatograph equipped with a Shimadzu QP-2010 Plus quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). Separation of VOCs was performed on a DB-WAXETR capillary column of 30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness (J&W Scientific Inc., Folsom, CA, USA). The oven time-temperature programme was as follows: initial temperature 40 °C held for 5 min, from 40ºC to 180°C at the rate of 5 °C min^{-1}, and then to 240°C at the rate of 10°C min^{-1}, which was held for 5 min. The carrier gas was helium at a

constant flow of 1 ml min^{-1} with the splitless GC inlet mode. GC inlet and transfer lines were set at 260 and 240 °C, respectively. The MS fragmentation was performed by electron impact ionization mode (70 eV), and the temperature of the ion source and quadrupole was 240°C. The data were recorded in full-scan mode in the mass acquisition range of 30–450 *m/z* with 0.30 s scan time.

2.3.2. Qualitative and quantitative analysis

The identification of the volatile organic compounds, focused on 101 molecules described in Table 1, was performed by comparing the EI-MS fragmentation pattern of each compound with those available on the National Institute of Standards and Technology (NIST05) mass-spectral library and on our home-based library. The compounds in trace whose similarity was more than 75% were considered (tentative identification) even if higher similarity was achieved for the identified compounds. Additionally, the confirmation of molecule identity was performed by comparing the gas chromatographic retention indices (RI) of volatile compounds, determined after injection of a series of *n*-alkane homologues (C7–C30) under the same GC-qMS analytical conditions described above, with literature data. The semi-quantitative concentrations of the VOCs identified were calculated as the area of the volatile marker component divided by the response factor of the ISTD 5-nonanoland expressed as micrograms of 5-nonanol equivalents per kg of sample (μg 5-nonanol Eq. kg^{-1} of CBS). Data were acquired and analysed by using GC-qMS Solution Workstation software (version 4.3) (GC-qMS Solution, Shimadzu Corporation, Kyoto, Japan).

2.4. E-nose analysis

E-nose analyses were performed using a portable electronic nose system PEN3 (Airsense Analytics GmbH., Germany). The system consists of a sampling unit and the gas detection system composed of 10 Metal Oxide Semiconductor (MOS) sensors, which are differentially sensitive to each characteristic volatile compound as follows: S1, aromatic; S2, broad range; S3, aromatic; S4, hydrogen; S5, aromatic andaliphatics; S6, broad range and methane; S7, sulfurorganic; S8, broad-range alcohol; S9, sulphur and chlorinate; and S10, methane and aliphatics [262]. These sensors are made up of a thin sheet of a certain metal oxide semiconductor. After exposure, a change in the conductance of the material takes place. This difference in the conductance is used to characterize the volatile substance.

For the analysis, 2 g of CBS powder was placed in a 20-mL glass vial and capped with a PTFE septum. Then, each vial was incubated at 30ºC for 30 min to reach the headspace

equilibrium. The gas headspace was injected into the E-nose carried by air for 90 s at a constant flow rate of 400 mL min^{-1}, and during this time, the sensor signals were recorded at each second. After each analysis, the sensor system was purged with filtered air for 120 s, to allow reestablishment of the instrument baseline prior to the next sample injection. The sensor response, G/G_0 (G and G_0 stand for the conductance of the MOS connected with the sample and clean gas, respectively), is expressed as resistivity (Ohm) and changed accordingly to the composition of volatile compounds. Data were collected by the pattern recognition software (WinMuster, v.1.6., Airsense Analytics GmbH., Germany). Three replicates of each CBS sample were independently analysed, and the average of sensor responses (area under the curve) was used for the subsequent statistical analysis.

2.5. Chemometric analysis

A total of 44 CBS samples obtained from the continents Africa and America were used to perform chemometric analysis. From these 44 samples, two batches of cocoa beans provided from the same producer were available (see Table 1, Chapter 1.1). All samples were analysed in triplicate with a final number of 264 analyses for each methodology used (GC-qMS (101 VOCs each) and E-nose (10 sensors each)). To discriminate the CBS samples as a function of geographical origin of production or cultivar principal component analysis (PCA) based on the normalized data (log10) was performed by using the *made4* package of R (https://www.r-project.org) and the function *dudi.pca.* Analysis of similarity based on the VOCs and E-nose table was applied with 999 permutations to detect significant differences as a function of the continent, macroarea, latitude, country of production, or cultivar, by using the anosim function in *vegan* package of R. Non-parametric Kruskal-Wallis as well as Wilcoxon tests were carried out in order to find VOCs differentially abundant between all the variables. Data were visualized as box plots, which represented the interquartile range between the first and the third quartiles, with the error bars showing the lowest and the highest values. Pairwise Spearman's non-parametric correlations (*corr.test* function in *psych* package of R) were used to study the relationships between VOCs and sensors. The correlation plots were visualized in R using the *made4* package of R. P-values were adjusted for multiple testing and a false discovery rate (FDR) < 0.05 or lower was considered as statistically significant.

3. Results and Discussion

The study of volatile constituents (VOCs) of the CBS is very important to define the quality and flavour of the product to be used as a food ingredient. The present study was divided into two main parts: the first one was dedicated to the analyses of all samples using GC-qMS and E-nose to define the volatile profile and fingerprint of CBS, followed by the classification of samples, using PCA analysis, and the identification of key compounds that differentiate the samples classes.

3.1. Volatile profile of the CBS characterized by HS-SPME/GC-qMS

The volatile components of CBS samples extracted and identified by HS-SPME/GC-qMS are described in Table 1. Each compound (VOC) is characterised by its retention index (RI), odour description as reported in literature, and the different semi-quantitative concentration ranges determined in the group of samples analysed. A total of 101 compounds, comprising aldehydes, ketones, sulphur compounds, esters, hydrocarbons, furans, pyrazines, alcohols, pyrroles, terpenes, isoprenoids and terpene alcohols, acids, lactones, and others were semi-quantified as μg kg^{-1} of 5-nonanol equivalents. The the sum of each class of compound, and the total amount of VOCs presented in a single sample are shown in detail in Table 2 described by Barbosa-Pereira et al. [29] (Chapter 1.2.1) .The total amount of VOCs ranged between 4.92 μg g^{-1} (VEN3) and 16.10 μg g^{-1} (VEN10), both from Venezuela, and these concentrations represent 10–20% of that total amount described by Tran et al. [255] for roasted cocoa beans (20.6 to 142.5 μg g^{-1}). In general, the most representative classes of compounds in the CBS were aldehydes (35.8%), pyrazines (18.7%), acids (11.0%), alcohols (7.9%), ketones (7.7%), and furan derivates (6.4%). The process of roasting has a great impact on cocoa aroma, and the alkyl pyrazines and Strecker aldehydes increased significantly at this stage in cocoa and consequently in the CBS, which is a main by-product produced during this stage. This distribution is slightly different from that found in literature for roasted cocoa beans, which presented acids and alcohols as the main compounds at high concentrations, or for roasted cocoa liquor, which displayed higher amounts of aldehydes, alcohols, and ketones [255,257,263]. However, the amounts of the several classes of compounds change with the cultivar and geographical origin of the cocoa bean [255,264].

In general, the CBSs from *Trinitario*, *Criollo*, and *Nacional* cocoa cultivars are those with the higher amounts of VOCs compared with those of the *Forastero* group. *Criollo* and *Nacional* cultivars display, on average, higher amounts of pyrazines, acids, alcohols, and

ketones than the *Trinitario* and *Forastero* cultivars. This data is in accordance with that found in literature for cocoa beans [265].

Since no data are available in the literature, the results of the present work will be discussed by comparing with studies performed for roasted cocoa beans and cocoa products, such as dark chocolate and cocoa powder, described in numerous reports [255,264,266-268].

Aldehydes were the most representative aroma compounds in the CBS with total amounts up to 5123.89 µg kg^{-1} quantified in samples yielded from cocoa beans from Dominican Republic (DOR1),with similar or higher amounts than that found in roasted cocoa beans (1.22–3.84 µg g^{-1}) [255]. Among aldehydes, 2-methylpropanal, 3-methylbutanal, nonanal, benzaldehyde, and phenylacetaldehyde were the most abundant in the CBS, as in cocoa beans [255,264]. The Strecker aldehydes, 2-methylpropanal, 3-methylbutanal, and phenylacetaldehyde, formed during fermentation and roasting processes, are described in the literature as flavour-active compounds and as key-aroma markers having a strong chocolate character with malty and buttery notes for the first two compounds and pleasant honey-like and nutty notes for phenylacetaldehyde [268]. Other aldehydes identified in CBSs, such as 2-phenyl-2-butenal, nonanal, 5-methyl-2-phenyl-2-hexenal, and 2-isopropyl-5-methyl-hex-2-enal (isomers 1and 2), have also been described as contributors to the cocoa odour and quality of final products conferring cocoa and fruity notes [264,266,267].

Pyrazines were one of the most representative groups of VOCs present in CBSs with concentrations up to 5285.68 µg kg^{-1} for the Venezuela (VEN9) samples, as observed in several cocoa beans. In this study, 2,3,5,6-tetramethylpyrazine was the most abundant pyrazine in the CBS that represented more than 50% of the total amount of pyrazines present in all CBS samples. 2,3,5,6-Tetramethylpyrazine is one of the main components of CBS aroma that exhibits chocolate flavour notes as described in the literature for dark chocolate [268]. Other pyrazines identified in the CBS were 2,3,5-trimethylpyrazine, 2,3-dimethyl-5-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2-methylpyrazine, and 2,6-dimethylpyrazine. All these compounds, derived from Maillard reactions, are characteristic and responsible for the cocoa aroma, providing the CBS samples with the essential notes of cocoa. 2,3,5-Trimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine are recognised as key-aroma compounds for cocoa and cocoa products and therefore also for the CBS [269,270]. A tentative identification was also performed for the pyrazine 2,5-dimethyl-3-isopentylpyrazine present insignificant amounts, which is described for the first time in this work for cocoa products.

Table 1. Volatile compounds (VOCs) identified in cocoa bean shell (CBS) powders by HS-SPME/GC-qMS with their experimental retention index (RI_{exp}), odour description and concentration range (µg kg^{-1})

Peak no.	VOC [a]	RI_{Exp}	Odour description[b]	Concentration range (µg kg^{-1})
Aldehydes				
1	**2-Methylpropanal**	<1000	Cocoa, chocolate, malty, roasted	55.87 - 470.62
2	**3-Methylbutanal**	<1000	Cocoa, chocolate, malty, roasted	100.97 - 891.32
3	Hexanal	1085	Green, fermented	26.26 - 321.47
4	Heptanal	1193	Green, Oily, fatty	1.64 - 174.35
5	Octanal	1298	Orange peel, oily, fatty, soapy	6.86 - 160.77
6	2-Isopropyl-5-methyl-hex-2-enal (isomer 1)	1369	Cocoa	11.99 - 357.04
7	2-Isopropyl-5-methyl-hex-2-enal (isomer 2)	1369	Cocoa	4.40 - 65.16
8	Nonanal	1405	Tallowy, soapy-fruity, fatty, waxy, pungent	40.31 - 1420.63
9	(*E*)-2-Octenal	1440	Fatty, waxy	4.13 - 29.61
10	Decanal	1511	Sweet, orange, waxy	1.49 - 56.53
11	Benzaldehyde	1536	Bitter, almond-like, burnt sugar, grass, earthy	243.98 - 1318.30
12	(*E*)-2-Nonenal	1548	Green	2.77 - 21.83
13	**Phenylacetaldehyde**	1658	Flowery, honey, sweet, rose, green, berry, nutty	260.13 - 2331.19
14	2-Phenyl-2-butenal	1898	Flowery, sweet, cocoa, roasted, rum	5.46 - 83.31
15	5-Methyl-2-phenyl-2-hexenal	2078	Sweet, roasted cocoa	0.04 - 113.09
Ketones				
16	2,3-Butanedione	<1000	Buttery, sweet	97.77 - 556.04
17	2-Heptanone	1189	Fruity, banana-like, green	29.31 - 362.87
18	6-Methyl-5-heptene-2-one	1348	Pungent, green	12.91 - 111.32
19	2-Nonanone	1399	Flowery, fatty	30.42 - 293.89
20	2-Decanone*	1505	Floral	8.22 - 109.41
21	3-Methyl-2-cyclohexen-1-one*	1605	Sweet, nutty	0.00 - 84.86
22	2-Undecanone*	1611	Waxy, fruity	3.07 - 67.13
23	Acetophenone	1665	Floral, sweet, almond, must, pungent	22.28 - 169.75
24	Phenylacetone*	1739	Almond	0.64 - 9.90
Sulfur compounds				
25	Dimethyl disulfide	1074	Rubbery, onion, meaty, sulphurous	5.52 - 291.23
26	**Dimethyl trisulfide**	1389	Onion, cabbage, sweaty, sulphurous	3.86 - 284.33
27	3-Phenylthiophene*	2126	–	0.12 - 10.96
28	2,3-Dihydrothiophene*	2333	–	0.35 - 11.82
Esters				
29	3-Methylbutyl acetate	1131	Banana, pear, sweet, fruity	6.57 - 449.32
30	Ethyl octanoate	1447	Fruity, floral	1.51 - 35.33
31	Methyl 2-phenylacetate	1774	Honey, sweet, jasmine	9.43 - 124.49
32	Ethylbenzeneacetate	1800	Floral, honey, rose, fruity, sweet, green, cucumber	2.21 - 63.97
33	**2-Phenylethyl acetate**	1829	Honey, sweet, flowery	1.69 - 506.70
34	Phenylethyl butyrate*	1877	Musty, sweet, floral	0.00 - 7.15

Peak no.	VOC [a]	RI_{Exp}	Odour description[b]	Concentration range (µg kg^{-1})
35	2-Methylpropyl benzoate	2045	Fruity, balsamic	0.25 - 6.45
36	Methyl hexadecanoate	2210	Fruity	0.96 - 11.41
Hydrocarbons				
37	Dodecane	1203	–	7.29 - 1105.30
38	3-Phenylpentane*****	1710	–	0.54 - 26.72
Furan derivates				
39	2-*n*-Pentylfuran	1241	Musty, green bean	21.69 - 409.93
40	Furfural	1478	Almond, caramel, sweet, woody, flowery, green	18.97 - 3911.55
41	Acetylfuran	1518	Sweet, balsamic, cocoa, green, grassy	4.71 - 191.66
Pyrazines				
42	2-Methylpyrazine	1273	Cocoa, chocolate, roasted nuts, green, hazelnut	11.49 - 200.16
43	2,6-Dimethylpyrazine	1334	Nutty, coffee, green, roasted, earthy	12.25 - 107.00
44	2-Ethylpyrazine	1341	Peanut-butter, musty, nutty, rum, earthy	7.77 - 57.30
45	2,3-Dimethylpyrazine	1352	Caramel, cocoa, sweet, baked, hazelnut, roasted, earthy	11.98 - 310.35
46	2-Ethyl-6-methylpyrazine	1393	Potato, earthy, halznut, cocoa, roasted, green	6.91 - 891.63
47	**2,3,5-Trimethylpyrazine**	1411	Cocoa, roasted nuts, peanut, earthy, green	41.66 - 891.63
48	**2-Ethyl-3,5-dimethylpyrazine**	1443	Earthy	1.66 - 106.10
49	2,5-Dimethyl-3-ethylpyrazine	1453	Potato, roast, earthy	3.84 - 309.79
50	2,3-Dimethyl-5-ethylpyrazine	1470	Cocoa, chocolate, praline	8.51 - 316.88
51	2,3,5,6-Tetramethylpyrazine	1482	Chocolate, cocoa, roasted, green, earthy	69.38 - 3298.06
52	2-Isobutyl-3-methylpyrazine	1497	Caramellic	0.68 - 32.66
53	2,3,5-Trimethyl-6-ethylpyrazine	1522	Candy, sweet, chocolate, cocoa, hazelnut, roasted	2.59 - 383.29
54	3,5-Dimethyl-2-isobutylpyrazine*	1543	Cocoa, hazelnut, musty, earthy, roasted, nutty	0.26 - 12.26
55	3-Methylbutylpyrazine*	1592	–	0.47 - 13.27
56	2-Isobutyl-3,5,6-trimethylpyrazine*	1602	–	0.40 - 43.65
57	2-Isoamyl-6-methylpyrazine*	1614	–	1.62 - 94.77
58	2,5-Dimethyl-3-isopentylpyrazine*	1669	–	0.65 - 321.09
59	2,3,5-Trimethyl-6-(2-methylbutyl)-pyrazine*	1682	–	0.00 - 24.12
60	2,5-Dimethyl-3-(1-methylpropyl)-pyrazine*	1710	–	0.00 - 74.33
61	2,3,5-Trimethyl-6-isopentylpyrazine*	1714	Green, floral	0.39 - 109.62
62	2-Acetyl-3,5-dimethylpyrazine*	1777	Nutty, roasted hazelnut	0.18 - 4.90
Alcohols				
63	**2-Heptanol**	1328	Citrus, earthy, oily	19.36 - 1655.07
64	1-Hexanol	1362	Fruity, green, herbaceous	2.58 - 98.75
65	2-Nonanol	1529	Waxy, green, creamy, citrus orange	6.79 - 137.23
66	1-Octanol	1568	Sharp, fatty, waxy, citrus, moss, nut, mushroom	11.90 - 141.35
67	1-Phenylethanol	1817	Honey, floral, rose, fragant	1.11 - 25.62
68	Benzyl Alcohol	1871	Sweet, fruity, floral	2.84 - 43.57
69	**2-Phenylethanol**	1892	Flowery, honey-like, rose, lilac, caramel, sweet	52.01 - 936.97
Pyrroles				
70	1-Pentyl-1H-pyrrole*	1381	Nutty	0.00 - 2.85

Peak no.	VOC [a]	RI_{Exp}	Odour description[b]	Concentration range (µg kg^{-1})
71	2-Acetylpyrrole	1968	Hazelnut, cocoa, chocolate, roasted, popcorn, musty, bread	14.98 - 223.47
72	1H-Pyrrole-2-carboxaldehyde	2025	Nutty, honey, candy	12.11 - 437.06
73	1-Methyl-1H-pyrrole-2-carboxaldehyde*	2102	Nutty	1.43 - 12.31
Terpenes/Isoprenoids				
74	Linalool oxide (*cis*-Furanoid)	1451	Sweet, nutty, fruity, floral/flowery	3.12 - 256.77
75	Linalool	1559	Rose, flowery, floral, citrus	4.79 - 132.09
76	α-Terpineol	1705	Herbaceous, Fruity	1.19 - 21.15
77	ß-Cadinene*	1757	Green, woody	0.00 - 7.84
78	ß-Damascenone*	1833	Floral	0.00 - 2.55
79	α-Ionone*	1848	Floral	0.47 - 4.48
80	Geranylacetone*	1858	Rose, floral	7.28 - 88.69
81	ß-Ionone*	1928	Woody, sweat, fruity	1.63 - 10.92
82	*trans*-Nerolidol*	2056	Floral, green and citrus like	0.00 - 3.10
83	*cis*-Methyl dihydrojasmonate*	2305	Sweet, fruity, floral, citrus lemon	0.33 - 1.86
Acids				
84	**Acetic acid**	1463	Sour, vinegar-like, pungent	21.76 - 1612.24
85	**2-Methylpropanoic acid**	1580	Acidic, sour, cheese, dairy, buttery, rancid, hammy	2.07 - 454.27
86	**3-Methylbutanoic acid**	1674	Old cheese, acidic, rancid, hammy	36.27 - 2154.25
87	3-Methylpentanoic acid*	1803	Sour, cheesy, fresh, fruity	0.70 - 26.07
88	4-Methylpentanoic acid	1809	Cheesy	2.14 - 45.19
89	2-Butylbutanoic acid	1956	–	1.43 - 33.13
90	Heptanoic acid	1964	Rancid, sour, sweaty	5.21 - 91.06
91	Octanoic Acid	2059	Sweat, cheese, oily, fatty	9.05 - 403.22
92	Nonanoic acid	2160	Green, fat, sweat, waxy	0.23 - 130.83
93	2-Phenylacetic acid	2487	Sweet, floral, chocolate, honey, tobacco	0.49 - 43.55
Lactones				
94	(S)-Massoialactone*	2009	Coconut, nutty	0.49 - 258.64
95	(R)-Massoialactone*	2242	Coconut, nutty	0.93 - 364.54
96	4,5,7,7a-Tetrahydro-4,4,7a-trimethyl-2(6H) benzofuranone	2338	Nutty	0.78 - 6.19
Others				
97	Styrene*	1266	Balsamic, sweet, floral	5.70 - 42.09
98	2-Acetylpyridine	1616	Caramel-like, sweet, Popcorn	0.80 - 3.21
99	*o*-Guaiacol	1867	Woody, spicy, smoky, burnt, musty	1.87 - 37.08
100	*p*-Cresol	2096	Smoke	0.76 - 93.71
101	3-Methyl-1H-Indole	2477	Mothball, faecal	0.36 - 3.94

[a]Volatile organic compounds (VOC) identified by probability based matching of mass spectra available in commercial libraries (NIST, Wiley) and the retention index (RI).The key-aroma markers are shown highlighted in bold according to [251,269,270].

[b] Odour descriptors according to literature [253,255,263,264,267,268,270-272] and from online databases: Flavornet (http://www.flavornet.org, accessed November 2018) and TGSC (The Good Scents Company) (http://www.thegoodscentscompany.com, accessed June 2018).

*Aromatic compounds described for the first time for cocoa and its related products.

Another important group of VOCs consists of short- and branched-chain fatty acids such as acetic acid, 2-methylpropanoic acid, and 3-methylbutanoic acid, which are key-aroma compounds used as markers of cocoa and cocoa products. The total amount of acids found in CBS samples were lower than that described by Tran et al. [255] for cocoa beans. Although acetic acid was found to be a major compound in the CBSs, the concentrations of this acid were lower than those described for cocoa beans. However, the concentrations found for 2-methylpropanoic acid and 3-methylbutanoic acid were similar to those described for cocoa products in the literature [255,264]. Although these acids are generally associated with unpleasant odour because of their rancid, sour-vinegar, and hammy notes in cocoa products, some acids present in the CBSs, such as octanoic acid and nonanoic acid, could present a pleasant odour with sweet notes.

Concerning alcohols, the total amount semi-quantified for this group of VOCs was lower than that described by Tran et al. [255] for cocoa beans. The main alcohols found in the CBS were two key-aroma compounds of cocoa: 2-heptanol and 2-phenylethanol that were present at higher concentrations than those found in cocoa beans and cocoa powder [255,264].

2,3-Butanedione, 2-heptanone, and 2-nonanone were the main ketones present in *Criollo* and *Nacional* CBS samples from Ecuador (ECU4, ECU5, and ECU7) that contribute to the aroma with sweet, fruity, and flowery notes.

Esters were other key VOCs present in the CBS associated with the fruity, floral, and sweet notes attributed to cocoa aroma. The key-aroma marker, 2-phenylethyl acetate was the main ester present in the CBS followed by 3-methylbutyl acetate, methyl 2-phenylacetate, and ethyl benzene acetate. Although the total amount in this group of VOCs was lower than that found in cocoa products, the main esters identified in CBS samples from specific origins (e.g., Peru, Tanzania, Togo, and Venezuela), were found in similar concentrations to that found for cocoa beans [255].

Other VOCs derived from roasted cocoa identified in the CBS were the terpenes linalool and linalool oxide, both with characteristic key chocolate flavours [264,268]. Furthermore, the pyrroles (1H-pyrrole-2-carboxaldehydeand 2-acetylpyrrole) were identified and quantified in the CBS in noteworthy amounts. An additional compound that contributes to the CBS flavour of cocoa is acetylfuran. Likewise, in the furan derivate group, furfural was identified and quantified at high concentrations in *Criollo* CBS samples from Venezuela (VEN10). Finally, dimethyl trisulfide, also described as a key-aroma compound for cocoa products, and dimethyl disulfide were identified and quantified. The highest amounts of both compounds

were detected in CBS samples from the Dominican Republic (DOR1) of the *Trinitario* cultivar.

The present study identified other VOCs, present in lower concentrations, which are described for the first time for cocoa-related products and may contribute to the total pleasant aroma of the CBS (see Table 1, compounds highlighted with asterisk symbol, *). Some of these molecules were ketones such as 2-decanone, 3-methyl-2-cyclohexen-1-one, 2-undecanone; pyrazines such as 2,3,5-trimethyl-6-isopentylpyrazine; several terpenes; and Massoialactone (S and R).

In this study, the roasting process was performed under standardized conditions to avoid the influence of this process on the volatile profile of CBS and to better evaluate the effect of the cultivar and/or the geographical origin. However, the CBS is a by-product produced at industrial scale by several companies with specific roasting treatments that could result in different volatile profiles for the same batch of cocoa beans. Therefore, further research needs to be done in order to access the volatile profile under different roasting conditions and evaluate the effect of this key-step on the final flavour of CBS.

3.2. Classification of CBSs based on VOCs determined by SPME-HS-GC-qMS

3.2.1. Classification of CBSs according to the cultivar and continent of origin – first approach

Figure 1 shows the principal component analysis (PCA) based on volatile fingerprinting of the CBS that was used to find differences among types of cultivars (Fig. 1a) and the continent of provenience (Fig. 1b). The PCA clearly showed a separation ($p<0.001$) of the *Criollo* CBS compared with the other cultivars that clustered together (Fig. 1a), and this was confirmed by the ANOSIM statistical test. By taking into account the continent of provenience, it was possible to observe a clear separation ($p<0.007$) of American and African CBS samples (Fig. 1b). However, it can be noted that the marked separation of samples from *Criollo* cultivar makes difficult the differentiation of *Forastero* samples from the others "fine aroma" cultivars (*Trinitario* and *Nacional*). Furthermore, *Criollo* CBS is mainly from the American continent and may also interfere in the classification according to the geographical origin.

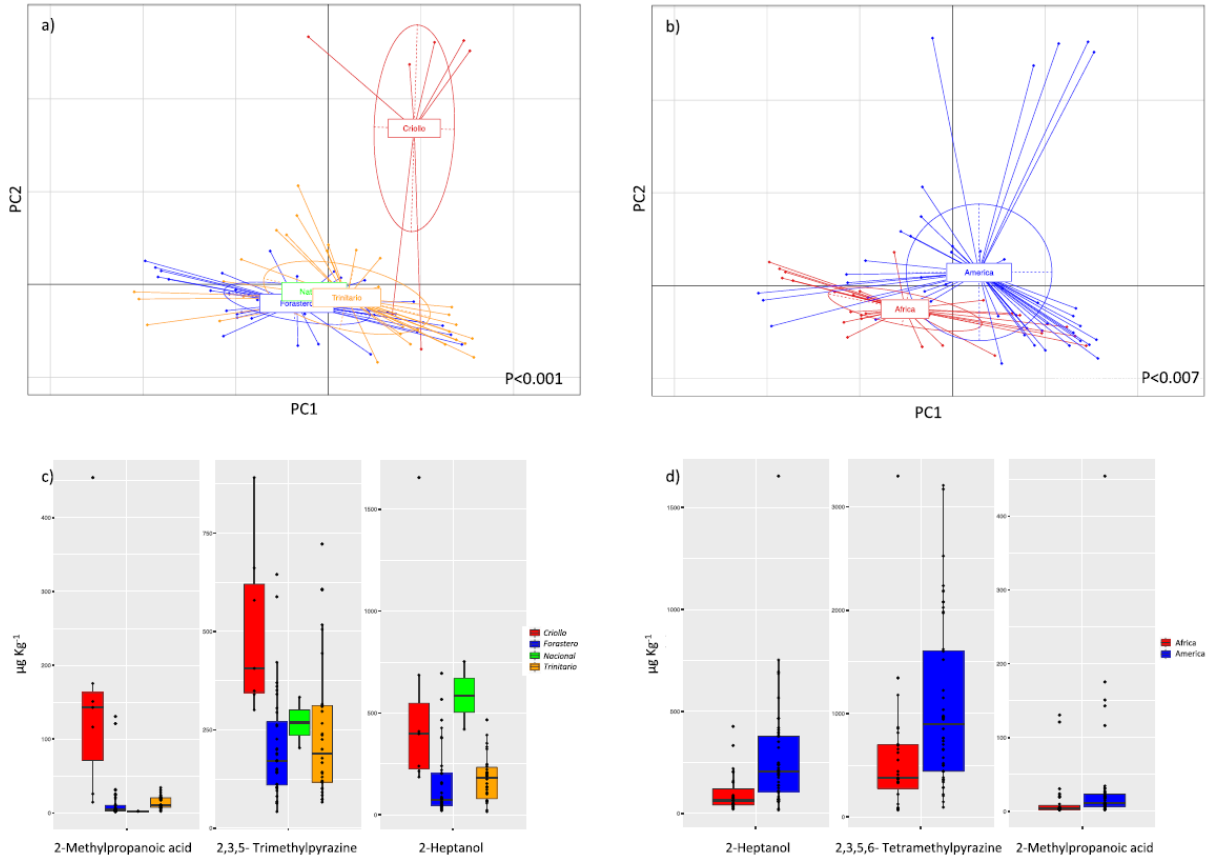

Figure 1. PCA based on the VOCs (µg kg-1) identified by HS-SPME/GC-qMS in all CBS samples as a function of: (a) cultivar, (b) continent of origin. The variance explained by the first component of PCA (PC1) was 28.54%, while the second component (PC2) explained 15.10%.Box plots showing abundance of key VOCs that can be used as possible markers for (c) cultivar and (d) geographical origin. For interpretation of the legends, see Table 1, Chapter 1.1.

Going more deeply into the volatile composition, the level of diversity of the VOCs was clearly different based on the CBS cultivar. Several compounds (48 VOCs) were found to be significantly different according to the cultivar (FDR<0.001 (10 VOCs), FDR<0.01 (17 VOCs), and FDR<0.05 (21 VOCs)), as shown in Table S1.1 (see supplementary material). Key aroma compounds, such as 2-methylpropanal, phenylacetaldehyde, 2,3,5-trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-heptanol, 2-phenylethanol, 2-methylpropanoic acid, and 3-methylbutanoic acid, were found to be significant for the CBS discrimination of the *Criollo* cultivar from the *Forastero* and *Trinitario* cultivars. Other compounds such as benzaldehyde, methyl-2-phenylacetate, 2,3-dimethylpyrazine, and 2,3,5,6-tetramethylpyrazine were present at high concentrations in *Criollo* CBSs and can also be putative markers of the *Criollo* cultivar. Tetramethylpyrazine was the most abundant pyrazine present in CBSs yielded from *Criollo* cocoa beans, as described by Tran et al. [255] for cocoa beans of the same cultivar. The 3-methylbutanoic acid was found to be a potential marker for the *Forastero* CBS, while 2-phenylethanol and 2-heptanol were found to be potential markers for the *Trinitario* CBS, as already described for cocoa beans [265]. The

boxplot of three volatile compounds highly significant for the classification of CBS according to cultivar is shown in Fig. 1c. 2-methylpropanoic acid was found at high concentrations in *Criollo* CBSs and allowed for the discrimination of this cultivar from *Forastero* and *Trinitario* cultivars (FDR<0.001) and distinguished the CBS *Trinitario* from the *Forastero* and *Nacional* cultivars (FDR<0.01 and FDR<0.05, respectively). Also 2-heptanol allowed for the differentiation of *Trinitario* CBSs from the other cultivars (FDR<0.05) and additionally distinguished *Forastero* CBSs from *Criollo* and *Nacional* (FDR<0.01 and FDR<0.05, respectively). Finally, 2,3,5-trimethylpyrazine was found to be highly significant, differentiating *Criollo* CBSs from *Forastero* and *Trinitario* cultivars (FDR<0.01).

Taking into account the geographical origin, several compounds (47 VOCs) were found to be significant in the classification of CBSs according to the continent of origin (FDR<0.001 (11 VOCs), FDR<0.01 (19 VOCs), and FDR<0.05 (17 VOCs)) as shown in Table S1.2 (see supplementary material). Considering the key aroma markers identified in cocoa samples [270], the boxplot showed that 2-heptanol (FDR<0.001) and 2-methylpropanoic acid (FDR <0.001) were those volatiles with the highest concentration in CBSs of *Criollo* cocoa from the American continent. Moreover, the most abundant pyrazine detected in CBS, 2,3,5,6-tetramethylpyrazine (FDR<0.01),was also associated with American samples (Fig. 1d).

3.2.2. Classification of Forastero CBS samples according to their geographical origins

PCA analysis was performed to evaluate the sample separation according to the CBS origin of the *Forastero* cultivar among 14 countries from Africa and America (see Figure 2). A clear separation (p<0.001) was observed according to macroarea (Fig. 2a), latitude (Fig. 2b), and the country of origin (Fig. 2c). For the classification according to latitude, the countries of production were distributed into four main groups: L1 (5ºS–5ºN); L2 (5ºS–20ºS); L3 (5ºN–10ºN); and L4 (10ºN–20ºN). By taking into account the macroarea as a discriminant factor, it was possible to observe that West African and South American CBS samples cluster together and were well-separated (p<0.001) from samples from East Africa and Central America (Fig. 2a). Moreover, the different volatile profile drove the impressive cluster separation (p<0.001) according to the latitudes (Fig. 2b). In particular, the CBSs from West Africa and latitude L3 (5ºN–10ºN) were the samples with low amounts of total VOCs, mainly aldehydes, pyrazines, and sulphur compounds and high amounts of acids. Going more deeply into the classification of the CBS samples as a function of the geographical origin, it was possible to differentiate (p<0.001) the CBS sample according to the country of origin (Fig.

2c). We observed that Congo clustered together with Uganda, and the two South American countries, Ecuador and Colombia, formed a central group in the centre of the PCA. The CBSs from cocoa beans produced in countries located at the latitude L2 (5ºS–20ºS), such Madagascar, Peru, and Tanzania, were those with high amounts of VOCs among *Forastero* cultivars (see Table 2 in Barbosa-Pereira et al., [29] (Chapter 1.2.1). For these samples, cocoa key aroma compounds were found at high concentrations such as acids (acetic acid), aldehydes (benzaldehyde, 2-methylpropanal, and 3-methylbutanal), esters (3-methylbutyl acetate and 2-phenylethyl acetate), and pyrazines such as 2,3,5-trimethylpyrazine and 2,3,5,6-tetramethylpyrazine. It should be pointed out that CBSs from Madagascar were well-separated from Sao Tomé. Even though both countries are African, they belong to different macroareas and grow at different latitudes. However, they are both islands, with specific climate conditions that may affect the volatile profile of cocoa beans and their products [251]. CBS samples from these countries were characterised by low concentrations of alcohols and by the presence of 3-methylbutanoic acid, 3-methylbutanal, phenylacetaldehyde, dimethyl trisulfide, trimethylpyrazine, and tetramethylpyrazine present at high concentrations conferring important flavour characteristics to the CBS that can valorise the product. CBSs from the Central American country the Dominican Republic (L4 10ºN–20ºN) were characterized by the presence of high amounts of aldehydes and pyrazines. Therefore, among the *Forastero* cultivar, CBS samples from Sao Tomé, Madagascar, the Dominican Republic, and Peru were those with high amounts of VOCs and could be distinguished from the rest of the samples (Fig.2c).

By taking into account the key VOCs of *Forastero* that drove this separation (Table S2.1, see supplementary material), 2-methylpropanal, 3-methylbutanal, phenylacetaldehyde, dimethyl trisulfide, 2-phenethyl acetate, 2-heptanol, 2-phenylethanol, 2-methylpropanoic acid, 3-methylbutanoic acid, 2,3,5-trimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine, were found to be putative markers of *Forastero* CBS according to the country of origin. In detail, it was possible to identify two main components that drove the separation among the different origins: phenylacetaldehyde and furfural. Particularly, phenylacetaldehyde was most present in CBSs from the Dominican Republic, Madagascar, Peru, and Sao Tomé, while furfural was in CBSs from Sierra Leone, Togo, and Tanzania (Fig. 2d).

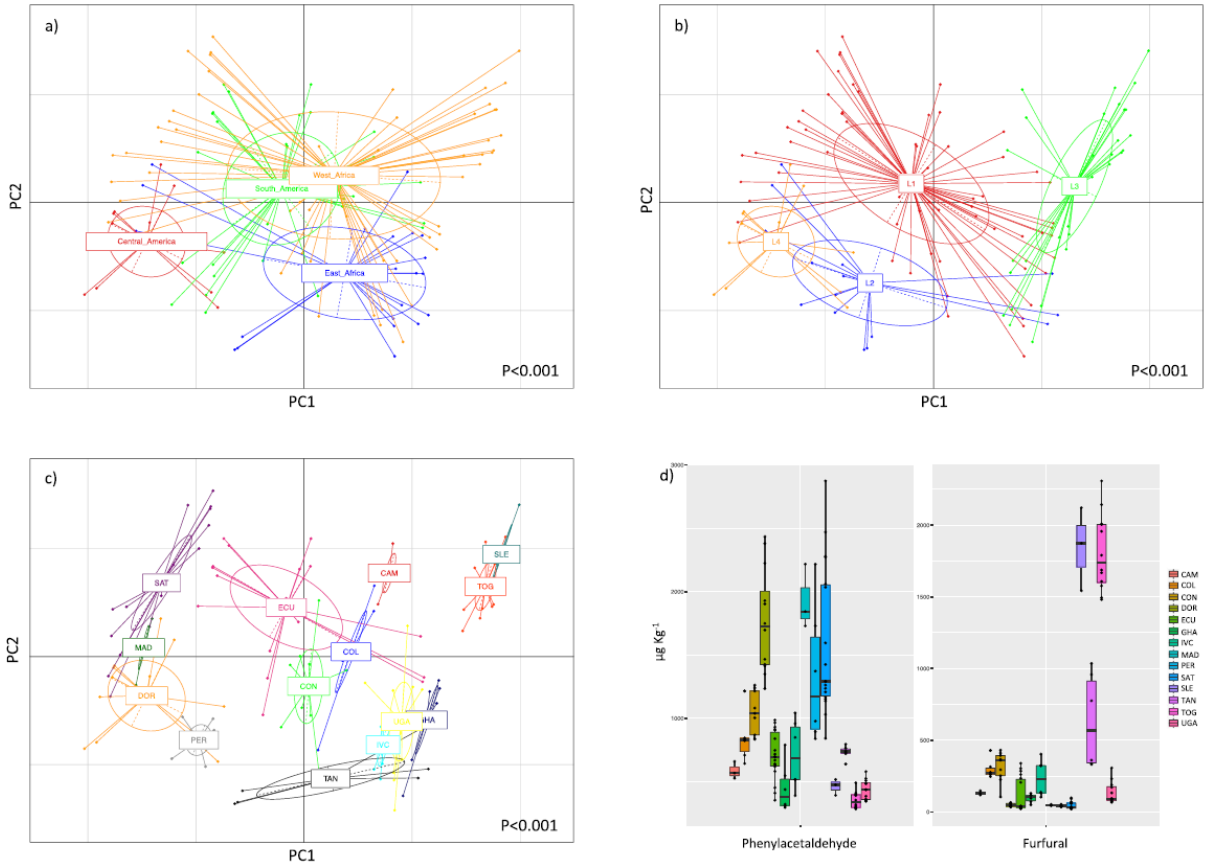

Figure 2. PCA based on the VOCs (µg kg^{-1}) identified by HS-SPME/GC-qMS in CBS samples of the *Forastero* cultivar according to: (a) macroarea, (b) latitude, and (c) country of origin. The variance explained by the first component of PCA was 32.02%, while the second component explained 13.71%. (d) Boxplot showing abundance of VOCs that can be used as potential markers of origin: phenylacetaldehyde and furfural. For interpretation of the legends, see Table 1 in Chapter 1.1).

3.2.3. Classification of Trinitario CBS samples according to their geographical origins

Considering *Trinitario* CBS samples (see Figure 3), we clearly observed a separation ($p<0.001$) between samples from Central America and South America (Fig. 3a). Moreover, taking into the account the latitude, we observed that samples from L1 (5ºS–5ºN) and L2 (5ºS–20ºS) clustered together and were well-separated ($p<0.001$) from L3 (5ºN–10ºN) and L4 (10ºN–20ºN) (Fig. 3b). Taking into account the CBS origin, we observed that CBSs yielded from cocoa beans grown in Central America, the Dominican Republic, Jamaica, and Mexico, located at the latitude L4 (10ºN–20ºN), clustered together. For South America, CBS samples were divided into three latitudes, L1 (5ºS–5ºN) comprising Ecuador and Colombia, L2 (5ºS–20ºS) comprising Brazil and Peru that were not separated among them, and finally CBSs from Venezuela at the latitude L3 (5ºN–10ºN) that were separated from the other three groups (Fig. 3c). However, the high number of samples from Venezuela respect to the other countries of origin could also contribute to this separation, since the sample set is not completely balanced.

CBSs from the Dominican Republic, Mexico, and Peru were those with high amounts of total VOCs among *Trinitario* CBSs, including pyrazines (e.g., 2,3,5-trimethylpyrazine, see Fig. 3d), ketones (2-nonanone), acids (acetic acid), and aldehydes (phenylacetaldehyde). Also, CBSs from Colombia and Jamaica displayed intermediate amounts of ketones (2-nonanone), terpenes, and aldehydes (3-methylbutanal) (See Fig. 3d and see Table 2 in Barbosa-Pereira et al., [29], Chapter 1.2.1). However, Jamaican CBSs were also characterized by low amounts of esters, furan derivates, and acids. CBSs yielded from cocoa beans grown in Brazil and Ecuador were characterized by low amounts of 2-methylpropanal, 2-methylbutanal, and phenylacetaldehyde; high amounts of nonanal and heptanal; low amounts of pyrazines, esters, and acids; and the presence of high concentrations of furans (furfural and acetylfuran). CBSs from Ecuador were separated from Brazilian samples based on the high content of alcohols, mainly 2-heptanol and 2-phenylethanol (See Table 3 in Barbosa-Pereira et al., [29], Chapter 1.2.1).

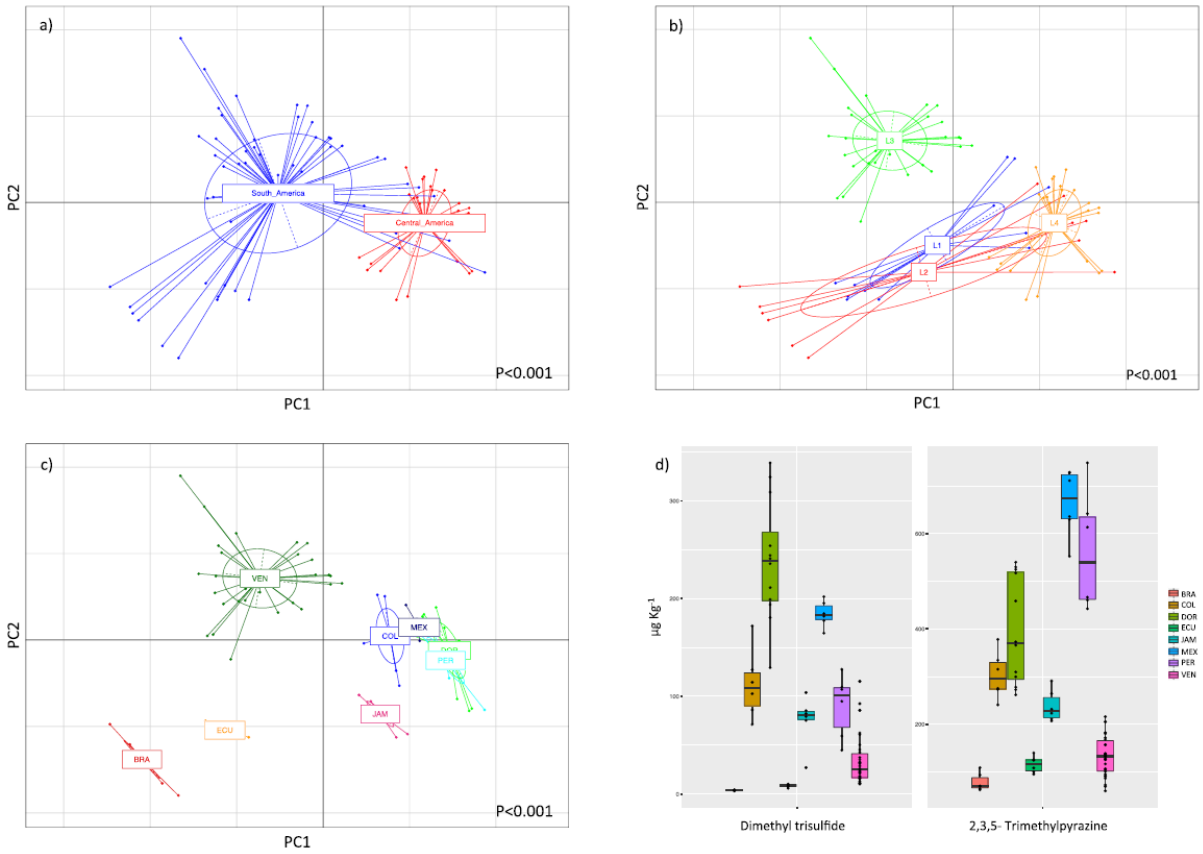

Figure 3. PCA based on the VOCs (µg kg^{-1}) identified by HS-SPME/qGC-MS in CBS samples of *Trinitario* cultivar according to: (a) macroarea, (b) latitude, and (c) country of origin. The variance explained by the first and second principal component was 35.63% and 15.26%, respectively. (d) Boxplot showing abundance of VOCs that can be used as potential markers of origin: dimethyl trisulfide and 2,3,5-trimethylpyrazine.For interpretation of the legends, see Table 1 in Chapter 1.1.

As for *Forastero* CBSs, several VOCs were found as potential markers for the classification of the *Trinitario* CBSs. The volatile compounds that had significant differences (FDR<0.05 or lower) among American countries are shown in Table S2.2 (see supplementary material). Figure 3d shows the boxplot of two key aroma compounds for cocoa, dimethyl trisulfide and 2,3,5-trimethylpyrazine,which contributed significantly (FDR<0.05 or lower) to the classification of CBSs. Specifically, dimethyl trisulfide was most present in CBSs from the Dominican Republic, Colombia, and Mexico, while 2,3,5-trimethylpyrazine was in CBSs from Mexico and Peru.

3.3. Volatile profile of the CBSs characterized by E-nose and classification based on the E-nose data set

For all CBS samples, the changes in the variation of signals were found to be similar (data not shown). The sensors, S2 (broad), S6 (broad-methane), S7 (sulphur organic), S8 (sensitive broad alcohol), and S9 (sensitive to aromatics and organic sulfides) were those that displayed high response intensity (See Table 3 in Barbosa-Pereira et al., [29], Chapter 1.2.1). The PCA analysis of E-nose data showed that the most significant classification of CBS was according to cultivar and country of origin (Figure 4). A clear separation of CBS ($p<0.001$) samples yielded from cocoa beans of *Criollo* cultivar from the other cultivars was observed (Fig. 4a). In this case, the potentials of several sensors that compose the E-nose were considered for the classification of CBSs. The sensors that displayed significant differences (FDR<0.05 or lower) among the cultivars are shown in Table S3 (see supplementary material). All sensors indicated highly significant separation of the CBSs from the *Criollo* cultivar from *Trinitario* and *Forastero* cultivars. These results confirm the discrimination of CBS samples from the *Criollo* cultivar obtained with GC-qMS data. Considering all cultivars, the most representative sensors for the classification of CBSs were S5 (sensitive to aromatic and aliphatics), S6 (broad-methane), S7 (sensitive to terpenes and sulfur-containing organic compounds), and S10 (methane and aliphatics).

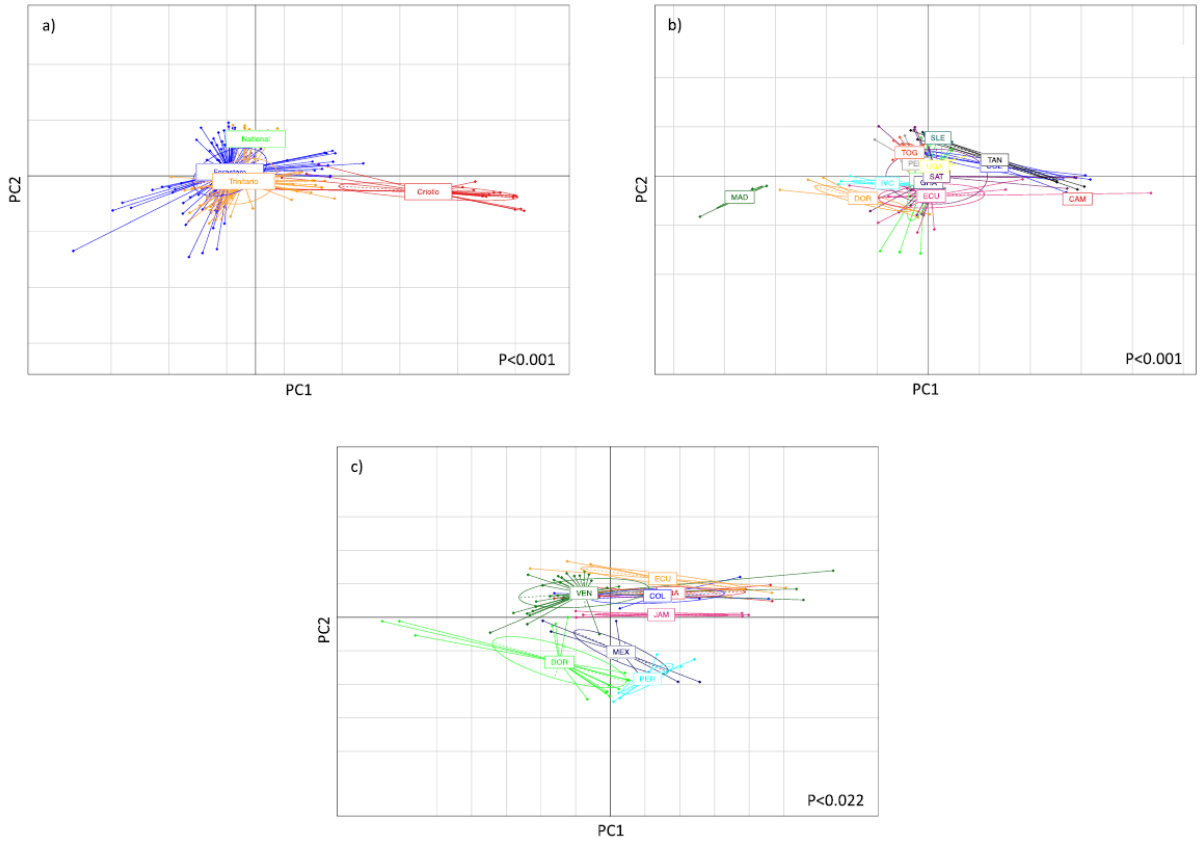

Figure 4. PCA based on E-nose data set for: (a) all CBS samples as a function of cultivar (the variance explained by the first and second principal component was 78.94% and 11.06%, respectively); (b) CBS samples of *Forastero* according to country of origin (the variance explained by the first and second principal component was 74.89% and 13.51%, respectively); (c) CBS samples of *Trinitario* cultivar according to country of origin (the variance explained by the first and second principal component was 68.30% and 13.97%, respectively). For interpretation of the legends, see Table 1 in Chapter 1.1.

Taking into account the geographical origin, the two groups of CBS samples from cultivars *Forastero* and *Trinitario* are shown in Fig. 4b and Fig. 4c, respectively. For the *Forastero* cultivar, CBSs from Madagascar were well-separated ($p<0.001$) from the rest of the samples. Likewise, samples from Sierra Leone and Togo were separated from the CBSs from the Dominican Republic and Ecuador as observed by the GC-qMS data. However, E-nose was not able to separate Peru or Sao Tomé, as observed from the GC-qMS data (see Fig. 2c). Considering the *Trinitario* cultivar samples, displayed in Fig. 4c, a significant separation ($p<0.022$) of CBS samples was observed. However, the efficacy of separation was lower than that observed for the GC-qMS analysis. The results highlighted that E-nose can be used as a tool for rapid discrimination of CBS samples from different cultivars, mainly for *Criollo* cultivar. Nevertheless, this methodology presented limitations for the classification of CBS for a single country compared to GC-qMS.

3.4. GC-qMS vs. E-nose – two case studies

In this section, CBSs from two representative countries of cocoa production with the most representative numbers of samples, Venezuela (n=10) and Ecuador (n=7), were taken into account to verify whether both GC-qMS and E-nose were able to classify CBSs among the same country of origin. A significant separation ($p<0.001$) of CBSs from Venezuela and Ecuador was observed using both analytical techniques, as shown in Figure 5.

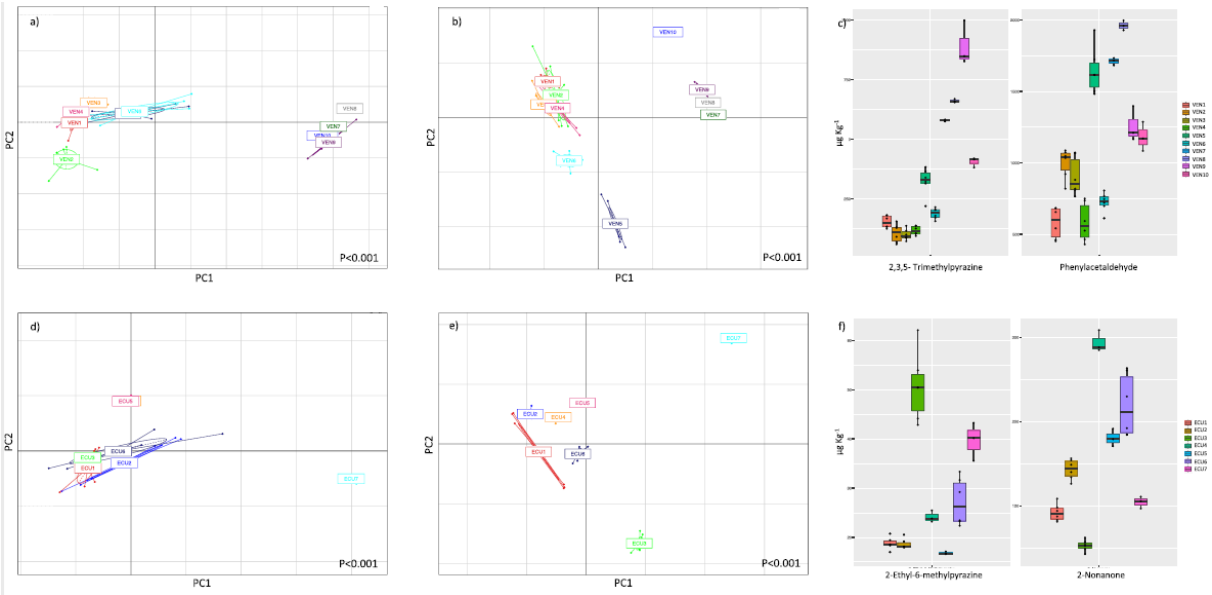

Figure 5. PCA based on the VOCs (µg kg^{-1}) identified by HS-SPME/GC-qMS and E-nose data set for CBS samples from different regions of Venezuela: (a) PCA based on E-nose data set (the variance explained by the first and second principal component was 93.63% and 3.04%, respectively); (b) PCA based on HS-SPME/GC-qMS data set (the variance explained by the first and second principal component was 32.88% and 22.41%, respectively). (c) Boxplot showing abundance of VOCs that can be used as potential markers of origin: 2,3,5-trimethylpyrazine and phenylacetaldehyde.
PCA based on the VOCs (µg kg^{-1}) identified by HS-SPME/GC-qMS and E-nose data set for CBS samples from different regions of Ecuador: (d) PCA based on E-nose data set (the variance explained by the first and second principal component was 82.23% and 10.76%, respectively); (e) PCA based on HS-SPME/GC-qMS data set (the variance explained by the first and second principal component was 27.76% and 24.13%, respectively). (f) Boxplot showing abundance of VOCs that can be used as potential markers of origin; 2-ethyl-6-methylpyrazine and 2-nonanone.
For interpretation of the legends, see Table 1 in Chapter 1.1.

In the case of CBSs from Venezuela (Fig. 5a and 5b), both techniques allowed for the separation of samples from the *Criollo* cultivar (VEN7, VEN8, VEN9, and VEN10) from other cultivars, except for samples from Canoabo (VEN5), which were not separated with E-nose (see Fig. 5a). However, E-nose was capable of differentiating CBSs from the Caucagua region (VEN2) of the *Trinitario* cultivar from the other regions of the same cultivar (VEN1, VEN3, VEN4, and VEN6), which was not accomplished with GC-qMS. Figure 5b shows a clear separation of CBS samples of the *Criollo* cultivar from the other cultivars using GC-qMS data. Considering the GC-qMS data, CBS samples from the Ocumare region (VEN6 and

VEN10) were separated according to cultivar. Moreover, CBSs from Canoabo (VEN5) were also separated from the samples clustered in the *Criollo* varietal group. As observed from the E-nose data, this technique allowed for the separation of a CBS from the rest of the samples of the *Trinitario* cultivar, but in this case, the CBS was from the Ocumare region (VEN6). Therefore, the use of GC-qMS coupled with the E-nose technique could be an interesting approach for the classification of Venezuelan CBSs. Volatile molecules such as 2,3,5-trimethylpyrazine and phenylacetaldehyde were identified as potential markers for the classification of CBSs from Venezuela, as shown in the boxplot represented in Fig. 5c. These and other volatile compounds that had significant differences (FDR<0.05 or lower) among Venezuelan regions of production are shown in Table S4.1 (see supplementary material).

The PCA of CBSs from Ecuador using E-nose and GC-qMS data sets is shown in Fig. 5d and Fig. 5e, respectively. As shown in Fig. 5d, the E-nose technique allowed for the classification of CBS according to the cultivar. Samples from the *Criollo* cultivar (ECU7) and samples from the *Nacional* cultivar (ECU4 and ECU5) were clearly separated from the *Forastero* cultivar. However, this technique was not able to separate both *Nacional* cultivar CBSs (ECU4 and ECU5), neither the *Trinitario* cultivar CBS (ECU2) from *Forastero* cultivar samples (ECU1, ECU3, and ECU6), which were separated by GC-qMS. PCA based on GC-qMS data of CBSs from Ecuador showed a significant separation (FDR<0.001) of the samples ECU7 (*Criollo* cultivar) and ECU3 (*Forastero* cultivar) among them and from the rest of the CBS samples. A clear separation between CBS samples from the *Forastero* cultivar (ECU1, ECU3, ECU6) and CBS yielded from cocoa beans of "fine aroma" (*Nacional*, *Trinitario*, and *Criollo* cultivars) was also observed. The GC-qMS technique allowed for a high separation of the CBSs compared to the E-nose that presented some limitations. Several VOCs were identified as potential markers for this classification (FDR<0.05 or lower) of CBSs from Ecuador, such as 2-ethyl-6-methylpyrazine and 2-nonanone, as shown in Fig. 5f (see Table S4.2, supplementary material).

3.5. HS-SPME-GC-qMS vs E-nose – correlation

According to the results described above, GC-qMS and E-nose could classify the CBSs at different levels. GC-MS methodology consents the identification and quantification of single molecules, it is more sensible and accurate and has demonstrated a remarkable discrimination potential with applicability for food authentication. However, the diffusion and practical application in the food industry of this technology is limited due to the high operational costs and personnel commitment required. Instead, E-nose is economical, non-destructive and

easier to work with due to the operational simplicity and allows bulk sampling. This technology has already application on food industry for a rapid screening and identification of samples and verification of variety for traceability purposes. However, it presents some drawbacks including the low sensitivity and specificity and less accuracy in comparison with GC-MS.As described before in section 3.4 for the classification of Venezuelan CBS, the combination of both techniques improved the ability for sample discrimination and therefore could represent a good possibility for CBS classification.

VOCs found at higher concentrations than 100 µg kg^{-1} at least for one sample were selected for the correlation, since this is the limit of detection of E-nose according to the manufacturer (Airsense Analytics GmbH., Germany). The correlation between VOCs and sensors is shown in Figure 6.

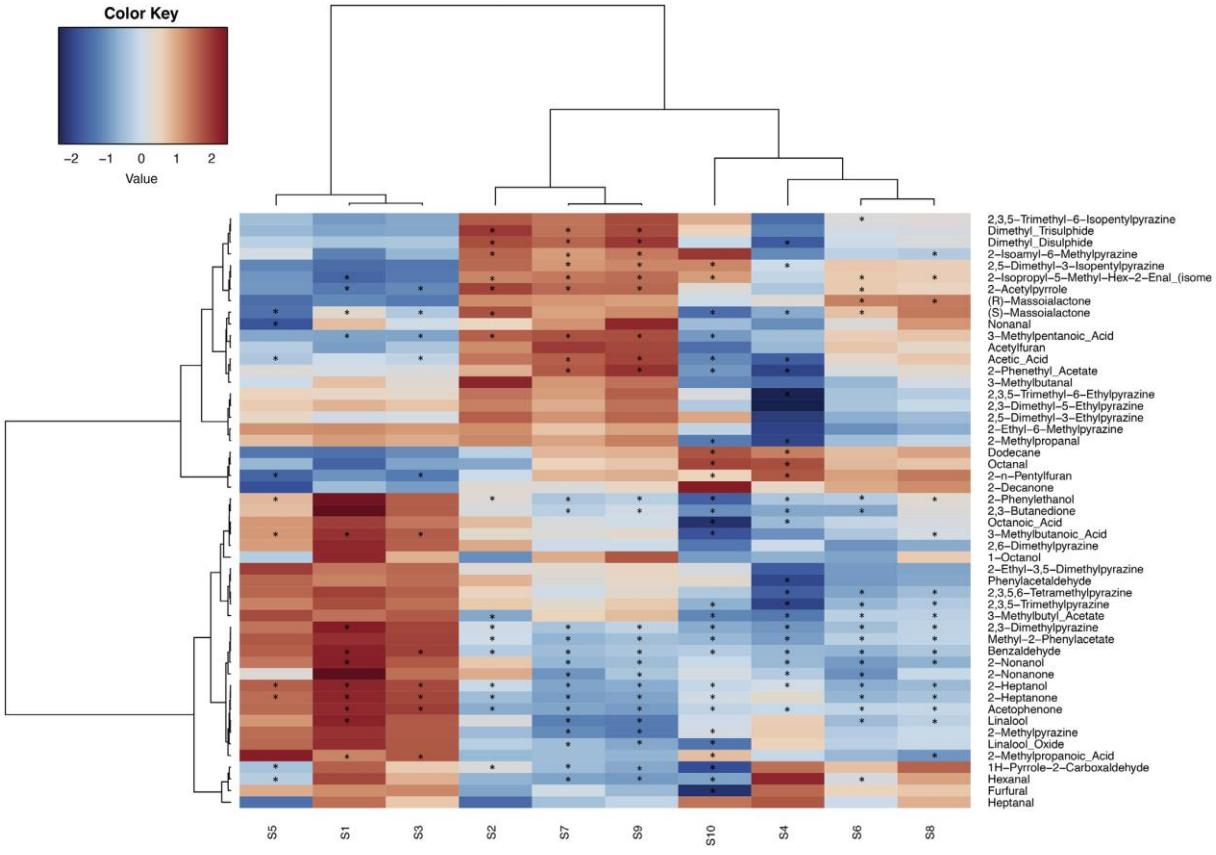

Figure 6. Correlation between the abundance of VOCs (µg kg^{-1}) and E-nose sensors. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colours represents the degree of correlation between the samples and VOCs as measured by the Spearman's correlations. Asterisks denote significant correlations after P value corrections (FDR < 0.05).

The heatmap shows clearly three main clusters of sensors: S1, S3, and S5 (Cluster 1); S2, S7, and S9 (Cluster 2); and S10, S4, S6, and S8 (Cluster 3). For Cluster 1, S1, S3, and S5 were found to correlate positively with aromatic molecules (S1, S3, and S5), such as benzaldeyde and 2-phenylethanol, aliphatic compounds (S5), such as pyrazines (2,3-

dmethylpyrazine), alcohols (2-heptanol and 2-nonanol), and ketones (2-heptanone and acetophenone), and acids (2-methylpropanoic acid and 3-methylbutanoic acid). These molecules were found at high concentrations for *Criollo* CBS and at lower concentration for samples from countries as Madagascar (*Forastero* classification). The response of the sensors of Cluster 1 showed the same behaviour with high response intensity for *Criollo* CBS and low intensity of response for Madagascar CBS (see Table 3 in Barbosa-Pereira et al., [29], Chapter 1.2.1). Cluster 2 results from the positive correlation of sensors S2, S7, and S9 with sulphur organic compounds representative of cocoa flavour: dimethyl trisulfide and dimethyl disulfide. These VOCs were found at high concentrations in samples from Mexico, Dominican Republic and Peru. The profile of these samples also showed high response intensity to Cluster 2 and confirm the classification provided by the E-nose (see Fig. 4 c). Finally, Cluster 3 exhibited a high correlation between sensors related with long chain aliphatic compounds (mainly S4 and S10) such as dodecane (hydrocarbons) and octanal.

4. Conclusion

This study provides information, for the first time, on the volatile fingerprint of the CBS determined by HS-SPME/GC-qMS and identifies the molecules responsible for differences among a feasible number of samples yielded from cocoa beans collected from different geographical regions and cultivars.

The presence of high amounts of cocoa key aroma markers in CBS samples, such as 2-methylpropanal, 3-methylbutanal, phenylacetaldehyde, dimethyl trisulfide, 2-phenylethyl acetate, 2,3,5-trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-heptanol, 2-phenylethanol, 2-methylpropanoic acid, and 3-methylbutanoic acid, valorises this by-product as a food ingredient. CBSs yielded form cocoa beans of the *Criollo* cultivar were those with high amounts of “fine aroma” molecules and, therefore, with more potential as a source of cocoa flavour.

GC-qMS-fingerprinting and E-nose data allowed for the identification and discrimination of fine flavour cocoa *Criollo*. Several markers, such as 2-methylpropanoic acid, 2,3-dimethylpyrazine, and 2-heptanol, were found to be mandatory for the classification of CBSs according to the cultivar. It was also possible to classify the CBS samples based on their different geographical origins using GC-qMS. Markers such as phenylacetaldehyde and furfural were associated with the CBSs of the *Forastero* cultivar from different countries from America and Africa. While for the *Trinitario* cultivar, dimethyl trisulfide and 3-

methylbutanal, among others, were found to be markers capable of classifying CBSs from the American territory.

The results highlighted remarkable diversity in the volatile profile of CBSs and confirmed the potential applicability of GC-qMS and E-nose for classification and future traceability of CBSs. The capability to identify common trends, leading variables and general indications through rapid and simple technology as E-nose is an encouraging result in this field. However, this technique easy to manipulate still requires the support of more accurate analytical techniques (GC-qMS) for comparison and calibration. Indeed, this study correlates, for the first time, the E-nose and GC-qMS data using a representative number of samples and contributes with new insights for this research field. Nevertheless, our findings are incomplete and further exhaustive investigation needs to be done considering a larger number of samples. The geographical traceability of CBS based on chemical analysis is complex and several intrinsic factors such as climatic conditions and processing conditions (e.g. roasting) should be considered in future studies.

Similar to cocoa beans, the CBS by-product might be considered for selective collection to yield a food ingredient with good aroma and specific flavour characteristics that could be recycled inside the cocoa industry, utilizing the concept of circular economy for a high add-value product.

Supplementary material of the article forming Chapter 1.2 was composed by:

- Table S1.1. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS that could be used as potential markers among cultivars.
- Table S1.2. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS that could be used as potential markers among continent of origin.
- Table S2.1. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS that could be used as potential markers among countries of origin (considering the cultivar Forastero).
- Table S2.2. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS that could be used as potential markers among countries of origin (considering the cultivar Trinitario).
- Table S3. Pairwise comparison of E-Nose sensors among CBS cultivars
- Table S4.1. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS, which could be used as potential markers among Venezuela geographical regions.
- Table S4.2. Pairwise comparison of several VOCs quantified in CBSs by HS-SPME/GC-qMS, which could be used as potential markers among Ecuador samples.

These data sets were extremely large and were considered of minimal importance for the well understanding of the present chapter. Due to space issues and for ecology purposes, they were not included in the present manuscript. However, if the reader desires to consult them, these data sets can be downloaded following the next link:

https://ars.els-cdn.com/content/image/1-s2.0-S0963996919303540-mmc1.xlsx

CHAPTER 1.2.1

Analytical Dataset on Volatile Compounds of Cocoa Bean Shells from Different Cultivars and Geographical Origins

Chapter 1.2.1 was conceived as a complementary chapter to Chapter 1.2. Extra information about the volatile profile of the CBS samples forming the sample set is presented here, as well as other results for different CBS properties such as moisture, pH, titratable acidity, and CIELab color parameters.

This chapter was issued and adapted from a published scientific article:

Analytical Dataset on Volatile Compounds of Cocoa Bean Shells from Different Cultivars and Geographical Origins

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If the reader desires it, the original paper can be consulted in this link

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1. Data

The dataset collected for 44 CBS samples from different cultivars and geographical origins is presented in four segments of data: A) Samples information regarding the cultivar and geographical origin is labeled in Table 1 of Chapter 1.1; B) Physicochemical characterization of CBS samples is described in Table 1 of this chapter; C) The experimental retention index, names and contents of the volatile organic compounds (VOCs) identified among the CBSs determined by HS-SPME/GC-qMS are described in Table 2 of this chapter and with more detail in Table S1 (Microsoft Excel Worksheet in supplementary material); and D) The dataset obtained from E-nose sensors for CBS samples is described in Table 3.

The data reported in Tables 2, S1 and 3 were used for the assessment of volatile fingerprint and classification of CBSs from different cultivars and geographical origins using chemometrics reported by Barbosa-Pereira et al. [66]

2. Experimental Design, Materials and Methods

2.1. Samples – CBS

Cocoa beans (n=44) from different cultivars and countries across the world (Table 1, Chapter 1.1) were purchased from several local chocolate enterprises. Cocoa bean shells were obtained from the cocoa beans after a standardized roasting process according to the procedure described by Barbosa-Pereira et al. [66] (Chapter 1.2). After separation from the respective cocoa beans, the CBS samples were ground into powders with a 250 µm mesh size and stored under a vacuum at −20 °C prior to further analysis. More detailed information related to the origin and description of samples can be consulted in Section 2.2. (CBS Samples) and Table 1 in Chapter 1.1.

2.2. Physicochemical Analysis

2.2.1. Moisture Content

Themoisture content of the CBS samples was determined by gravimetry, at 110 °C until constant weight, using a Gibertini Eurotherm electronic moisture balance (Gibertini Elettronica, Novate Milanese MI, Italy).

2.2.2. Determination of pH and titratable acidity

Titratable acidity (TA) and pH of the CBS were determined according to AOAC official method described by Nazaruddin et al. [273]. Five grams of CBS powder were homogenized in 100 ml of boiled distilled water by stirring for 30 s and filtered through Whatman no. 4 filter under vacuum. An aliquot (25 mL) was used to measure pH using a pH meter Knick Portamess® 913 (Knick, Berlin, Germany). Then, the same aliquot was titrated with 0.1 mol L^{-1} NaOH standard solution to an endpoint pH of 8.2. All determinations were performed in triplicate. The results of titratable acidity (TA) were expressed as g of acetic acid equivalents / 100 g of CBS.

2.2.3. Color Analysis – CIELab

The color analysis of CBSs was performed in transmittance mode on a CM-5 spectrophotometer (Konica Minolta, Tokyo, Japan) as reported by Rojo-Poveda et al. [13]. *L**, *a**, and *b** CIELab parameters were used to measure the color, where *L** is a coefficient of lightness ranging from 0 (black) to 100 (white), *a** indicates the colors red-purple (when positive *a**) and bluish-green (when negative *a**), and *b** denotes the colors yellow (when positive *b**) and blue (negative *b**).

2.3. HS-SPME/GC-qMS analysis – Insrument and Analytical conditions

The VOCs from the CBS samples were analysed using a headspace solid phase micro extraction (HS-SPME) coupled with gas chromatography/quadrupole mass spectrometry (GC/qMS) as described by Barbosa-Pereira el al. [66] (Chapter 1.2).

Table 1. Humidity, pH, titratable acidity, and CIELab values of the CBSs powders obtained from cocoa beans from different origins and cultivars.

Sample	Moisture[a]	pH	Titratable Acidity[b]	*L**	*a**	*b**
BRA	6.97 ± 0.38	4.06 ± 0.02	0.69 ± 0.00	40.15 ± 1.19	11.46 ± 0.61	22.98 ± 1.98
CAM1	5.46 ± 0.68	5.32 ± 0.01	0.22 ± 0.00	41.63 ± 0.87	11.96 ± 0.13	21.21 ± 0.45
CAM2	9.15 ± 0.27	6.31 ± 0.02	0.11 ± 0.00	36.23 ± 1.36	12.32 ± 0.15	21.01 ± 0.54
COL1	7.48 ± 0.25	4.83 ± 0.44	0.55 ± 0.24	42.88 ± 2.20	12.21 ± 0.34	21.78 ± 1.31
COL2	7.80 ± 0.75	5.42 ± 0.14	0.43 ± 0.05	36.94 ± 0.91	14.92 ± 1.01	26.24 ± 4.93
CON1	7.52 ± 0.30	4.90 ± 0.01	0.38 ± 0.00	43.72 ± 0.94	12.48 ± 0.17	24.58 ± 0.53
CON2	7.25 ± 0.34	5.20 ± 0.07	0.33 ± 0.03	40.22 ± 0.64	12.70 ± 0.23	23.28 ± 1.73
DOR1	6.44 ± 0.12	5.51 ± 0.03	0.47 ± 0.00	40.86 ± 1.42	14.03 ± 0.69	23.47 ± 0.77
DOR2	7.19 ± 0.48	5.58 ± 0.01	0.31 ± 0.05	45.91 ± 0.78	11.63 ± 0.71	22.16 ± 0.98
DOR3	7.85 ± 0.18	4.61 ± 0.01	0.42 ± 0.00	44.84 ± 0.83	12.15 ± 0.17	23.33 ± 0.47
DOR4	7.47 ± 0.27	5.42 ± 0.03	0.37 ± 0.01	32.66 ± 2.37	16.02 ± 1.18	32.20 ± 2.47
ECU1	8.45 ± 0.37	5.05 ± 0.18	0.32 ± 0.02	41.65 ± 1.21	13.61 ± 1.59	23.94 ± 1.30
ECU2	6.89 ± 0.41	4.97 ± 0.04	0.29 ± 0.01	45.81 ± 1.46	10.95 ± 0.77	21.29 ± 1.87
ECU3	6.74 ± 0.96	5.71 ± 0.01	0.34 ± 0.01	42.22 ± 2.01	14.62 ± 0.79	25.29 ± 0.62
ECU4	6.05 ± 0.14	5.71 ± 0.01	0.18 ± 0.00	35.98 ± 5.83	15.13 ± 0.67	30.74 ± 2.83
ECU5	7.12 ± 0.19	5.68 ± 0.01	0.23 ± 0.00	39.48 ± 9.47	14.19 ± 1.60	28.73 ± 4.87
ECU6	8.13 ± 0.38	4.96 ± 0.03	0.38 ± 0.00	47.01 ± 1.62	12.85 ± 0.71	26.22 ± 2.59
ECU7	6.46 ± 0.35	6.34 ± 0.01	0.18 ± 0.00	35.58 ± 0.93	12.89 ± 0.57	18.09 ± 0.89
GHA	9.18 ± 0.33	5.40 ± 0.08	0.18 ± 0.02	40.44 ± 3.39	12.54 ± 1.19	23.63 ± 1.18
IND	7.01 ± 0.18	5.49 ± 0.01	0.20 ± 0.01	42.33 ± 0.22	11.11 ± 0.35	20.72 ± 0.64
IVC	7.39 ± 1.44	5.46 ± 0.11	0.18 ± 0.01	35.45 ± 4.45	11.38 ± 0.29	19.45 ± 2.38
JAM	7.19 ± 0.35	6.32 ± 0.02	0.27 ± 0.00	35.68 ± 0.68	13.05 ± 0.23	21.06 ± 1.65
MAD	6.97 ± 0.36	4.96 ± 0.01	0.53 ± 0.01	43.12 ± 0.78	14.25 ± 0.06	23.92 ± 0.82
MEX	6.02 ± 1.46	5.24 ± 0.09	0.64 ± 0.03	36.34 ± 1.13	15.19 ± 0.55	23.88 ± 2.12
PER1	7.80 ± 1.03	5.57 ± 0.46	0.36 ± 0.23	36.90 ± 5.93	14.41 ± 2.43	24.72 ± 2.78
PER2	6.37 ± 0.67	5.17 ± 0.27	0.64 ± 0.15	39.61 ± 1.88	14.81 ± 0.52	25.29 ± 3.36
SAT1	6.95 ± 0.88	4.97 ± 0.03	0.40 ± 0.01	39.15 ± 1.70	15.92 ± 0.73	28.96 ± 5.04
SAT2	7.99 ± 0.39	5.32 ± 0.03	0.43 ± 0.01	42.36 ± 1.21	13.29 ± 0.41	24.15 ± 0.84
SAT3	6.87 ± 0.40	5.78 ± 0.05	0.35 ± 0.01	38.79 ± 0.92	13.49 ± 0.31	21.76 ± 1.15
SLE	7.12 ± 0.51	4.14 ± 0.02	0.70 ± 0.00	42.30 ± 2.24	11.03 ± 0.26	23.17 ± 0.61
TAN	6.70 ± 0.71	4.68 ± 0.11	0.42 ± 0.01	42.84 ± 0.80	11.93 ± 0.75	23.84 ± 1.61
TOG1	6.67 ± 0.90	4.33 ± 0.05	0.60 ± 0.01	41.48 ± 2.05	12.10 ± 0.76	24.58 ± 0.70
TOG2	7.68 ± 0.24	4.40 ± 0.19	0.28 ± 0.11	41.31 ± 2.21	12.56 ± 0.49	26.00 ± 1.41
UGA1	9.22 ± 0.79	5.19 ± 0.31	0.23 ± 0.09	43.63 ± 1.18	10.89 ± 0.54	22.25 ± 1.10
UGA2	7.92 ± 0.65	5.42 ± 0.04	0.16 ± 0.01	44.47 ± 1.32	11.01 ± 0.64	22.91 ± 1.11
VEN1	6.01 ± 0.81	4.82 ± 0.10	0.32 ± 0.01	46.28 ± 1.73	11.44 ± 0.65	23.08 ± 1.00
VEN2	6.23 ± 1.01	4.58 ± 0.09	0.48 ± 0.01	36.63 ± 0.44	14.34 ± 0.81	30.32 ± 1.63
VEN3	7.43 ± 0.88	6.02 ± 0.14	0.21 ± 0.02	37.89 ± 0.40	14.71 ± 0.21	26.46 ± 0.77
VEN4	6.30 ± 1.44	5.01 ± 0.31	0.36 ± 0.02	37.81 ± 1.22	13.85 ± 0.39	28.72 ± 1.56
VEN5	7.34 ± 0.80	5.94 ± 0.04	0.22 ± 0.03	35.36 ± 1.06	12.26 ± 0.36	20.83 ± 2.23
VEN6	7.44 ± 1.27	5.26 ± 0.02	0.39 ± 0.02	39.29 ± 2.82	14.29 ± 0.98	26.71 ± 4.44
VEN7	5.46 ± 0.45	5.95 ± 0.01	0.25 ± 0.00	37.97 ± 0.50	13.64 ± 0.28	19.86 ± 0.41
VEN8	5.63 ± 0.11	6.02 ± 0.01	0.27 ± 0.00	36.63 ± 1.02	13.56 ± 0.22	18.48 ± 0.54
VEN9	5.68 ± 0.11	5.49 ± 0.01	0.32 ± 0.00	40.78 ± 0.91	12.95 ± 0.13	21.35 ± 0.26
VEN10	5.74 ± 0.12	4.65 ± 0.02	0.55 ± 0.00	40.83 ± 0.87	12.37 ± 0.22	20.69 ± 0.41

[a]Moisture expressed as % wt/wt
[b]Titratable acidity (% acetic acid wt/wt) =((N*V*Eqwt)/(wt*1000))*100

2.3.1. Qualitative and quantitative analysis

The identification of the volatile organic compounds, focused on 101 molecules, was performed by comparing the EI-MS fragmentation pattern of each compound with those available on the National Institute of Standards and Technology (NIST05) mass-spectral library and on our home-based library as reported by Barbosa-Pereira et al (see section 2.3.2 and Table 1 in Chapter 1.2). The semi-quantitative concentrations of the VOCs identified were calculated as the area of the volatile marker component divided by the response factor of the ISTD 5-nonanol and expressed as micrograms of 5-nonanol equivalents per kg of sample (μg 5-nonanol Eq. kg^{-1} of CBS). CBS sample were analysed in triplicate and the data of the sum of each class of compound are shown in Table 2, while the data for a single molecule are detailed in Table S1 (Microsoft Excel Worksheet in supplementary material).

Table 2. Total contents of the different categories of volatile compounds determined in CBS powders from different geographical origins and cultivars.

Sample	Concentration* (µg kg-1 of CBS)													
	∑ Aldehydes	∑ Ketones	∑ Sulfur compounds	∑ Esters	∑ Hydrocarbons	∑ Furan derivates	∑ Pyrazines	∑ Alcohols	∑ Pyrroles	∑ Terpenes/ Isoprenoids	∑ Acids	∑ Lactones	∑ Others	∑ Total
BRA	2621.6	411.0	18.6	106.1	324.0	2225.5	405.4	348.5	467.5	349.3	462.9	99.2	25.8	7865.3
CAM1	2151.5	574.5	39.6	83.5	268.3	299.0	746.1	383.6	112.8	77.9	210.5	58.9	20.5	5026.7
CAM2	2353.0	995.8	23.1	90.9	569.1	279.6	499.0	696.8	143.6	68.5	100.4	133.5	60.2	6013.3
COL1	3088.4	638.0	68.3	282.6	119.0	418.5	865.1	682.2	167.3	126.2	1351.6	79.2	52.0	7604.7
COL2	4164.0	705.4	227.7	207.7	348.4	162.1	2240.4	619.5	112.6	111.9	490.5	32.9	116.4	9268.4
CON1	2948.6	763.4	82.4	297.8	205.8	436.2	1140.8	903.4	185.1	136.7	1189.4	87.2	27.8	7849.5
CON2	3773.8	665.6	76.5	199.4	290.5	534.8	1125.7	428.5	157.6	109.6	526.8	68.2	30.7	7907.8
DOR1	5123.9	546.1	577.4	158.1	242.5	193.9	2671.0	477.5	133.7	139.7	818.2	12.6	47.9	11013.4
DOR2	3820.2	605.5	181.4	178.3	244.0	304.0	1902.3	424.9	114.4	166.5	543.3	33.8	36.0	8478.2
DOR3	1894.5	597.4	11.9	359.7	298.2	677.7	505.5	759.2	111.5	270.2	795.3	49.5	18.2	5938.1
DOR4	4544.6	695.8	365.1	188.3	309.8	137.6	3272.7	824.6	214.3	120.6	1807.6	227.3	47.1	12279.3
ECU1	2244.0	564.8	37.6	199.1	261.3	345.0	689.3	514.1	142.5	93.4	871.6	33.3	20.6	5851.0
ECU2	2520.8	624.0	21.0	167.0	376.5	474.5	685.4	869.6	165.1	103.8	442.0	65.5	29.4	6023.6
ECU3	2871.3	431.1	184.3	176.2	137.7	196.6	1070.3	329.9	70.5	87.3	359.1	5.4	21.5	5959.7
ECU4	3883.3	990.1	113.4	312.5	251.7	164.4	1377.1	1908.8	200.6	107.3	1692.7	201.7	32.5	9675.7
ECU5	2772.7	1181.0	50.3	288.7	191.9	129.9	2784.3	1068.1	86.6	134.2	1255.1	30.8	33.8	9287.9
ECU6	2596.9	746.0	44.0	582.2	248.7	117.6	2112.0	779.9	92.1	52.1	1012.7	49.6	16.2	8018.6
ECU7	4405.9	1362.2	103.4	524.9	10.9	116.2	1916.0	2310.2	62.5	228.0	2124.0	4.4	37.6	11244.4
GHA	2020.7	832.8	29.3	112.2	413.7	233.7	940.0	667.3	116.2	221.8	1159.9	452.8	28.4	6909.8
IVC	2542.1	1076.1	31.3	73.4	1108.5	404.1	1263.5	876.3	235.3	124.1	1154.3	565.7	29.5	8956.4
JAM	4244.8	818.7	159.1	148.1	176.4	135.8	1697.8	1000.3	91.1	186.0	327.2	18.4	151.1	8503.0
MAD	4398.3	628.4	371.2	478.6	292.4	166.0	5140.7	277.1	198.8	66.5	1895.2	20.3	82.8	14087.7
MEX	4425.8	797.5	280.1	586.7	584.1	157.8	4353.5	319.2	79.3	48.8	1299.0	14.2	88.8	13034.6
PER1	4112.6	1044.0	197.8	1036.6	128.7	193.4	3031.1	1309.3	180.8	87.8	2155.1	214.8	34.1	13726.0
PER2	4951.5	979.8	281.1	830.8	319.8	227.0	4515.2	1084.9	183.6	74.4	1257.7	241.2	41.4	14988.4
SAT1	3163.1	343.8	150.7	127.9	200.2	213.0	1009.9	135.8	175.5	76.1	508.2	6.4	77.5	6188.1
SAT2	3669.2	514.0	158.6	312.9	145.7	153.7	1851.9	384.0	61.1	70.6	747.8	12.7	87.9	8170.2
SAT3	5122.6	846.7	284.3	195.8	370.9	174.3	2801.4	364.2	71.0	65.6	436.0	14.1	116.1	10863.0
SLE	1698.4	461.5	26.3	236.9	349.1	2004.0	198.3	474.0	157.0	133.7	1148.1	74.2	14.9	6976.5
TAN	3335.2	642.2	97.4	868.4	391.6	920.7	1131.5	934.5	277.2	154.8	1494.7	100.1	32.5	10380.7
TOG1	1712.2	291.7	29.3	316.6	318.2	2081.0	309.4	428.6	203.9	222.4	1404.0	367.8	16.9	7701.9
TOG2	1444.8	375.3	24.7	434.8	218.8	1918.9	272.5	439.5	182.0	183.7	1121.5	572.7	20.8	7210.2
UGA1	2666.4	747.9	33.6	385.6	635.9	399.8	1359.1	1032.2	161.7	119.4	1404.7	32.7	27.9	9007.0
UGA2	2849.8	826.1	28.5	201.8	227.7	354.2	792.6	880.5	142.4	114.8	421.1	13.5	26.5	6879.5
VEN1	1731.1	373.8	40.0	255.6	162.9	486.3	1176.0	266.6	112.0	65.7	832.5	25.4	20.8	5548.7
VEN2	2657.9	275.9	166.9	259.4	282.6	449.3	838.7	296.3	267.9	92.7	826.6	46.8	18.1	6479.0
VEN3	2621.5	421.0	43.3	146.1	142.8	101.1	665.2	302.6	48.7	43.4	338.6	6.7	29.6	4910.2
VEN4	1690.7	419.0	58.9	205.9	161.5	330.0	1138.0	514.6	118.4	124.0	503.6	11.8	16.5	5292.9
VEN5	4536.5	1115.8	115.4	308.1	536.7	294.3	3167.8	1046.2	114.0	174.5	329.7	49.9	55.3	11844.2
VEN6	2309.5	467.9	87.2	230.6	89.4	98.8	1702.0	492.5	67.3	102.3	484.1	11.2	20.5	6163.3
VEN7	4260.7	726.7	186.3	356.7	20.3	153.2	3918.3	1355.8	119.0	91.8	1654.9	10.3	40.6	12894.7
VEN8	4843.8	715.6	146.2	604.7	16.7	112.1	3731.4	891.2	84.6	47.0	1354.1	3.3	52.6	12603.4
VEN9	2977.5	593.9	132.5	760.0	21.0	487.9	5285.7	895.2	101.9	66.7	2100.8	29.2	26.8	13479.0

*The total amounts of each category of VOCs semi-quantified as 5-nonanol equivalents (µg kg-1 of CBS). Data are presented as the sum (∑) of the means of the different molecules for each category (n=6)

2.4. E-nose analysis

E-nose data were recorded using a portable electronic nose system PEN3 (Airsense Analytics GmbH., Germany). The system consists of a sampling unit and the gas detection system composed of 10 Metal Oxide Semiconductor (MOS) sensors, which are differentially sensitive to each characteristic volatile compound or group of compounds [262]. The analyses were performed as described by Barbosa-Pereira et al. [66] (Chapter 1.2). Each CBS sample was independently analysed in triplicates and the average of sensor responses (area under the curve) is shown in Table 3.

Table 3. Average of E-nose sensor responses G/G0 (area under the curve; G and G0 stand for the conductance of the MOS connected with the sample and clean gas, respectively), expressed as resistivity (Ohm), for CBS powders from different geographical origins and cultivars.

Sample	E-nose Sensors									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
BRA	21.67 ± 2.71	7066.91 ± 1413.30	25.19 ± 3.39	92.05 ± 1.00	28.58 ± 4.00	1640.40 ± 280.07	2553.81 ± 476.52	662.21 ± 154.87	1335.16 ± 217.20	101.07 ± 2.77
CAM1	24.32 ± 0.54	6471.08 ± 88.20	28.25 ± 0.42	89.67 ± 0.46	31.76 ± 0.37	1319.07 ± 16.36	1820.72 ± 68.53	481.43 ± 3.10	968.75 ± 47.29	98.44 ± 0.79
CAM2	19.44 ± 0.95	7476.53 ± 298.55	22.38 ± 0.72	93.80 ± 0.77	25.06 ± 0.59	1845.28 ± 72.22	2545.55 ± 249.02	764.69 ± 127.89	1291.37 ± 135.09	104.36 ± 0.74
COL1	21.82 ± 3.63	7363.27 ± 2195.01	25.39 ± 4.30	89.32 ± 0.98	28.52 ± 5.11	1586.49 ± 310.26	2746.75 ± 698.52	642.11 ± 166.67	1446.12 ± 309.88	97.98 ± 1.68
COL2	21.56 ± 2.58	7984.51 ± 1821.07	25.27 ± 3.04	90.98 ± 0.76	28.77 ± 3.38	1671.50 ± 269.72	2684.13 ± 479.19	634.39 ± 98.26	1423.07 ± 177.29	105.14 ± 3.46
CON1	19.13 ± 0.19	9443.36 ± 142.67	21.85 ± 0.17	89.55 ± 0.15	24.02 ± 0.12	1783.55 ± 5.68	3202.33 ± 74.91	698.94 ± 4.27	1655.81 ± 39.61	95.44 ± 0.29
CON2	18.68 ± 0.32	7708.13 ± 391.41	22.23 ± 0.74	92.90 ± 3.38	24.37 ± 0.96	2028.79 ± 338.99	2747.88 ± 198.67	788.78 ± 110.15	1375.48 ± 99.26	102.99 ± 8.56
DOR1	16.56 ± 2.26	11829.35 ± 2456.92	19.06 ± 2.66	92.44 ± 1.10	20.69 ± 3.62	2214.38 ± 211.63	3502.73 ± 442.91	924.91 ± 174.36	1687.11 ± 144.54	104.98 ± 2.07
DOR2	17.31 ± 2.33	10941.95 ± 2082.60	19.88 ± 2.78	93.02 ± 1.15	21.94 ± 3.82	2103.86 ± 257.59	3287.51 ± 478.67	892.91 ± 218.62	1601.99 ± 193.46	107.83 ± 1.92
DOR3	18.92 ± 0.09	8003.06 ± 28.39	21.80 ± 0.10	89.65 ± 0.19	23.68 ± 0.12	1790.95 ± 7.24	2610.91 ± 9.35	689.22 ± 4.38	1352.05 ± 12.65	93.67 ± 0.46
DOR4	18.70 ± 0.30	9687.95 ± 151.09	21.39 ± 0.28	89.04 ± 0.17	22.98 ± 0.32	1688.86 ± 15.24	2735.65 ± 138.98	723.46 ± 39.29	1474.48 ± 93.49	94.81 ± 0.42
ECU1	19.25 ± 0.66	8914.25 ± 235.90	22.29 ± 0.66	92.37 ± 0.95	25.19 ± 1.02	1896.56 ± 51.70	2971.79 ± 263.95	770.30 ± 119.21	1484.32 ± 125.55	108.43 ± 4.02
ECU2	21.48 ± 2.38	7361.28 ± 1716.64	25.02 ± 2.93	92.16 ± 1.60	28.38 ± 3.22	1627.18 ± 224.41	2601.30 ± 687.30	674.54 ± 171.02	1328.67 ± 327.70	104.41 ± 5.57
ECU3	19.20 ± 0.17	9074.20 ± 990.59	21.98 ± 0.27	92.43 ± 0.32	24.84 ± 0.49	1861.96 ± 33.03	3179.05 ± 173.82	745.17 ± 86.90	1625.42 ± 92.66	102.99 ± 0.65
ECU4	19.31 ± 0.35	9609.19 ± 149.24	21.66 ± 0.36	90.40 ± 0.06	22.98 ± 0.35	1601.76 ± 9.61	2313.98 ± 33.13	682.87 ± 3.95	1278.82 ± 26.81	93.15 ± 0.33
ECU5	19.67 ± 0.24	9341.42 ± 548.90	22.11 ± 0.22	89.85 ± 0.48	23.72 ± 0.18	1560.93 ± 21.72	2513.20 ± 60.96	699.18 ± 40.12	1434.21 ± 79.14	93.54 ± 0.42
ECU6	21.94 ± 5.00	8693.98 ± 1899.47	25.73 ± 5.29	90.12 ± 1.78	29.65 ± 6.17	1681.91 ± 457.92	3156.50 ± 804.72	673.04 ± 249.26	1631.93 ± 327.72	105.49 ± 4.77
ECU7	55.71 ± 3.48	1514.15 ± 270.69	57.24 ± 2.92	89.38 ± 0.15	55.23 ± 2.88	574.67 ± 47.85	1646.82 ± 105.21	242.67 ± 12.20	620.72 ± 11.39	90.93 ± 0.14
GHA	18.93 ± 0.36	8274.79 ± 644.82	21.89 ± 0.35	91.25 ± 0.60	24.46 ± 0.64	1890.14 ± 35.03	2797.61 ± 134.52	750.80 ± 85.80	1437.51 ± 99.64	102.10 ± 1.30
IND	25.30 ± 0.38	4874.39 ± 190.58	29.38 ± 0.17	89.00 ± 0.18	33.11 ± 0.16	1267.68 ± 21.82	1860.06 ± 176.65	490.07 ± 58.38	1011.30 ± 94.65	95.67 ± 1.32
IVC	17.69 ± 1.56	10417.91 ± 1928.62	20.13 ± 2.10	92.39 ± 1.03	21.78 ± 3.07	1984.29 ± 149.63	3021.47 ± 257.57	806.68 ± 112.71	1463.84 ± 52.88	103.01 ± 1.00
JAM	21.28 ± 2.66	8305.77 ± 890.69	24.61 ± 3.32	90.71 ± 1.13	27.63 ± 3.90	1657.18 ± 277.15	2551.96 ± 390.84	625.90 ± 106.84	1318.46 ± 165.16	101.70 ± 1.82
MAD	13.60 ± 0.84	14944.91 ± 211.24	15.86 ± 0.72	96.09 ± 2.33	16.73 ± 0.61	2685.12 ± 243.43	4464.91 ± 292.33	1188.71 ± 126.59	2072.15 ± 209.74	110.69 ± 2.45
MEX	21.34 ± 2.60	8754.26 ± 1136.11	25.38 ± 2.72	88.62 ± 1.49	29.06 ± 2.95	1768.73 ± 244.89	3489.61 ± 318.57	711.20 ± 127.84	1833.66 ± 136.42	103.28 ± 4.36
PER1	18.77 ± 0.74	9253.63 ± 1623.86	21.81 ± 0.70	90.92 ± 1.92	24.29 ± 0.89	1880.98 ± 57.96	3181.73 ± 472.45	747.68 ± 78.93	1615.90 ± 224.08	99.39 ± 1.56
PER1	20.64 ± 1.96	7902.55 ± 518.35	24.35 ± 2.86	89.04 ± 1.60	27.48 ± 4.08	1660.89 ± 130.46	3012.20 ± 370.89	648.06 ± 62.76	1603.76 ± 215.80	97.88 ± 2.80
SAT1	19.27 ± 0.64	10015.39 ± 226.20	22.80 ± 0.67	92.18 ± 0.53	25.83 ± 0.67	1935.85 ± 74.23	3239.05 ± 294.67	739.29 ± 96.39	1618.85 ± 135.67	107.17 ± 4.15
SAT2	17.86 ± 0.31	8806.54 ± 316.45	21.02 ± 0.21	89.04 ± 0.21	23.17 ± 0.18	1891.34 ± 58.48	3252.55 ± 346.96	756.60 ± 81.73	1685.34 ± 174.02	95.69 ± 0.61
SAT3	21.71 ± 3.10	7747.48 ± 2194.60	25.27 ± 3.67	90.38 ± 2.17	28.59 ± 3.98	1629.68 ± 315.13	2614.14 ± 612.93	616.95 ± 146.59	1367.30 ± 262.75	104.55 ± 7.31
SLE	19.64 ± 0.41	8540.30 ± 429.64	22.36 ± 0.36	89.93 ± 0.12	24.78 ± 0.41	1710.06 ± 9.25	3138.62 ± 263.82	725.62 ± 94.95	1671.07 ± 158.77	93.93 ± 0.38
TAN	21.13 ± 3.55	7226.23 ± 1776.82	24.68 ± 4.17	89.28 ± 0.24	27.58 ± 5.02	1579.49 ± 237.78	2577.40 ± 619.43	631.37 ± 130.42	1381.09 ± 342.89	96.31 ± 0.57
TOG1	18.42 ± 0.25	9096.71 ± 226.91	21.47 ± 0.19	90.55 ± 0.32	23.92 ± 0.24	1949.76 ± 22.78	3190.16 ± 163.26	759.22 ± 68.24	1616.83 ± 85.04	100.49 ± 0.46
TOG2	18.04 ± 0.38	9144.50 ± 648.34	21.21 ± 0.40	90.01 ± 0.38	23.57 ± 0.57	1913.86 ± 42.76	3269.53 ± 256.43	754.92 ± 72.44	1690.98 ± 143.82	96.44 ± 0.62
UGA1	18.67 ± 0.59	8883.83 ± 605.31	21.60 ± 0.53	90.86 ± 0.23	23.98 ± 0.65	1910.96 ± 51.92	2972.22 ± 200.97	750.44 ± 74.80	1489.62 ± 96.00	100.91 ± 0.10
UGA2	18.81 ± 0.40	7748.48 ± 512.48	21.64 ± 0.39	91.36 ± 0.84	23.57 ± 0.61	1741.24 ± 33.06	2514.97 ± 110.86	696.66 ± 56.16	1331.73 ± 92.41	94.93 ± 0.70
VEN1	18.49 ± 0.29	9322.05 ± 407.03	21.54 ± 0.40	92.34 ± 0.21	24.50 ± 0.71	2058.22 ± 45.29	3233.22 ± 257.10	808.82 ± 99.68	1629.05 ± 148.69	105.62 ± 0.72
VEN2	18.76 ± 1.04	12279.52 ± 340.46	22.22 ± 0.95	91.30 ± 0.68	25.54 ± 1.24	1943.82 ± 153.76	3815.66 ± 368.50	758.72 ± 112.61	1886.10 ± 163.19	107.07 ± 0.89
VEN3	19.30 ± 0.45	8369.49 ± 276.09	22.62 ± 0.37	91.98 ± 0.41	25.87 ± 0.43	1915.56 ± 59.49	2746.92 ± 218.96	754.34 ± 106.30	1400.97 ± 108.24	108.49 ± 0.97
VEN4	18.93 ± 0.36	9151.03 ± 178.30	22.15 ± 0.43	92.52 ± 0.27	25.34 ± 0.62	1964.55 ± 25.49	3058.53 ± 264.09	782.13 ± 116.28	1526.11 ± 132.82	109.71 ± 0.38
VEN5	21.03 ± 2.71	7408.06 ± 1578.26	24.48 ± 3.39	90.95 ± 1.43	27.63 ± 3.90	1710.84 ± 300.91	2658.24 ± 496.27	689.56 ± 168.85	1381.97 ± 217.53	101.56 ± 2.34
VEN6	23.26 ± 4.43	8058.15 ± 1845.73	26.77 ± 4.80	91.10 ± 1.07	30.30 ± 5.32	1561.91 ± 395.52	2547.69 ± 615.04	610.96 ± 145.44	1340.39 ± 228.82	105.14 ± 5.51
VEN7	57.71 ± 1.33	4055.88 ± 116.29	58.42 ± 1.25	88.99 ± 0.31	55.57 ± 1.39	582.72 ± 25.23	1746.05 ± 47.27	216.82 ± 7.81	727.72 ± 21.74	90.95 ± 0.04
VEN8	56.64 ± 2.34	2684.49 ± 119.20	57.39 ± 2.30	88.57 ± 0.06	54.95 ± 2.17	583.64 ± 40.85	1657.69 ± 78.70	226.60 ± 10.67	654.89 ± 19.80	91.00 ± 0.24
VEN9	55.58 ± 1.67	4277.87 ± 253.80	57.58 ± 1.79	88.54 ± 0.33	56.97 ± 4.62	577.43 ± 77.01	1997.31 ± 324.73	227.75 ± 14.88	756.53 ± 205.78	91.00 ± 0.09
VEN10	57.01 ± 0.86	3909.49 ± 255.19	57.15 ± 0.80	89.07 ± 0.17	54.64 ± 0.84	608.08 ± 16.61	1938.36 ± 36.70	226.49 ± 3.61	798.33 ± 19.07	91.35 ± 0.16

Data are presented as the mean (n=6) ± standard deviation

Supplementary material of the article forming Chapter 1.2.1 was composed by:

- Table S1. Content of identified volatile compounds (RI and MS confirmations) in CBS powders from different geographical origins and cultivars.

This data set was extremely large and was considered of minimal importance for the well understanding of the chapter. Due to space issues and for ecology purposes, it was not included in the present manuscript. However, if the reader desires to consult it, the data set can be downloaded following the next link:

https://ars.els-cdn.com/content/image/1-s2.0-S2352340919306225-mmc1.xlsx

PART 2 – COCOA BEAN SHELL-BASED FUNCTIONAL FOODS

After confirming the cocoa-similar aroma profile of CBS and its richness in polyphenols with biofunctional potential, **Part 2** was devoted to the development and study of some CBS-based functional foods, and is composed by four different chapters issued from four different published scientific articles. Specifically, some CBS-based functional beverages and biscuits with potential to be adapted as antidiabetic food products were proposed and studied from different points of view. These two different types of food were proposed because of the differences in their food matrices, so different parameters could be considered and lately compared. Foods were studied from a technological point of view in order to understand the influence of CBS addition in the technological factors linked to the food production and the final product. Also, consumer acceptance tests and sensory evaluations of the model foods were performed. On the other hand, the CBS-based functional foods were analyzed to assess their biofunctional potential and the influence of the food matrix in the way that this potential is conserved along the digestion processs. It is worth mentioning that safety assays on CBS were not performed in the present study as those were already performed by our suppliers and the prepared foods were therefore considered as safe for human consumption.

Chapter 2.1 addresses the development of some CBS functional beverages and the optimization of the preparation technique and CBS presentation to obtain the most functional and polyphenol-rich beverages. Among the preparation techniques, two were selected because of their presence and common use among consumers: capsule (percolation) and tea bag (maceration). These selected beverages were sensory optimized in **Chapter 2.2** and a study of their polyphenol and methylxanthine bioaccessibility after an *in vitro* digestion was performed. In addition, the changes in two functional properties (antiradical and α-glucosidase inhibition capacities) after gastrointestinal digestion were also assessed.

After confirming the antidiabetic potential of CBS by the inhibition of the α-glucosidase enzyme, some CBS-functional biscuits were envisaged and adapted to diabetic consumers by utilizing tagatose (a low-glycemic index sugar) instead of sucrose. **Chapter 2.3** reports the influence of CBS addition and the utilization of tagatose in the technological qualities of the biscuits, as well as a sensory study of the developed biscuits. Besides, the dietary fiber content provided to the biscuits by the CBS powder is assessed in this chapter. **Chapter 2.4** was devoted to the study of the biscuits with a nutritional approach. Again, bioaccessibility and changes in functional properties after *in vitro* gastrointestinal digestion were studied, and the influence of a more complex food matrix was assessed. In addition to this, the intestinal permeability of some target compounds within the digested biscuits was also studied.

CHAPTER 2.1

Effects of Particle Size and Extraction Methods on Cocoa Bean Shell Functional Beverage

This chapter is focused on the development and optimization of CBS-based functional beverages with antioxidant and antidiabetic properties. The purpose of this work was to evaluate the effects of the CBS particle size and extraction methods on the chemical composition and consumer acceptance of a functional beverage, in order to find the best combination of technological parameters and health benefits. Besides, the analysis of the prepared beverages allowed confirming the antidiabetic potential of CBS.

This chapter was issued and adapted from a published scientific article:

Effects of Particle Size and Extraction Methods on Cocoa Bean Shell Functional Beverage

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If the reader desires it, the original paper can be consulted in this link

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1. Introduction

According to the International Cocoa Organization (ICCO), each year, more than 4.7 million tons of cocoa beans are processed and consumed worldwide [250]. Considering that the main products of cocoa are obtained from its roasted bean, which represents only 10% of the total weight of the fruit, cocoa processing produces a large amount of vegetal residue. Besides being expensive, the disposal of these by-products can be harmful for the environment because they contain potentially phytotoxic polyphenols [14] and high concentrations of theobromine, which may be toxic for non-human mammals [15]. Such underutilization of residual biomass can be overcome by the development of an added-value foodstuff based on cocoa by-products, particularly the cocoa bean shell (CBS), which represents 12% to 20% of the cocoa bean [220]. CBS has been reported to be a considerable source of proteins and dietary fiber, with low fat content in comparison with cocoa beans [28] but with a similar profile of volatiles [72]. Considering that CBS is a final-stage by-product from cocoa processing, it appears to be an economical, organoleptic, and nutritionally rewarding substance for the transformation of cocoa bean industries. Most of the research into CBS utilization is related to animal feeding and, despite the presence of theobromine, CBS has positive effects on fortified diets for ruminants, pigs, and poultry [15], among other animals. Besides, CBS has been applied as an additive in organic fertilizer [274], as biomass for biogas production [145], or as a pectin source [28], among other applications. The application of this cocoa by-product to food has attracted some attention due to its nutritional characteristics and high concentration of phenolic compounds, mainly flavonoids [84]. In recent years, some studies in the food research field were published suggesting CBS as a food ingredient [220]. This research interest can be linked to the sustainability and bioeconomy framework of the modern food and agricultural industries leading to the valorization of functional foods developed from by-products, as an opportunity to make healthier foods. Functional foods are similar to conventional ones in that they are part of a standard diet and consumed on a regular basis and in regular amounts. A functional food is claimed to have

proven benefits for the maintenance or promotion of a state of well-being or health or a reduction in the risk of a pathological process or disease [275]. Although this market niche is not well defined, by influencing the data on global sales, functional foods irrefutably represent a top trend in the food industry.

Because of their antioxidant capacity, phenolic compounds may be capable of protecting cell components from oxidative damage, thus limiting the risk of several diseases associated with oxidative stress, for instance, diabetes [276]. The relevance of polyphenols to the management of blood glucose is mainly due to their inhibition of digestive enzymes involved in the metabolism of carbohydrates (α-glucosidase and α-amylase) [84]. Even if these phenolic compounds are poorly absorbed, they can still act on membrane-bound enzymes located in the intestinal epithelium. Moreover, other mechanisms of action of polyphenols on glucose uptake, after ingestion of carbohydrate-rich meals, are being studied [277]. The presence of bioactive components in CBS and its sustainable character may arouse interest in such a product as a potential ingredient in the functional-beverage industry. Despite their resource-rich matrix, the effective extraction of plant bioactive compounds and their concentrations in the final product can be influenced by processing or preparation methods.

Unground CBS has already been utilized as one of the ingredients for commercialized herbal infusion bags, such as the ‘ChocoTea’ infusion bags from Valberbe® or the ‘Choco’ tisane bags from YogiTea®. Nonetheless, other preparations destined for the use of this by-product in home-based beverage-making techniques have not been proposed yet. In this paper, several CBS preparations were developed to be employed in six diffused techniques for coffee home-preparation available at a consumer level, such as the Moka, Neapolitan flip, American, Espresso, Capsule, and French press coffee makers. Instead of coffee powder, CBS at different grinding degrees (GDs) was employed to find the optimal GD for each extraction technique. The aim of this study was to find the best combination of the CBS GD and the beverage preparation technique in order to obtain a new functional beverage with the optimal chemical composition, biological effects (antioxidant and antidiabetic properties), and sensory characteristics.

2. Materials and Methods

2.1. Chemicals

Folin & Ciocalteu's phenol reagent, sodium carbonate (≥99.5%), 2,2'-diphenyl-1-picrylhydrazyl (95%) (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%; trolox), vanillin (99%), (+)-catechin hydrate (>98%), methanol (≥99.9%), hydrochloric acid (fuming 37%), aluminium chloride (99%), sodium nitrite (≥99%), α-glucosidase from intestinal acetone powders from rat, p-nitrophenyl-α-D-glucopyranoside (≥99%; p-PNG), acarbose (≥95%), potassium phosphate monobasic (≥99%), formic acid (≥98%), quercetin-3-O-glucoside (≥90%; Q-3-G), theobromine (≥98.5%), caffeine (≥98.5%), and quercetin (≥98.5%) were provided by Sigma-Aldrich (Milan, Italy). Potassium phosphate dibasic (≥ 98%) was acquired from Carlo Erba (Milan, Italy). Gallic acid, ethanol (≥99.9%), sodium hydroxide (1 M), (-)-epicatechin (>90%), procyanidin B1 (≥98.5%; PCB1), procyanidin B2 (≥98.5%; PCB2), protocatechuic acid (>97%), caffeic acid (≥95%), and vanillic acid (≥99%) were supplied by Fluka (Milan, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

2.2. Samples

CBS from São Tomé cocoa beans (Forastero variety) was kindly supplied by Pastiglie Leone S.r.l. (Turin, Italy). The CBS was divided into different grain sizes using a BA200N vibrating sieve (CISA, Barcelona, Spain). Five GDs were obtained: above 4000 μm (GD1), 2000–4000 μm (GD2), 1000–2000 μm (GD3), 500–1000 μm (GD4), and 250–500 μm (GD5).

The chemical and nutritional characterization of CBS was carried out according to Bertolino et al. [278].

2.3. Preparation of beverages

The beverages were prepared by six techniques available to consumers, i.e. Moka Express 6 cups (Bialetti, Brescia, Italy), Neapolitan flip coffee pot (Ilsa, Turin, Italy),

American coffee maker Cucina HD 7502 (Phillips, Milano, Italy), Espresso Saeco HD 8423/11 (Phillips, Milano, Italy), Capsule LM 3100 (AEG, Milano, Italy), and French press Kaffe (Ikea, Collegno, Italy). Still mineral water (Valmora, Luserna San Giovanni, Italy) was used for the beverage production. Each of the GDs was tested with all the coffee makers, resulting in 30 beverages. Three production batches were generated for each beverage.

The water quantities employed varied for each technique following the established rules for the use of the different machines. For Espresso and Capsule techniques, the initial volumes of water are unknown because both machines operate with a continuous inlet of water and therefore it is possible to define only the final volume of the obtained beverage. In addition, the quantities of CBS powders used for the beverage preparations were adapted to each technique and GD to obtain a technologically viable formulation.

All the beverages were centrifuged on a MPW-260R centrifuge (MPW, Warsaw, Poland) at 3075 × g for 10 minutes and then passed through a 0.45 μm cellulose acetate filter (Carlo Erba, Milan, Italy) before analyses.

2.4. Analytical procedures

2.4.1. Physico-chemical analysis

A pH meter MICROpH 2002 (CRISON, Carpi, Italy) served for pH measurement.

Determination of total acidity (expressed in grams of acetic acid per liter of a beverage) was performed by potentiometric titration of 5 mL of a beverage (diluted to 50 mL with distilled water) by means of 0.01N NaOH up to pH 8.2.

The dry extract of each beverage was analyzed gravimetrically for a 5 mL sample of each beverage dried in an oven at 110°C until constant weight.

The color analysis was conducted in transmittance mode on a CM-5 spectrophotometer (Konica Minolta, Tokyo, Japan). *L**, *a**, and *b** CIELab parameters were used to measure the color, where *L** is a coefficient of lightness ranging from 0 (black) to 100 (white), *a** indicates the colors red-purple (when positive *a**) and bluish-green (when negative *a**), and

*b** denotes the colors yellow (when positive *b**) and blue (negative *b**). The ΔE parameter, which represents the difference between two colors [279] and its perceptibility by the human eye when ΔE > 2.5, was calculated according to the following equation:

$$\Delta E = \sqrt{(L*_a - L*_b)^2 + (a*_a - a*_b)^2 + (b*_a - b*_b)^2}$$

2.4.2. Total phenolic, tannin, and flavonoid Contents

The total phenolic (TPC), total flavonoid (TFC), and total tannin (TTC) contents were determined according to the methods described by Barbosa-Pereira et al. [57], in 96-well microplates, using a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy), as described in Chapter 1.1. All the measurements were performed in triplicate. For the TPC analysis, a calibration curve of gallic acid (20–100 mg/L) was constructed to quantify the concentration, which was expressed in milligrams of gallic acid equivalents per liter of a beverage (mg GAE/L). The quantification of both TFC and TTC was performed based on a standard curve of catechin (5–500 mg/L), and the concentrations were expressed in milligrams of catechin equivalents per liter of a beverage (mg CE/L).

2.4.3. Antioxidant capacity

The antioxidant capacity of the beverages was assessed by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH•) radical–scavenging method described by Barbosa-Pereira et al. [57], as described in Chapter 1.1. All the assays were conducted in triplicate in 96-well microplates with the BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments). Antioxidant capacity was calculated as the inhibition percentage (IP) of the DPPH radical as follows:

$\text{IP}\ (\%) = \frac{(A_0 - A_{30})}{A_0} \times 100$, where A0 is absorbance at the initial time point, and A30 is the absorbance after 30 minutes.

A standard curve of trolox was constructed (12.5–300 μM) for assessment of the radical-scavenging activity values, which were expressed as millimoles of trolox equivalents per liter of a beverage (mmol TE/L).

2.4.4. Antidiabetic capacity

The antidiabetic activity was determined based on the method described by Kwon et al. [280], adapting it to a 96-well microplate. The principle of this assay is based on the inhibition of the enzyme α-glucosidase, which belongs to the class of hydrolases and is specific for the α (1 → 4) glycosidic bonds, thus acting in the non-reducing ends of the polysaccharide chain, allowing the release of α-D-glucose (Figure 1).

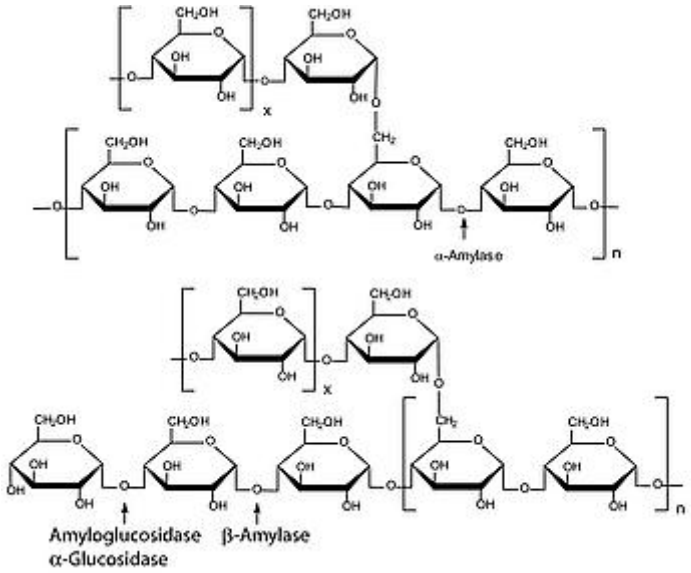

Figure 1. Site of action of α-glucosidase in the polysaccharide chains.

Acarbose is a molecule of microbial origin. It is an oligosaccharide with a saccharide-like structure that belongs to the category of α-glucosidase inhibitors. This molecule can act selectively in the intestine, reducing the activity of the enzyme and limiting in this way the availability and absorption of glucose. Therefore, it has an antidiabetic activity since it decreases the post-prandial glycemic index. Natural inhibitors with activities similar to that of acarbose can be assumed with the diet.

For the study of the antidiabetic effects of the beverages, an aliquot (50 µL) of the sample was mixed with 100 µL of α-glucosidase (10 mg/mL), prepared in 0.1 M phosphate buffer pH 6.9, and incubated for 5 minutes. After that, 50 µL of substrate *p*-PNG (4-Nitrophenyl α-D-Glucopyranoside) at 4 mM (prepared in the phosphate buffer) was added, and the solution was mixed. The solution was incubated for 30 minutes at 37°C, and then absorbance was measured at 405 nm against a blank control. The substrate in the presence of the enzyme that has not been inhibited by the sample reacts according to the following reaction:

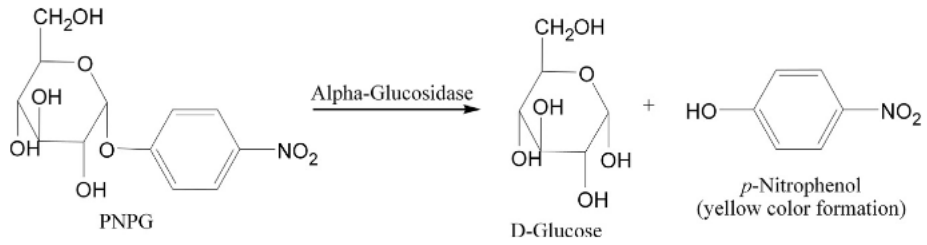

Figure 2. Reaction of the *p*-PNG subtract and the α-glucosidase enzyme

The *p*-nitrophenol is an indicator that turns the solution yellow at alkaline pH and allows for the evaluation of the residual enzyme activity. The no-apparition of yellow color in the solution (α-glucosidase inhibited by polyphenols and not by p-nitrophenol) can be then measured at 405 nm and compared to a standard of acarbose.

Acarbose at 0.5 mM (half-maximal inhibitory concentration, IC_{50}) served as a positive control for this assay, and the antiabetic capacity was expressed as the α-glucosidase inhibition percentage. All the measurements were conducted in triplicate in 96-well microplates, using the BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments).

2.4.5. RP-HPLC-PDA analysis

Characterization of the polyphenols contained in the beverages was performed by means of reversed-phase high-pressure liquid chromatography with a photodiode array detector (RP-HPLC-PDA) as described in section 2.4 of Chapter 1.1.

Detection was carried out via continuous scanning of wavelengths between 200 and 400 nm. Methylxanthines (theobromine and caffeine) were quantified at 272 nm, protocatechuic acid at 293 nm, caffeic acid at 325 nm, flavan-3-ols (catechin, epicatechin, and catechin-3-O-glucoside) and procyanidins B (type B procyanidin and procyanidin B2) were quantified at 280 nm, and flavonols (quercetin, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside) at 365 nm.

The quantification was performed based on external linear calibration curves analyzed under the same conditions and the following correlation coefficients were obtained: R^2 = 0.9995 for theobromine, R^2 = 0.9996 for caffeine, R^2 = 0.9999 for catechin, R^2 = 0.9998 for epicatechin, R^2 = 0.9997 for protocatechuic acid, R^2 = 0.9999 for caffeic acid, R^2 = 0.9998 for procyanidin B1 (PB1), R^2 = 0.9999 for procyanidin B2 (PB2), R^2 = 0.9996 for quercetin-3-O-glucoside (Q-3-G), and R^2 = 0.9988 for quercetin. For catechin-3-O-glucoside, type B procyanidin, and quercetin-3-O-rhamnoside, concentrations were expressed as catechin, procyanidin B1, and quercetin-3-O-glucoside equivalents, respectively.

2.5. Consumer acceptance evaluation

For each beverage, a consumer test was carried out with 20 tasters where appearance, odor, taste, flavor, texture, overall liking, and purchase predisposition were evaluated on a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like) [281]. The tests were performed in an air-conditioned room with white light at approximately 21°C.

2.6. Statistical analysis

All the obtained results were subjected to analysis of variance (ANOVA) with Duncan's post hoc test at 95% confidence level and to linear regression analysis in the Windows software called STATISTICA, version 13.3 (StatSoft Inc., Tulsa, OK, USA).

Values obtained by the consumer test were analyzed by the Kruskal–Wallis test (test H).

3. Results and Discussion

3.1. Cocoa bean shell – chemical and nutritional composition

The chemical and nutritional composition of the cocoa bean shell employed in beverage preparation, expressed for 100 g of dried product, was as follows: protein: 20.9 g, fat: 2.3 g, carbohydrates: 7.85 g, dietary fiber: 55.1 g (42.3 g of insoluble fiber and 12.8 g of soluble fiber), water: 5.9 g, and ash: 7.9 g.

3.2. Beverage yield

Thirty formulations were developed, resulting in 30 beverages with different yields, mostly depending on the CBS GD (Table 1). Yields ranged from 72.0% to 93.3% when GDs above 500 μm were used, and a notable substantial decrease in the recovery percentage was observed with a reduction in the GD, thereby leading to such values as 29.6% for the beverage prepared with the Moka and GD5. This decrease could be mostly due to the water-holding capacity of the insoluble fiber present in the CBS; this fiber became more available when the surface-to-volume ratio of the CBS powders increased. In some cases, also the larger CBS powder quantities used in order to obtain technologically realistic preparations influenced on water-holding capacity. Nevertheless, this influence may not affect the beverages obtained with other coffee makers such as the American pot or French press where the CBS quantities remain the same at all the GDs, and the reduction in the yield is due to the lower GD only.

Table 1. Amounts of CBS powder and water utilized for the beverage preparations, volume of the obtained beverages, and process yield registered for each production technique and GD. ANOVA was performed on the process yields among GDs and extraction techniques.

		Moka	**Neapolitan**	**American**	**Espresso**	**Capsule**	**French press**	*Sig.*
> 4000 µm	Water (mL)	230.00	400.00	200	n/a	n/a	100.00	
	CBS powder (g)	10.00	14.00	8.00	7.00	2.00	6.00	
	Beverage (mL)	188.00 ± 3.46	373.33 ± 2.89	172.00 ± 1.73	123.67 ± 1.53	59.33 ± 0.58	82.67 ± 0.58	
	Yield (%)	81.74 ± 1.51^{bC}	93.33 ± 0.72^{aA}	86.00 ± 0.87^{aB}	n/a	n/a	82.67 ± 0.58^{aC}	***
2000-4000 µm	Water (mL)	230.00	400.00	200.00	n/a	n/a	100.00	
	CBS powder (g)	18.00	17.00	8.00	13.00	2.80	6.00	
	Beverage (mL)	190.00 ± 2.00	363.33 ± 2.89	159.67 ± 6.81	125.67 ± 2.08	60.33 ± 0.58	83.00 ± 1.00	
	Yield (%)	82.61 ± 0.87^{abBC}	90.83 ± 0.72^{aA}	79.83 ± 3.40^{bC}	n/a	n/a	83.00 ± 1.00^{aB}	***
1000-2000 µm	Water (mL)	230.00	400.00	200.00	n/a	n/a	100.00	
	CBS powder (g)	22.00	30.00	8.00	16.00	6.50	6.00	
	Beverage (mL)	188.33 ± 1.53	329.66 ± 1.53	152.67 ± 1.15	122.67 ± 0.29	60.33 ± 0.58	78.67 ± 1.15	
	Yield (%)	81.88 ± 0.66^{bA}	82.42 ± 0.38^{aA}	76.33 ± 0.58^{cC}	n/a	n/a	78.67 ± 1.15^{bB}	***
500-1000 µm	Water (mL)	230.00	400.00	200.00	n/a	n/a	100.00	
	CBS powder (g)	26.00	30.00	8.00	18.00	8.00	6.00	
	Beverage (mL)	186.67 ± 2.31	316.67 ± 5.77	148.67 ± 4.16	119.67 ± 0.58	60.33 ± 0.58	72.00 ± 1.73	
	Yield (%)	81.16 ± 1.00^{bA}	79.17 ± 1.44^{aA}	74.33 ± 2.08^{cB}	n/a	n/a	72.00 ± 1.73^{cC}	***
250-500 µm	Water (mL)	230.00	400.00	200.00	n/a	n/a	100.00	
	CBS powder (g)	26.00	30.00	8.00	18.00	8.00	6.00	
	Beverage (mL)	68.00 ± 13.86	200.00 ± 81.85	146.67 ± 2.31	71.00 ± 9.64	36.35 ± 0.28	71.33 ± 1.53	
	Yield (%)	29.57 ± 6.02^{cC}	50.00 ± 20.46^{bB}	73.33 ± 1.15^{cA}	n/a	n/a	71.33 ± 1.53^{cA}	***
Sig.		***	**	***	n/a	n/a	***	

n/a not applicable

Means followed by different lower case superindexes within the same column (different grinding degrees) and by upper case superindexes within the same row (different techniques) are significantly different at $p < 0.05$

Significance: ** $p < 0.01$; *** $p < 0.001$

Data are expressed as mean values (n=3) ± standard deviation

For the lowest GD (GD5, 250–500 µm), the beverage yields decreased considerably, and this parameter, in general, lost the repeatability observed for larger GDs, mostly owing to the technological problems during the beverage preparation such as machine blockage in cases where the CBS absorbed too much water or with extremely long preparation periods. Due to the aforementioned problems, the beverages obtained by the Moka, Neapolitan, Espresso, and Capsule techniques with GD5 were assumed to be not technologically viable and it was not possible to proceed with further analyses. Only the beverages obtained with the French press and American techniques were considered for GD5.

3.3. Physico-chemical characterization

3.3.1. Acidity, dry matter and color

The pH and the titratable acidity results obtained for the functional beverages are shown in Table 2. The beverages had pH and titratable acidity ranging from 4.84 to 5.19 and from 0.12 to 1.64 g of acetic acid equivalents per liter of a beverage, respectively. In general, lower pH and higher acidity were observed in beverages produced with a lower GD, except for the beverages produced with the Moka and the Neapolitan techniques, which showed nonsignificant differences in pH when the GD was varied. Nevertheless, they still showed the same tendency for acidity, which increased with a decrease in particle size, probably owing to a major acid extraction when the CBS surface-to-volume ratio was increased. This tendency was not observed for the beverage produced with the French press technique where pH and acidity were found to be independent of the CBS particle size.

For all the beverages obtained by percolation techniques (Moka, Neapolitan, American, Espresso, and Capsule), the quantity of dry matter increased when the GD was reduced (Table 2) because the extraction seemed to be more effective at low GD. For French Press, the quantity of dry matter did not correlate with the GD of CBS, suggesting that the extraction by this maceration technique was barely affected by the particle size of the CBS powder.

Table 2. pH, titratable acidity, dry matter, and CIELab values of the beverages obtained by each extraction technique and GD of CBS, and ANOVA among GDs for each technique.

Technique	Grinding Degree (μm)	pH	Titratable acidity (g acetic acid eq/L)	Dry weight (%)	*L**	*a**	*b**
Moka	> 4000	4.91 ± 0.05^{a}	0.40 ± 0.05^{d}	0.52 ± 0.06^{d}	82.73 ± 1.51^{a}	5.69 ± 1.36^{d}	50.84 ± 3.99^{c}
	2000-4000	4.88 ± 0.01^{a}	0.61 ± 0.05^{c}	0.84 ± 0.11^{c}	75.91 ± 0.64^{b}	11.65 ± 1.06^{c}	64.77 ± 2.83^{b}
	1000-2000	4.89 ± 0.03^{a}	0.95 ± 0.06^{b}	1.53 ± 0.06^{b}	61.20 ± 2.83^{c}	25.76 ± 1.85^{b}	80.92 ± 0.54^{a}
	500-1000	4.93 ± 0.01^{a}	1.64 ± 0.09^{a}	2.70 ± 0.17^{a}	33.21 ± 1.96^{d}	37.75 ± 1.12^{a}	55.08 ± 3.30^{c}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	ns	***	***	***	***	***
Neapolitan	> 4000	4.92 ± 0.03^{a}	0.29 ± 0.03^{c}	0.41 ± 0.01^{c}	82.76 ± 0.70^{a}	5.52 ± 0.50^{c}	48.74 ± 1.33^{c}
	2000-4000	4.91 ± 0.04^{a}	0.37 ± 0.04^{c}	0.49 ± 0.06^{c}	80.56 ± 2.80^{a}	7.33 ± 2.19^{c}	54.14 ± 5.51^{c}
	1000-2000	4.88 ± 0.03^{a}	0.95 ± 0.08^{b}	1.50 ± 0.15^{b}	56.56 ± 4.34^{b}	28.45 ± 2.89^{b}	79.31 ± 0.68^{a}
	500-1000	4.93 ± 0.01^{a}	1.23 ± 0.04^{a}	2.00 ± 0.13^{a}	39.04 ± 2.66^{c}	37.24 ± 0.70^{a}	63.97 ± 4.02^{b}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	ns	***	***	***	***	***
American	> 4000	4.96 ± 0.01^{b}	0.26 ± 0.01^{d}	0.36 ± 0.01^{c}	87.98 ± 0.53^{a}	1.98 ± 0.18^{e}	38.34 ± 0.68^{e}
	2000-4000	5.07 ± 0.04^{a}	0.37 ± 0.02^{c}	0.51 ± 0.04^{bc}	85.17 ± 0.64^{b}	4.02 ± 0.51^{d}	48.39 ± 1.86^{d}
	1000-2000	5.07 ± 0.01^{a}	0.40 ± 0.01^{b}	0.69 ± 0.01^{b}	80.12 ± 0.34^{c}	8.35 ± 0.26^{c}	59.79 ± 0.60^{c}
	500-1000	4.99 ± 0.02^{b}	0.55 ± 0.01^{a}	1.01 ± 0.26^{a}	73.99 ± 1.33^{d}	14.54 ± 1.37^{b}	71.05 ± 1.92^{b}
	250-500	4.87 ± 0.01^{c}	0.57 ± 0.01^{a}	1.00 ± 0.04^{a}	64.30 ± 0.74^{e}	23.80 ± 0.72^{a}	80.12 ± 0.31^{a}
	Significance	***	***	***	***	***	***
Espresso	> 4000	5.09 ± 0.07^{a}	0.20 ± 0.02^{d}	0.28 ± 0.04^{d}	91.14 ± 1.37^{a}	0.58 ± 0.69^{d}	29.45 ± 3.67^{d}
	2000-4000	4.98 ± 0.04^{b}	0.37 ± 0.04^{c}	0.51 ± 0.05^{c}	86.15 ± 1.81^{b}	3.32 ± 1.29^{c}	44.39 ± 4.08^{c}
	1000-2000	4.98 ± 0.01^{b}	0.61 ± 0.05^{b}	0.89 ± 0.07^{b}	77.63 ± 2.16^{c}	10.61 ± 2.13^{b}	64.10 ± 3.74^{b}
	500-1000	4.87 ± 0.02^{c}	1.08 ± 0.02^{a}	1.62 ± 0.05^{a}	65.87 ± 0.52^{d}	22.10 ± 0.51^{a}	79.52 ± 0.69^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	**	***	***	***	***	***
Capsule	> 4000	5.19 ± 0.04^{a}	0.12 ± 0.01^{d}	0.15 ± 0.00^{d}	94.73 ± 0.12^{a}	-0.47 ± 0.05^{c}	18.15 ± 0.56^{d}
	2000-4000	5.15 ± 0.04^{a}	0.17 ± 0.01^{c}	0.24 ± 0.00^{c}	92.73 ± 0.21^{a}	-0.08 ± 0.06^{c}	25.28 ± 0.90^{c}
	1000-2000	5.15 ± 0.02^{a}	0.43 ± 0.00^{b}	0.66 ± 0.01^{b}	82.41 ± 0.67^{b}	6.22 ± 0.58^{b}	53.72 ± 1.56^{b}
	500-1000	4.84 ± 0.01^{b}	0.93 ± 0.03^{a}	1.33 ± 0.06^{a}	68.71 ± 1.92^{c}	19.06 ± 1.73^{a}	75.91 ± 1.66^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	***	***	***	***	***	***
French press	> 4000	4.91 ± 0.05^{ab}	0.62 ± 0.00^{a}	0.91 ± 0.06^{a}	77.73 ± 1.53^{b}	10.80 ± 1.47^{a}	67.09 ± 2.30^{a}
	2000-4000	4.94 ± 0.02^{ab}	0.65 ± 0.05^{a}	0.91 ± 0.05^{a}	76.48 ± 2.49^{b}	11.97 ± 2.55^{a}	68.31 ± 4.37^{a}
	1000-2000	4.94 ± 0.01^{ab}	0.50 ± 0.04^{b}	0.86 ± 0.10^{a}	77.42 ± 2.71^{b}	10.96 ± 2.65^{a}	65.83 ± 4.68^{a}
	500-1000	4.96 ± 0.01^{a}	0.40 ± 0.02^{c}	0.66 ± 0.05^{b}	82.23 ± 1.42^{a}	6.53 ± 1.27^{b}	56.03 ± 3.50^{b}
	250-500	4.90 ± 0.02^{b}	0.49 ± 0.03^{b}	0.83 ± 0.05^{a}	75.22 ± 0.36^{b}	12.75 ± 1.40^{a}	67.87 ± 3.18^{a}
	Significance	ns	***	**	*	*	*

n/a not applicable
Means followed by different letters are significantly different at $p < 0.05$
Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant
Data are expressed as mean values (n=3) ± standard deviation

Regarding the chromatic parameters (Table 2), generally, the brightness parameter decreased with the decreasing GD due to the increase in the surface-to-volume ratio of the CBS powder thereby allowing for better extraction of color pigments. The beverages having the lowest values of *L** and therefore, darker beverages, were those obtained at the GD4 with Moka and Neapolitan techniques. Generally, both parameters *a** and *b** rose with the

decreasing GD, as the beverages became browner. Always following a similar trend, various beverages showed significant differences when the GD was changed except for the beverages produced with the French press. This fact can be numerically explained by the ΔE parameter (data not shown), which determines whether two colors can be distinguished by the human eye (ΔE > 2.5). Considering the different CBS GDs within each technique, the beverages showed values of ΔE higher than 2.5 except for some beverages obtained with the French press technique, where $\Delta E_{GD1\text{-}GD2}$ = 2.10, $\Delta E_{GD1\text{-}GD3}$ = 1.31, and $\Delta E_{GD2\text{-}GD5}$ = 1.55, which means that these beverages had colors indistinguishable for the human eye.

3.3.2. Polyphenolic content

TPC, TFC, and TTC data are presented in Figure 3 (Fig. 3a, 3b, and 3c, respectively).

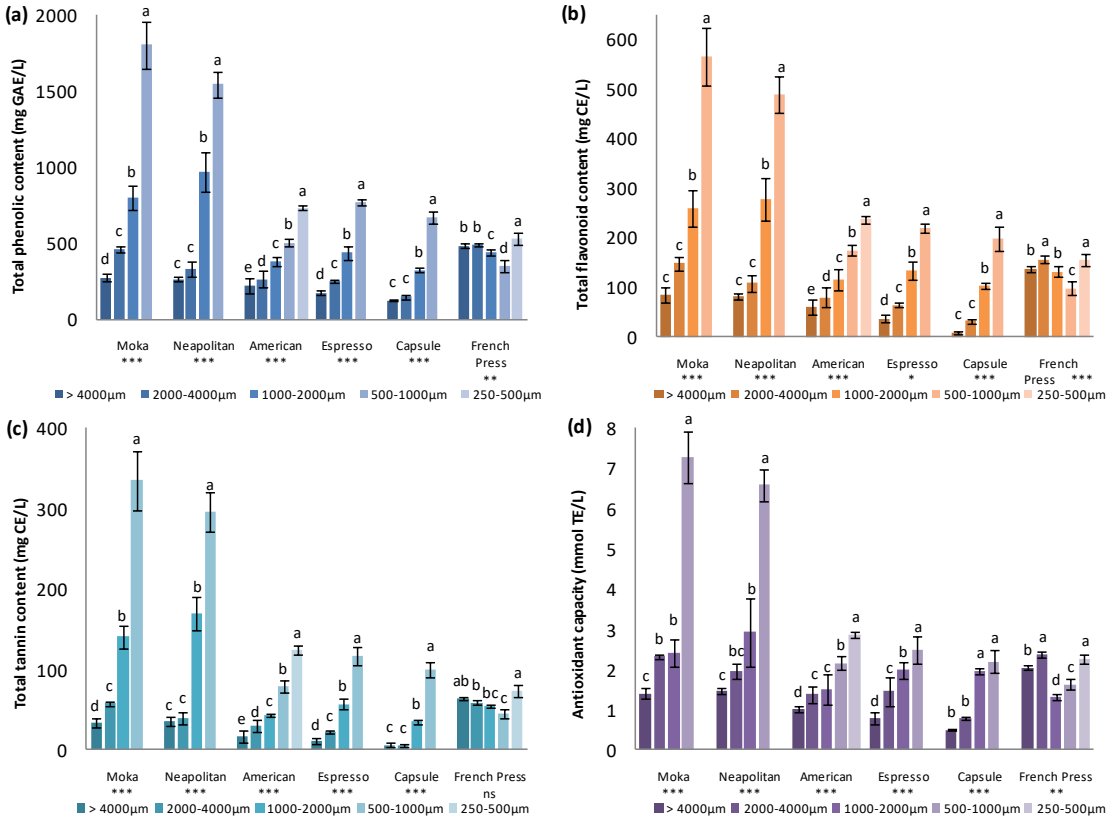

Figure 3. Total phenolic content (TPC), (**a**), total flavonoid content (TFC) (**b**), and total tannin content TTC (**c**), and antioxidant capacity (**d**) for the beverages produced by the six techniques at different CBS GDs; ANOVA among GDs for each technique. GAE = gallic acid equivalent, CE = catechin equivalent, and TE = trolox equivalent. Different letters indicate significant differences at $p < 0.05$. Significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = not significant.

TPC varied considerably among the beverages obtained by different techniques and at varied GDs, with values that ranged from 126.86 mg GAE/L for the beverage obtained by the Capsule technique at GD1 to 1803.83 mg GAE/L for the beverage obtained by the Moka technique using GD4. Except for the beverage obtained with the French press, large significant differences were detected for all the other beverages when the GD of the CBS was varied. For the beverages obtained by percolation techniques, TPC increased with a reduction in the GD, whereas for the beverage produced by the maceration technique (French press), TPC values were not influenced by the GD. The highest phenolic content was seen in beverages prepared by the Moka and Neapolitan techniques, where the results ranged from 276 mg GAE/L at GD1 to 1803.83 mg GAE/L at GD4 and from 263.67 mg GAE/L at GD1 to 1545.87 mg GAE/L at GD4, respectively. The beverage obtained by the Capsule technique manifested the lowest values of this parameter, ranging from 126.86 mg GAE/L at GD1 to 671.68 mg GAE/L at GD4. On the other hand, considering the intake, one cup (200 mL) of the beverage obtained by the French press technique at any GDs or by the American technique using CBS at GDs 3–5, provided the same quantity of polyphenols as one cup (60 mL) of the beverages obtained by the Moka or the Neapolitan techniques with CBS at GD4. The different values of TPC obtained for the beverages ranged between various values presented in previous studies, where beverages thought to have 'high-polyphenol content' were evaluated. The values of TPC in the present study were in most cases even higher than those found by Zujko & Witkowska [282] for drinking chocolate (600 mg GAE/L) or hot cocoa (300 mg GAE/L), but also higher in some cases than those of different tea types such as white tea (1040 mg GAE/L), green tea (850 mg GAE/L), black tea (720 mg GAE/L), and red tea (380 mg GAE/L). Reported TPC values for red wine (2410 mg GAE/L) and white wine (260 mg GAE/L) [282] were also within the range of the values obtained for the beverages studied in the present work. Regarding the values reported for some fruit juices by Gardner et al. [283] such as orange (755 mg GAE/L), apple (339 mg GAE/L), or pineapple juice (358 mg GAE/L), the levels of total phenols in CBS beverages were always between these values or even higher.

Flavonoids were the main compounds that contributed to TPC, constituting from 20.8% to 34.7% of this value, depending on the preparation technique but these contributions remained constant at different GDs within each technique. TFC and TPC highly correlated (r = 0.9965), and thus the former followed the same tendencies of abundance depending on the technique and GD. In this way, the highest value of TFC was seen in the beverages obtained by the Moka technique at GD4 (566.42 mg CE/L), followed by the beverage obtained by the Neapolitan technique with GD4 (489.43 mg CE/L). Beverages obtained by the two techniques had higher values at each GD compared with the other techniques except for the French press, for which the beverages showed slight differences with the variation of the GD. The TFC values of the beverages produced by the French press technique ranged between 97.98 and 155.63 mg CE/L and therefore had the highest values when larger CBS particle sizes were chosen. Again, the lowest values were observed for the Capsule beverage, which had TFC between 8.78 and 198.47 mg CE/L, which increased with a reduction in the CBS particle size.

Values of TTC accounted for 3.4% to 19.2% of TPC with a significantly high correlation (r = 0.9968). Contrary to what was observed between TPC and TFC, the percentage of TTC's contribution to TPC increased with a reduction in the GD within the values obtained for each technique, except for the beverages produced by the maceration technique, which maintained the contribution of the tannin content (12.0% to 13.9%) to TPC values independently of the GD. This fact can be noticed as the differences between big and small CBS particle sizes in TTC values become higher than those of TPC and TFC. CBS particle size could have an influence on selective extraction of some polyphenol groups for the percolation techniques, as could be the case for tannins. Tannins are normally larger molecules than flavonoids and therefore, a decrease in the particle size could facilitate their extraction compared to that of flavonoids, which were already extracted using the CBS powder with high particle sizes. In this way, greater increases were observed for TTC within the same technique when the particle size was reduced in comparison with those observed for TPC and TFC. Nevertheless, the highest concentrations of tannins were again detected in the Moka and Neapolitan

beverages at GD4 (334.64 and 296.06 mg CE/L, respectively) and the lowest values of TTC were observed in the Capsule beverage, which ranged from 5.18 to 99.86 mg CE/L.

More than 30 polyphenolic compounds that may contribute to the above values were detected and quantified by HPLC analysis. Only the concentrations determined for the main cocoa marker phenolic compounds and those showing the highest concentrations are given in Table 3. Belonging to the group of phenolic acids, protocatechuic acid and caffeic acid were found in CBS beverages, with the former being the most abundant. Both phenolic acids have already been quantified in cocoa bean and chocolate samples [237,241]. Nonetheless, even if the caffeic acid was present at lower concentrations than protocatechuic acid, in the CBS beverages it showed higher levels (with respect to protocatechuic acid) than those found in chocolate 100% cocoa made from Sao Tome cocoa beans (Forastero variety) studied by Rodríguez-Carrasco et al. [241]. These results indicate that the proportions of these two components diverge in the CBS with respect to the cocoa bean. For protocatechuic acid, the concentrations depending on the technique and GD used to produce the beverages followed a trend similar to the one already observed. The highest concentration was obtained in the beverage produced by the Moka technique using the minimum GD (18.14 mg/L) while the lowest values were found for the Capsule beverage (ranging from 1.32 to 10.80 mg/L) and the beverage produced by the American technique (ranging from 2.91 to 7.23 mg/L). Similar data were obtained for the French press beverage regardless of the particle size (ranging from 5.06 to 7.70 mg/L). As for caffeic acid, the lowest concentrations were seen in the beverage produced by the American technique, with values ranging from 0.15 to 0.33 mg/L. A slight decrease in the caffeic acid concentration was observed in the beverages prepared by the French press technique in comparison with the other techniques.

Table 3. Content (mg/L) of methylxanthines (theobromine and caffeine) and polyphenols (phenolic acids, flavan-3-ols, procyanidins B, and flavonols) evaluated by HPLC for the beverages obtained via the different techniques and GDs of the CBS and ANOVA among GDs for each technique.

Technique	Grinding Degree (μm)	Methylxanthines		Phenolic acids		Flavan-3-ol			Procyanidins B		Flavonols		
		Theobromine	Caffeine	Protocatechuic acid	Caffeic acid	Catechin-3-O-glucoside	Catechin	Epicatechin	Type B procyanidin	Procyanidin B2	Quercetin-3-O-glucoside	Quercetin-3-O-rhamnoside	Quercetin
Moka	> 4000	147.33 ± 12.08^{d}	25.44 ± 2.85^{d}	5.25 ± 0.66^{c}	0.17 ± 0.01^{c}	6.43 ± 0.76^{c}	1.01 ± 0.06^{b}	0.51 ± 0.03^{d}	8.64 ± 1.52^{c}	0.79 ± 0.04^{d}	0.19 ± 0.01^{c}	0.24 ± 0.02^{d}	0.88 ± 0.00^{d}
	2000-4000	261.85 ± 12.05^{c}	37.75 ± 2.38^{c}	5.98 ± 0.95^{c}	0.35 ± 0.05^{b}	6.16 ± 0.19^{c}	0.41 ± 0.02^{b}	1.77 ± 0.02^{c}	10.50 ± 1.80^{c}	1.43 ± 0.17^{c}	0.44 ± 0.02^{b}	0.39 ± 0.07^{c}	1.76 ± 0.01^{c}
	1000-2000	384.78 ± 13.15^{b}	68.07 ± 5.73^{b}	10.29 ± 1.00^{b}	0.41 ± 0.08^{b}	13.49 ± 0.43^{b}	0.81 ± 0.09^{b}	3.20 ± 0.18^{b}	20.38 ± 3.03^{b}	1.81 ± 0.32^{b}	0.50 ± 0.04^{b}	0.51 ± 0.02^{b}	1.80 ± 0.01^{b}
	500-1000	703.79 ± 52.64^{a}	124.84 ± 15.91^{a}	18.14 ± 1.38^{a}	0.81 ± 0.05^{a}	21.73 ± 1.98^{a}	1.92 ± 0.33^{a}	6.38 ± 0.10^{a}	36.30 ± 5.29^{a}	3.30 ± 0.13^{a}	0.93 ± 0.13^{a}	0.66 ± 0.04^{a}	3.54 ± 0.02^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Sig.	***	***	***	***	***	***	***	***	***	***	***	***
Neapolitan	> 4000	127.84 ± 3.97^{d}	19.17 ± 0.80^{c}	3.44 ± 0.15^{c}	0.16 ± 0.00^{d}	4.21 ± 0.06^{b}	0.59 ± 0.08^{bc}	0.45 ± 0.01^{d}	5.56 ± 0.06^{c}	0.74 ± 0.03^{d}	0.17 ± 0.01^{d}	0.17 ± 0.01^{d}	0.88 ± 0.01^{d}
	2000-4000	185.78 ± 12.03^{c}	26.11 ± 2.15^{c}	4.10 ± 0.38^{c}	0.31 ± 0.01^{c}	4.61 ± 0.27^{b}	0.36 ± 0.02^{c}	1.55 ± 0.04^{c}	7.29 ± 0.46^{c}	1.30 ± 0.04^{c}	0.36 ± 0.03^{c}	0.32 ± 0.00^{c}	1.75 ± 0.00^{c}
	1000-2000	418.97 ± 24.11^{b}	80.49 ± 8.83^{b}	10.94 ± 1.55^{b}	0.40 ± 0.04^{b}	13.67 ± 0.67^{a}	0.83 ± 0.20^{b}	3.61 ± 0.04^{b}	20.69 ± 2.15^{b}	1.59 ± 0.10^{b}	0.51 ± 0.04^{b}	0.52 ± 0.04^{b}	1.78 ± 0.00^{b}
	500-1000	583.12 ± 29.74^{a}	102.98 ± 7.30^{a}	13.23 ± 0.88^{a}	0.67 ± 0.01^{a}	13.58 ± 1.13^{a}	1.57 ± 0.20^{b}	5.24 ± 0.16^{a}	27.10 ± 1.90^{a}	2.88 ± 0.08^{a}	0.98 ± 0.07^{a}	0.75 ± 0.01^{a}	3.54 ± 0.02^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Sig.	***	***	***	***	***	***	***	***	***	***	***	***
American	> 4000	115.00 ± 3.29^{e}	14.57 ± 1.42^{e}	2.91 ± 0.24^{e}	0.15 ± 0.00^{c}	3.89 ± 0.28^{c}	0.36 ± 0.08^{c}	0.25 ± 0.03^{b}	5.71 ± 0.14^{c}	0.72 ± 0.03^{c}	0.16 ± 0.00^{d}	0.15 ± 0.00^{b}	0.87 ± 0.00^{b}
	2000-4000	145.57 ± 5.42^{d}	21.24 ± 1.26^{d}	4.20 ± 0.23^{d}	0.17 ± 0.01^{c}	5.95 ± 0.23^{b}	0.52 ± 0.04^{bc}	0.43 ± 0.07^{b}	7.84 ± 0.21^{b}	0.77 ± 0.06^{c}	0.19 ± 0.00^{c}	0.15 ± 0.01^{b}	0.87 ± 0.00^{b}
	1000-2000	233.01 ± 3.92^{a}	34.38 ± 0.97^{c}	5.54 ± 0.07^{c}	0.33 ± 0.03^{a}	6.17 ± 0.26^{b}	0.39 ± 0.03^{bc}	1.30 ± 0.06^{a}	10.54 ± 0.86^{a}	1.37 ± 0.04^{a}	0.37 ± 0.01^{a}	0.35 ± 0.03^{a}	1.75 ± 0.01^{a}
	500-1000	180.89 ± 1.11^{c}	37.09 ± 0.72^{b}	6.76 ± 0.30^{b}	0.22 ± 0.02^{b}	8.65 ± 0.26^{a}	1.08 ± 0.05^{a}	1.54 ± 0.43^{a}	12.19 ± 0.58^{a}	0.99 ± 0.09^{b}	0.20 ± 0.02^{c}	0.17 ± 0.01^{b}	0.88 ± 0.00^{b}
	250-500	193.03 ± 0.84^{b}	45.69 ± 0.91^{a}	7.23 ± 0.27^{a}	0.21 ± 0.01^{b}	8.44 ± 0.56^{a}	0.55 ± 0.16^{b}	1.65 ± 0.13^{a}	12.33 ± 2.33^{a}	0.97 ± 0.06^{b}	0.24 ± 0.02^{b}	0.16 ± 0.01^{b}	0.87 ± 0.00^{b}
	Sig.	***	***	***	***	***	***	***	***	***	***	***	***
Espresso	> 4000	92.34 ± 9.99^{d}	10.63 ± 0.81^{d}	2.10 ± 0.13^{d}	0.16 ± 0.01^{c}	2.67 ± 0.21^{d}	0.24 ± 0.05^{c}	0.04 ± 0.01^{c}	4.16 ± 0.27^{d}	0.65 ± 0.02^{c}	0.17 ± 0.00^{c}	0.17 ± 0.01^{d}	0.87 ± 0.00^{c}
	2000-4000	195.25 ± 20.65^{c}	24.91 ± 3.64^{c}	4.27 ± 0.54^{c}	0.32 ± 0.01^{b}	4.76 ± 0.54^{c}	0.28 ± 0.03^{c}	1.42 ± 0.10^{b}	7.25 ± 1.23^{c}	1.29 ± 0.02^{b}	0.36 ± 0.02^{b}	0.34 ± 0.02^{c}	1.74 ± 0.00^{b}
	1000-2000	292.82 ± 21.28^{b}	42.90 ± 4.16^{b}	7.16 ± 0.48^{b}	0.32 ± 0.00^{b}	9.43 ± 0.53^{b}	0.53 ± 0.01^{a}	1.84 ± 0.16^{b}	13.97 ± 0.97^{b}	1.34 ± 0.13^{b}	0.38 ± 0.05^{b}	0.45 ± 0.00^{b}	1.76 ± 0.01^{a}
	500-1000	392.36 ± 4.40^{a}	66.81 ± 1.55^{a}	12.59 ± 0.18^{a}	0.43 ± 0.00^{a}	13.98 ± 0.22^{a}	0.45 ± 0.04^{b}	2.87 ± 0.10^{a}	20.72 ± 0.66^{a}	1.60 ± 0.08^{a}	0.45 ± 0.03^{a}	0.56 ± 0.05^{a}	1.77 ± 0.00^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Sig.	***	***	***	***	***	***	***	***	***	***	***	***
Capsule	> 4000	59.82 ± 2.66^{d}	6.09 ± 0.50^{d}	1.32 ± 0.05^{d}	0.14 ± 0.00^{b}	1.71 ± 0.17^{d}	0.12 ± 0.04^{c}	0.00 ± 0.00^{c}	2.77 ± 0.11^{d}	0.60 ± 0.02^{b}	0.15 ± 0.01^{c}	0.17 ± 0.00^{c}	0.86 ± 0.00^{b}
	2000-4000	88.78 ± 3.27^{c}	9.75 ± 0.59^{c}	2.13 ± 0.11^{c}	0.15 ± 0.00^{b}	2.61 ± 0.03^{c}	0.22 ± 0.03^{bc}	0.14 ± 0.02^{c}	3.96 ± 0.31^{c}	0.68 ± 0.04^{b}	0.15 ± 0.01^{c}	0.13 ± 0.01^{c}	0.87 ± 0.00^{b}
	1000-2000	230.27 ± 9.07^{b}	33.46 ± 0.15^{b}	5.49 ± 0.36^{b}	0.40 ± 0.07^{a}	6.26 ± 0.33^{b}	0.31 ± 0.09^{ab}	1.27 ± 0.01^{b}	9.49 ± 0.25^{b}	1.31 ± 0.08^{a}	0.35 ± 0.01^{b}	0.39 ± 0.02^{b}	1.76 ± 0.00^{a}
	500-1000	361.79 ± 5.64^{a}	59.60 ± 1.99^{a}	10.80 ± 0.33^{a}	0.38 ± 0.01^{a}	12.21 ± 0.59^{a}	0.37 ± 0.03^{a}	2.53 ± 0.11^{a}	17.99 ± 1.03^{a}	1.39 ± 0.04^{a}	0.44 ± 0.02^{a}	0.49 ± 0.04^{a}	1.76 ± 0.01^{a}
	250-500	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Sig.	***	***	***	***	***	**	***	***	***	***	***	***
French press	> 4000	289.14 ± 10.27^{a}	43.07 ± 3.46^{ab}	7.51 ± 0.28^{a}	0.33 ± 0.01^{b}	8.40 ± 0.57^{a}	1.00 ± 0.03^{a}	2.35 ± 0.23^{a}	14.05 ± 0.79^{a}	1.55 ± 0.05^{a}	0.41 ± 0.02^{a}	0.44 ± 0.04^{a}	1.76 ± 0.01^{a}
	2000-4000	272.30 ± 11.82^{a}	40.47 ± 2.09^{b}	7.34 ± 0.24^{a}	0.34 ± 0.03^{b}	8.16 ± 0.59^{a}	0.54 ± 0.02^{b}	1.77 ± 0.18^{b}	13.58 ± 1.35^{a}	1.61 ± 0.10^{a}	0.41 ± 0.07^{a}	0.39 ± 0.04^{a}	1.76 ± 0.01^{a}
	1000-2000	172.48 ± 9.12^{b}	31.03 ± 4.31^{c}	6.54 ± 0.63^{b}	0.20 ± 0.00^{c}	8.82 ± 1.09^{a}	0.94 ± 0.08^{a}	0.88 ± 0.11^{c}	12.99 ± 1.82^{a}	0.87 ± 0.02^{b}	0.18 ± 0.02^{b}	0.20 ± 0.03^{b}	0.88 ± 0.00^{b}
	500-1000	154.85 ± 22.81^{b}	26.73 ± 2.38^{c}	5.06 ± 0.22^{c}	0.21 ± 0.01^{c}	5.68 ± 0.19^{b}	0.52 ± 0.05^{b}	0.63 ± 0.03^{c}	8.68 ± 0.74^{b}	1.02 ± 0.21^{b}	0.18 ± 0.01^{b}	0.18 ± 0.02^{b}	0.88 ± 0.01^{b}
	250-500	286.68 ± 16.56^{a}	46.65 ± 3.02^{a}	7.70 ± 0.33^{a}	0.36 ± 0.00^{a}	7.80 ± 0.65^{a}	0.57 ± 0.07^{b}	2.27 ± 0.09^{a}	12.59 ± 1.26^{a}	1.44 ± 0.08^{a}	0.42 ± 0.05^{a}	0.39 ± 0.02^{a}	1.76 ± 0.01^{a}
	Sig.	***	***	***	***	**	*	*	**	***	***	***	***

n/a not applicable
Means followed by different letters are significantly different at $p < 0.05$
Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
Data are expressed as mean values (n=3) ± standard deviation

As for flavan-3-ols, we detected and quantified catechin, epicatechin, and catechin-3-O-glucoside in all the beverages; these are three characteristic flavan-3-ols for cocoa beans and chocolate already reported in CBS [57]. They can be found as free monomers or forming condensed tannins as monomeric constituents [81]. Variable concentrations were noted for these compounds, with catechin-3-O-glucoside being the most abundant, followed by epicatechin and catechin. Nevertheless, the concentrations obtained for both catechin and epicatechin were lower than those obtained with other types of extraction procedures where organic solvents were used as in the work of Hérnandez-Hérnandez et al. [60]. Catechin and epicatechin showed low solubility in water, even when pressure and high temperatures were applied in the present work, whereas catechin-3-O-glucoside was present at higher concentrations, probably because of the water solubility ensured by the glycoside group. As a flavonoid compound, epicatechin significantly correlated with the results obtained for both TPC and TFC ($r = 0.9628$ and $r = 0.9574$, respectively).

Two procyanidins of type B were detected and quantified by HPLC analysis. Procyanidins are flavan-3,4-diols, generally forming condensation compounds with epicatechin at 4-8 or 4-6 bonds [81]. Type B procyanidin was found to be the polyphenolic compound at the highest concentration among those quantified in the studied samples. This compound was present at the highest concentrations in both Moka and Neapolitan beverages with the smallest CBS particle size (36.30 and 27.10 mg procyanidin B1 eq/L, respectively) and with the lowest values for the beverages produced by the Capsule and American techniques; the concentration increased with a decrease in the GD. As epicatechin, this compound highly correlated with both TPC and TFC ($r = 0.9621$ and $r = 0.9572$, respectively). Procyanidin B2 was also found at its highest concentrations in the Moka and Neapolitan beverages, and at lower concentrations in the Capsule and American ones. These results followed the trend of an increasing concentration when the GD was decreased for almost all the beverages obtained by the percolation techniques; and an intermediate constant concentration was observed for the maceration technique, independently of the GD.

Finally, three flavonols were quantified in the CBS beverages, quercetin and two of its glycoside derivates: quercetin-3-O-glucoside and quercetin-3-O-rhamnoside. Quercetin and its derivates are part of the more abundant and recurrent flavonoids in foods known for their bitter flavor [81].

All these polyphenolic compounds, which possess antioxidant properties, could be beneficial in terms of prevention of diseases related to oxidative stress. Therefore, it is highly important for humans to consume them with nutrition. The new beverages (based on CBS)

developed in this study may be an interesting source of these bioactive compounds with potential health benefits.

3.3.3. Methylxanthines

The concentrations of theobromine and caffeine evaluated by HPLC are given in Table 3. The amounts of theobromine were approximately 5–7-fold higher than those of caffeine. The concentrations of both methylxanthines significantly increased with a reduction in the GD for all the beverages obtained by percolation techniques. In general, the beverages that manifested the highest concentrations were those obtained by the Moka and Neapolitan methods, ranging from 147.33 to 703.79 mg theobromine/L and 25.44 to 124.84 mg caffeine/L for the former, and from 127.84 to 583.12 mg theobromine/L and 19.17 to 102.98 mg caffeine/L for the latter. The observed caffeine contents were lower than those observed for other kinds of beverages such as coffee (567 mg/L), mate (520 mg/L) [101], matcha (300 mg/L), loose leaf teas (99.21–296.86 mg/L), or bagged teas (151.73–246.71 mg/L) [284]. Nonetheless, the theobromine contents of CBS beverages are significantly higher than those observed for the same beverages, showing such amounts as 12.18 mg theobromine/L for matcha, 7.55–86.18 mg/L for loose leaf teas, and 21.58–66.91 mg/L for bagged teas [284].

Nevertheless, considering the expected intake of each type of beverage (60 mL for Moka, Neapolitan, Espresso, and Capsule and 200 mL for French press and American), the largest amounts of theobromine and caffeine would be consumed with the beverage produced by the French press technique, for which up to 60 mg of theobromine and 9 mg of caffeine would be consumed with each expected dose.

Cocoa is known for stimulating the brain due to the presence of theobromine and caffeine. As mentioned above, these two methylxanthines are also present in the CBS, the former being notably more abundant than the latter. They both influence alertness and mood in a positive way, acting on the central nervous system, and thus, may partly account for cocoa acceptance by consumers. Besides, several beneficial biological activities are linked to these methylxanthines such as the anticarcinogenic, antiobesity, antioxidant, antitumor, diuretic or energizer effects of caffeine or the cAMP-phosphodiesterase-inhibitory, diuretic (when consumed at 300–600 mg/day), stimulant, or myorelaxant activities of theobromine [101]. Theobromine is also linked to the beneficial effects of cocoa consumption because of various other benefits associated with it, all of them without some of the unwanted effects of caffeine [285]. Usmani et al. [286] observed that a single 1000 mg dose of theobromine has a significantly greater antitussive effect on humans than a single 60 mg dose of codeine, an

opioid drug whose clinical use is limited due to its unacceptable side effects, which could be avoided with an alternative theobromine treatment. Neufingerl et al. [287] reported that 850 mg of theobromine per day increases serum high-density lipoprotein cholesterol concentrations by 0.16 mmol/L. Another example of theobromine consumption benefits has been demonstrated by Kargul et al. [288], who showed that application of theobromine at concentrations of 100 and 200 mg/L to teeth could significantly protect enamel surface via a cariostatic effect, thus being an alternative to fluoride treatments. According to these data, CBS is likely to represent the proper combination of both methylxanthines in order to exert all the aforementioned beneficial actions without the secondary effects of big doses of caffeine, such as tachycardia or increased blood pressure if consumed at doses over 250 mg [289]. Regarding the above-mentioned examples of theobromine's benefits, one single expected dose of the CBS beverage would not yield the needed levels to observe the effects on high-density lipoprotein cholesterol level or the antitussive properties but it would clearly contribute to these benefits. Moreover, further optimizations or extract concentrations of the prepared beverages could be proposed to improve their functionality. Additionally, harmful levels of caffeine will not be an issue when expected doses of the beverages are consumed.

3.4. Biofunctional characteristics

Polyphenols, as antioxidants, chelators of divalent cations, or inhibitors of enzymatic activities, have been reported to have several possible beneficial effects, e.g. anti-carcinogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, anti-allergenic, immunomodulating, antimicrobial, vasodilatory, analgesic, or antidiabetic effects [81,280].
The beverages studied in the present work were found to contain considerably large quantities of various polyphenols and thus could exert a particular functional effect on the human body through the different properties of their polyphenols. Amongst these features, antioxidant and antidiabetic properties were studied here to evaluate the potential bioactivity of the beverages.

3.4.1. Antioxidant capacity

The development of some chronic diseases such as cancer, cardiovascular diseases, and diabetes is tightly related to oxidative stress. Therefore, new chemopreventive approaches have been developed for preventing the damaging effects of free radicals and oxidants, mostly based on acquisition of radical scavengers and antioxidants from the diet. It has been already demonstrated that polyphenols from cocoa products (mostly flavanols) can interfere with

these harmful processes and thus prevent the pathogenesis of the aforementioned diseases [169].

Results showing the radical scavenging or antioxidant capacity of the functional beverages expressed in mmol TE/L are presented in Figure 3d. The highest antioxidant capacity values were found in the beverages produced by the Moka and the Neapolitan methods at GD4 (7.29 and 6.58 mmol TE/L, respectively). These values were at least twice higher than those observed in the other beverages. A general trend was observed with a proportional increase in the antioxidant capacity with the decreasing CBS particle size for all the beverages produced by percolation techniques, whereas those obtained by the French press manifested no dependence on the GD, having the highest level of antioxidant capacity at the bigger CBS particle sizes, as reported about other parameters above. Antioxidant capacity showed significantly high correlations with the obtained values of TPC, TFC, and TTC ($r = 0.9656$, $r = 0.9716$, and $r = 0.9649$, respectively), even though lower correlations were seen between the antioxidant capacity and the single compounds detected by HPLC (ranging from $r = 0.7713$ for quercetin-3-O-rhamnoside to $r = 0.9348$ for procyanidin B2).

3.4.2. Antidiabetic capacity

Cocoa polyphenols, in particular flavanols, have been reported to possess several antidiabetic bioactivities such as the improvement of insulin secretion by protecting β-pancreatic cells and the improvement of insulin sensitivity by protecting insulin-sensitive tissues from oxidative damage, among other reasons [84]. However, the main and more extended antidiabetic property of polyphenols have been reported to be the inhibition of key enzymes involved in glucose metabolism and absorption such as α-glucosidase or α-amylase [280]. This effect could be achieved by means of some drugs such as acarbose, a potent α-glucosidase inhibitor that is in disuse because of its considerable side effects that affect quality of life, e.g. abdominal distension, flatulence, meteorism, and possibly diarrhea [280]. For all these reasons, there is great interest among researchers in the search for healthy and sustainable alternatives such as the CBS preparations for beverages presented in this work. We carried out the study of the antidiabetic capacity due to the α-glucosidase inhibition of the beverages and the results are depicted in Figure 4.

As expected, according to TPC, TFC, and TTC results and taking into account the potential influence of these compounds on the antidiabetic capacity, the beverage exerting the higher percentage of α-glucosidase inhibition was the one obtained with Moka at the smallest CBS particle size GD4 (52.0%), followed by the Neapolitan (36.8%), Espresso (32.0%), and Capsule (26.2%) beverages at the same GD. The smallest percentage of α-glucosidase inhibition was observed for the beverage produced by the Capsule technique at the biggest GD of the CBS (4.7% with GD1). In general, beverages produced with big particle sizes of the CBS are those exerting the smallest α-glucosidase inhibition, especially for the techniques that employ pressure for extraction, where the contact time between water and the CBS decreases, and so does extraction performance. The French press beverages instead showed an intermediate value of α-glucosidase inhibition independently of the CBS particle size. It is important to note that the contribution to the antidiabetic effect manifested by the new functional beverages is close to that of 0.5 mM acarbose serving as a control sample corresponding to the IC_{50} concentration for this drug. The α-glucosidase inhibition parameters showed a significantly high correlation with TPC (r = 0.9537) and some of the detected polyphenolic compounds such as protocatechuic acid (r = 0.9826), type B procyanidin (r = 0.9870), and, as expected, both flavan-3-ols catechin-3-O-glucoside and epicatechin (r = 0.9803 and r = 0.9500, respectively).

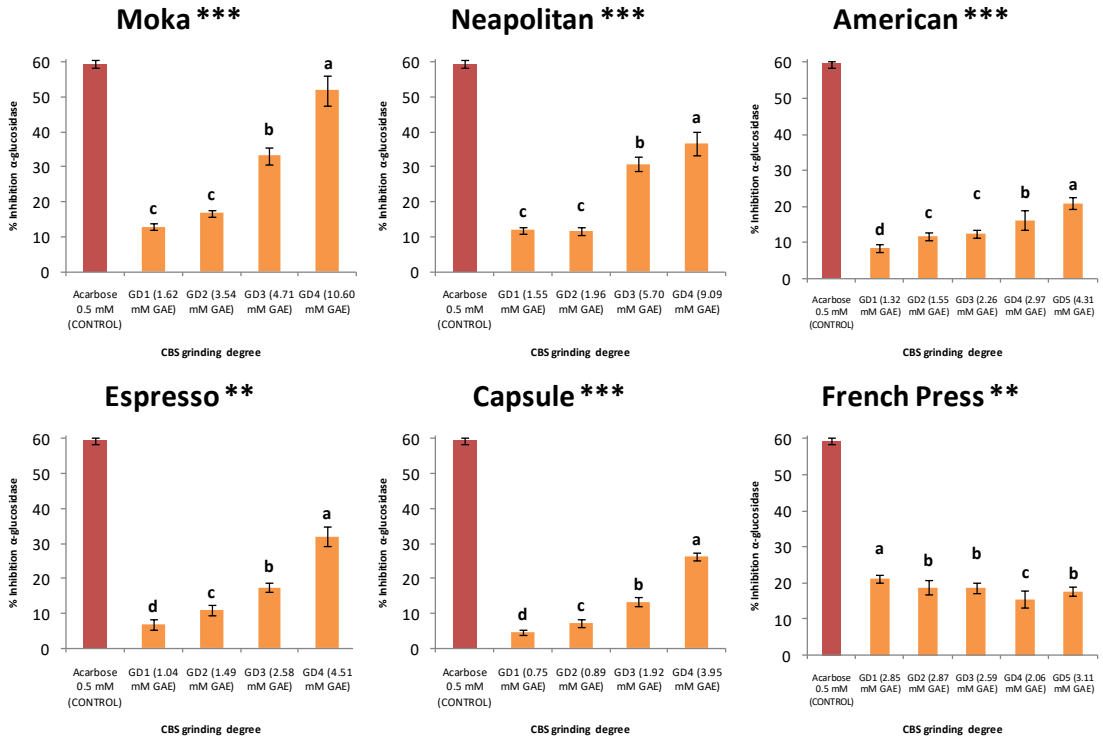

Figure 4. α-Glucosidase inhibition by the beverages produced via the six techniques at various CBS GDs related to the total phenolic content expressed in mM GAE (gallic acid equivalents); ANOVA among GDs for each technique. Acarbose at 0.5 mM (IC_{50}) served as a control. Different letters indicate significant differences among the GDs at $p < 0.05$. Significance: $^{**}p < 0.01$; $^{***}p < 0.001$.

This is the first study where the α-glucosidase inhibition due to polyphenolic content is reported for CBS. Nonetheless, the hypoglycemic effects of this by-product due to its fiber content have already been described by Nsor-Atindana et al. [39]. We did not evaluate this effect.

3.5. Consumer acceptance evaluation

Table 4 shows the sums of ranks values calculated for the consumer evaluation parameters for each beverage preparation technique and CBS particle size. These values were subjected to the Kruskal–Wallis test to highlight the differences in acceptance for the different beverages prepared by the same technique, comparing the GDs of the CBS.

As far as the aspect was concerned, slight differences were evidenced, and a general preference for the beverages obtained with smaller GDs was observed. Taking into account the results obtained for the beverages' color, where in general, the brightness decreased while the GD decreased and both parameters *a** and *b** rose when the CBS GD decreased, it could be assumed that darker and browner beverages were preferred over lighter ones. Regarding the odor, the beverages obtained by the Neapolitan and the Moka techniques were generally the most appreciated while those obtained by the Capsule and the French press techniques were the least appreciated. In the case of taste, the beverage obtained by the Capsule technique showed high levels of consumer acceptance for almost all the GDs. In some cases, even the beverages obtained by the Neapolitan and American methods were highly liked. The situation was again ambiguous for the flavor where the liking seems to be influenced by the technique–GD interaction, though in general, the beverages obtained by the Neapolitan or American technique were among the most appreciated. It was observed that overall, the beverages that had high values of titratable acidity were less liked in terms of taste and flavor. The overall rating obviously reflects all the previous variability for the various parameters of consumer acceptance, with the Neapolitan and the American techniques yielding the most liked beverages at the highest GDs, whereas the Capsule and Espresso methods seem to be the ones preferred at small GDs. Again, this pattern is reflected in the purchase predisposition, with a greater preference for Neapolitan technique beverages with large CBS particle sizes and for those generated by Capsule and Espresso at smaller GDs.

It should be remarked that the most active beverages (Moka and Neapolitan with small GDs) are between those most appreciated as far as the appearance or the odor are concerned. But, on the contrary, the appreciation of these beverages decreases considerably when evaluating taste and flavor, and so do the overall liking and the purchase predisposition. This

fact could be related to the unpleasant, bitter and, in some cases, astringent flavor related to the polyphenols and the methylxantines which are present in high amounts in these beverages.

Table 4. Consumer evaluation of the beverages and results of the Kruskal–Wallis test. Data are expressed as the sum of ranks of the results obtained from 20 tasters who filled out a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like).

Production Technique	Grinding Degree	Appearance	Odor	Taste	Flavor	Overall liking	Purchase predisposition
Moka	> 4000 µm	309.5^{ab}	234.0^{b}	482.5^{a}	473.0^{a}	486.5^{a}	395.0^{a}
	2000-4000 µm	188.0^{b}	220.0^{b}	335.0^{ab}	283.5^{b}	312.5^{b}	265.5^{a}
	1000-2000 µm	288.5^{ab}	311.5^{ab}	206.0^{bc}	188.0^{b}	177.5^{b}	279.0^{a}
	500-1000 µm	390.0^{a}	410.5^{a}	152.5^{c}	231.5^{b}	199.5^{b}	236.5^{a}
	250-500 µm	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	*	*	***	***	***	ns
Neapolitan	> 4000 µm	372.5^{a}	291.0^{a}	449.5^{a}	362.0^{ab}	433.5^{a}	439.5^{a}
	2000-4000 µm	230.5^{a}	344.5^{a}	361.0^{ab}	382.5^{a}	406.0^{a}	376.5^{a}
	1000-2000 µm	271.0^{a}	231.5^{a}	245.5^{bc}	202.5^{b}	181.5^{b}	205.0^{b}
	500-1000 µm	302.0^{a}	309.0^{a}	120.0^{c}	229.0^{ab}	155.0^{b}	155.0^{b}
	250-500 µm	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	ns	ns	***	**	***	***
American	> 4000 µm	138.5^{b}	338.0^{a}	400.5^{ab}	446.0^{a}	436.0^{ab}	467.5^{a}
	2000-4000 µm	510.5^{a}	444.0^{a}	595.0^{a}	557.5^{a}	569.5^{a}	403.0^{a}
	1000-2000 µm	380.5^{a}	288.0^{a}	328.0^{bc}	342.0^{a}	309.0^{bc}	318.0^{a}
	500-1000 µm	461.0^{a}	310.0^{a}	363.0^{bc}	396.0^{a}	392.0^{ab}	372.0^{a}
	250-500 µm	339.5^{ab}	450.0^{a}	143.5^{c}	88.5^{b}	123.5^{c}	269.5^{a}
	Significance	***	ns	***	***	***	ns
Espresso	> 4000 µm	120.0^{b}	138.0^{b}	277.0^{ab}	247.0^{ab}	172.5^{b}	214.0^{bc}
	2000-4000 µm	316.0^{a}	337.0^{a}	413.0^{a}	369.0^{a}	402.0^{a}	394.5^{a}
	1000-2000 µm	356.5^{a}	290.0^{ab}	366.5^{a}	391.5^{a}	351.5^{a}	368.5^{ab}
	500-1000 µm	383.5^{a}	410.0^{a}	119.5^{b}	168.0^{b}	250.0^{ab}	199.0^{c}
	250-500 µm	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	***	***	***	**	**	**
Capsule	> 4000 µm	125.5^{b}	151.0^{b}	431.0^{b}	292.5^{ab}	235.5^{b}	201.0^{b}
	2000-4000 µm	185.0^{b}	248.0^{b}	321.0^{b}	188.0^{b}	195.5^{b}	206.0^{b}
	1000-2000 µm	389.0^{a}	310.0^{ab}	172.0^{ab}	283.5^{ab}	300.0^{ab}	339.5^{ab}
	500-1000 µm	476.0^{a}	467.0^{a}	252.0^{a}	412.0^{a}	445.0^{a}	429.5^{a}
	250-500 µm	n/a	n/a	n/a	n/a	n/a	n/a
	Significance	***	***	***	**	***	***
French press	> 4000 µm	464.5^{a}	526.5^{a}	192.0^{c}	472.0^{a}	436.0^{a}	319.0^{ab}
	2000-4000 µm	312.0^{a}	172.0^{b}	237.5^{bc}	119.0^{b}	132.5^{b}	224.5^{b}
	1000-2000 µm	366.0^{a}	140.5^{b}	367.5^{abc}	290.0^{ab}	285.5^{ab}	493.0^{a}
	500-1000 µm	265.5^{a}	494.0^{a}	580.0^{a}	503.0^{a}	488.0^{a}	461.5^{a}
	250-500 µm	422.0^{a}	497.0^{a}	453.0^{ab}	446.0^{a}	488.0^{a}	332.0^{ab}
	Significance	ns	***	***	***	***	**

n/a not applicable
Means followed by different letters are significantly different at $p<0.05$
Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant
Data are expressed as sums of ranks

3.6. Technological efficiency of polyphenol extraction

To identify the technique allowing higher extraction yield of polyphenols, normalization of the TPC values was performed according to the different CBS powder amounts and water quantities employed. The new TPC values, expressed in milligrams of gallic acid equivalents per gram of CBS ranged between 2.72 and 16.32 mg GAE/g (Table 5). A significant increase in TPC was observed for the beverages obtained by the forced percolation techniques (Moka, Espresso, and Capsule) at GDs below 1000 µm and for the beverages produced by the Neapolitan method (natural percolation) at GDs below 2000 µm. For the beverage produced

by the American technique (natural percolation), TPC increased progressively with the decreasing GDs. On the contrary, for the beverage produced with the French press, no influence of the GDs was observed, thereby leading to the conclusion that the difference in GDs had only a minor impact on the polyphenol extraction by this technique based on a maceration process. Neapolitan was always the most effective technique when total phenol extraction was compared among the beverages obtained by all the techniques at the same GD, followed by those produced by the American, French press, or Moka techniques. The lowest TPC values belong to the beverages produced by the Capsule and Espresso. On the other hand, these techniques usually afforded higher extraction of essential oils because they produce more aromatic coffee. The highest TPC observed (16.32 mg GAE/g for the beverage obtained with Neapolitan using GD4) was significantly higher than those reported for other types of CBS extraction in previous works. Hernández-Hernández et al. [60] obtained a TPC value of 3 mg GAE/g when extracting 1 g of CBS at 500 μm in 6 mL of water at 70°C. Manzano et al. [53] observed a TPC value of 6.04 mg GAE/g using 2 g of CBS screened at 75 μm, which was processed in 50 mL of water with 5-minute reflux extraction. Nevertheless, in other studies in which assisted extraction was carried out, higher values of TPC were obtained as compared to those obtained for the beverages in the present work, as expected. Nsor-Atindana et al. [39] reported a value of 17.21 mg GAE/g after the extraction of 2 g of CBS ground up at 250 μm in 50 mL of water using microwaves. In any case, it is important to note that the solid/liquid ratio, which was different in all studies, could also have some influence on these results.

Table 5. Total phenolic content for the beverages after normalization considering CBS and water quantities. ANOVA among GDs and extraction techniques. Values are expressed in milligrams of gallic acid equivalents for each gram of CBS powder employed for the beverage preparation (mg GAE/g CBS).

	Moka	**Neapolitan**	**American**	**Espresso**	**Capsule**	**French Press**	*Sig.*
> 4000 μm	5.21 ± 0.54^{cB}	7.03 ± 0.47^{cA}	4.84 ± 0.28^{dB}	3.12 ± 0.30^{bC}	3.76 ± 0.25^{bC}	6.68 ± 0.64^{aA}	***
2000-4000 μm	6.37 ± 2.25^{bcAB}	7.14 ± 1.06^{cA}	5.26 ± 0.09^{dB}	2.72 ± 0.39^{bC}	3.25 ± 0.30^{cC}	6.74 ± 0.72^{aA}	***
1000-2000 μm	6.92 ± 0.72^{bB}	10.00 ± 1.33^{bA}	7.32 ± 0.34^{cB}	3.41 ± 0.33^{bD}	3.03 ± 0.11^{cD}	5.78 ± 0.30^{bC}	***
500-1000 μm	12.94 ± 0.96^{aB}	16.32 ± 1.04^{aA}	9.39 ± 0.92^{bC}	5.10 ± 0.15^{aD}	5.06 ± 0.25^{aD}	4.10 ± 0.31^{cE}	***
250-500 μm	n/a	n/a	13.45 ± 0.86^{aA}	n/a	n/a	6.29 ± 0.26^{aB}	***
Sig.	***	***	***	***	***	***	***

n/a not applicable
Means followed by different lower case superindexes within the same column (different grinding degrees) and by upper case superindexes within the same row (different techniques) are significantly different at $p<0.05$
Significance: *** $p < 0.001$
Data are expressed as mean values (n=3) ± standard deviation

4. Conclusions

The various extraction techniques used for CBS ground to different degrees allowed us to obtain beverages with different chemical characteristics and consumer-related parameters. Several compounds were identified and quantified by HPLC (phenolic acids, flavan-3-ols, quercetin-glycosides, catechin-glycosides, and procyanidins), which may underlie the high radical scavenging capacity and significant α-glucosidase inhibition results shown by the beverages. The GD was optimized for each extraction technique; the smallest GDs allowed us to obtain the most functional beverages when using percolation techniques, whereas the maceration technique (French press) in general, showed no dependence on the CBS particle size. This finding may be of great interest as with the French press technique, no further CBS grinding treatments will be needed to obtain a beverage having high-potential biological activities. In terms of consumer acceptance, it was found that, in general, the most active beverages were the least appreciated as far as taste and flavor are concerned, probably because of the bigger presence of polyphenols and methylxanhines. This fact could open a possibility for further research in order to optimize these beverages, aiming at a higher consumer acceptance. Such optimization could be achieved by bioactive compounds encapsulation or by adding new pleasant ingredients, among other options.

For the first time, it was demonstrated that the Moka and Neapolitan techniques may be the most effective methods for polyphenol extraction, affording the highest radical scavenging activity and α-glucosidase inhibition capacity, whereas the beverage produced by the Capsule technique showed the poorest extraction. Therefore, this work indicates that CBS may be an optimal ingredient for home-made functional beverages with potential health benefits for consumers, thereby reducing the environmental and economic impact of by-product disposal.

CHAPTER 2.2

In vitro Bioaccessibility and Functional Properties of Phenolic Compounds from Enriched Beverages Based on Cocoa Bean Shell

In this chapter, two of the beverage preparation techniques of the previous chapter were selected basing on a 'consumer acceptance/biofunctional potential/commonly used technique' compromise. Tea bag (maceration) and capsule techniques were selected and different sensory optimizations of these beverages were proposed by adding different flavorings. After a consumer acceptance evaluation, two of the sensory optimized beverages were proposed for the study of the *in vitro* bioaccessibility of the polyphenols and methylxanthines contained in them. This allowed an understanding of the amount of biofunctional compounds contained in the beverages which is really available for absorption at the intestinal level after the gastrointestinal digestion process. The changes in two functional properties (antiradical and α-glucosidase inhibition capacities) after gastrointestinal digestion were also assessed. This study allowed an assessment of the bioaccessibility behavior of the CBS-contained biofunctional compounds in the presence of a liquid and simple food matrix.

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In Vitro Bioaccessibility and Functional Properties of Phenolic Compounds from Enriched Beverages Based on Cocoa Bean Shell

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1. Introduction

The chocolate industry generates large amounts of by-products since the cocoa bean (CB) represents one-third of the total weight of the fruit while the remaining 67% is made up of the pod husk, placenta, shell, and germ [290]. The cocoa bean shell (CBS) is the thin and fibrous external tegument of the bean, which is removed with the germ before or after roasting [220]. The CBS represents 10–17% of the bean and, considering 4.7 million tons of cocoa beans are processed worldwide each year, its annual production amount is approximately 700,000 tons that are generally intended to be used as fuel, feed, or fertilizer [218]. The CBS has an interesting nutritional profile with more than 50% (w/w) of dietary fibre [220], almost three times higher than the CB. It also has a low fat content with physical and chemical characteristics identical to those of cocoa butter except for linoleic acid, which is higher in the CBS (7.49% in CBS fat vs. 3.93% in cocoa butter) [88,220]. Moreover, the CBS is also a source of dietary minerals (e.g., calcium, iron, and magnesium) [112] and key aromatic compounds (e.g., 3-methylbutanal and 2,3,5-trimethylpyrazine) that contribute to its cocoa flavour notes [29,66]. Finally, the CBS contains a high amount of polyphenols with a total phenolic content that ranges from 3.12–94.95 mg of gallic acid equivalents (GAE)/g of dried CBSs mainly resulting from flavanols, which include catechins, epicatechins, and procyanidins [218]. Recently, polyphenols have been widely studied for their health benefits, serving as antioxidants, chelators of bivalent cations, and enzyme activity inhibitors (e.g., enzymes responsible for ROS production) and modulators (e.g., nitric oxide synthase, cyclooxygenase, and lipoxygenase) [81]. Skenderidis et al. [291] demonstrated the capability of polyphenols from goji berry extract to enhance the antioxidant status of muscle cells C2C12 by increasing the levels of a crucial antioxidant molecule, the glutathione, and the protective effects against radical-induced DNA damage.

Many biofunctionalities and potential health benefits of cocoa polyphenols have been reviewed by Rojo-Poveda et al. [218], such as antidiabetic activity; anticariogenic, anticarcinogenic, and anti-inflammatory effects; and actions on cardiovascular health. In particular, flavonoids have been suggested as natural inhibitors of the α-amylase and α-glucosidase enzymes directly involved in the degradation of complex carbohydrates; in other words, they are able to lower the amount of glucose intake directly binding to amino acid residues in the enzyme active sites and exclude substrate binding [292]. The inhibition capacity of flavonoids depends on their molecular structure and is higher when planar structure of the rings, double bonds in the 2 and 3 positions in the C_3 part of the flavonoid

skeleton (C_6–C_3–C_6), and hydroxylation in the rings occur [293]. By reducing the post-prandial level of glucose in the bloodstream, flavonoids can therefore be used in diabetes treatment as substitutes for some drugs (e.g., acarbose) that cause side effects, such as abdominal distension, flatulence, meteorism, and diarrhoea [292].

In order to exert some of their physiological functions, polyphenols have to be absorbed in the gastrointestinal tract and reach the target tissue through the circulation system. So far, many studies have been conducted in order to elucidate the bioaccessibility of polyphenols, defined by Watson et al. [161] as the fraction of an ingested nutrient from the food matrix that is available for absorption. The bioaccessibility of polyphenols depends on several factors, which include their release from the food matrix, molecular size, hydrophilic/lipophilic balance as related to their glycosylation, and different pH-dependent transformations (degradation, hydrolysis, epimerization, and oxidation within the gastrointestinal tract) as well as solubility and interactions between polyphenols and food components [294]. In fact, polyphenols in liquid matrices are directly available for absorption while those in solid matrices have to first be extracted through mechanical, chemical, and enzymatic actions to make the absorption in the gastrointestinal tract easier [295].

Attention to the CBS has recently increased, and several studies [218,220] have focused on its uses as a source of bioactive compounds in food formulation. Numerous applications have been reviewed [218], mainly in order to take advantage of the antioxidant properties and high fibre content. In fact, CBS has been deemed suitable for use in the preparation of low-calorie and/or fibre-rich foods, such as chocolate biscuits, cakes, muffins, dietetic chocolate substitutes, and bread [218]. Furthermore, the antioxidant properties of the CBS were successfully tested to prevent lipid oxidation in cooked beef compared to synthetic BHT and β-tocopherol, and it has also been suggested for application in frying oils [218]. According to the literature, CBSs can also be used in the production of hot and cold beverages. Quijano-Aviles et al. [120] formulated a dairy drink enriched with CBSs, coffee silverskin, and orange peel aqueous extracts. Rojo-Poveda et al. [13] evaluated the functional properties of beverages obtained from CBSs with various particle sizes using six different extraction techniques (Moka, Neapolitan, American, Espresso, Capsule, and French Press). In the latter work, encouraging results were obtained for the beverages from a nutritional point of view, with a high total phenolic content (up to 1803.83 mg GAE/L) and proven antioxidant and antidiabetic properties (up to 7.29 mmol of Trolox equivalents per litre of beverage and up to 52.00% of α-glucosidase inhibition, respectively). Nevertheless, there were still opportunities for further enhancements in consumer acceptance. In fact, the high content in polyphenols and

methylxanthines and the acidity led to beverages with a bitter, astringent and unpleasant taste and flavour [13]. The addition of other ingredients beside CBS in the preparation would be beneficial to the taste, flavour and the overall liking of the beverages, especially if they are able to cover the acidity of the shell.

The main purpose of this study, as an extension of the Rojo-Poveda et al. [13] study (Chapter 2.1), was to assess the bioaccessibility of bioactive compounds and functional properties of two selected and sensorially enhanced beverages based on CBSs and enriched with different flavourings. In order to identify the most suitable beverages for potential commercialization, an evaluation of the sensory effects of the different flavouring combination together with a study of their polyphenolic content and functional properties was made prior to the bioaccessibility study.

2. Materials and Methods

2.1. Chemicals

Folin-Ciocalteu's phenol reagent, 2-2'-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (97%) (Trolox), methanol (≥ 99.9%), formic acid (≥ 98%), α-glucosidase from rat intestinal acetone powders, p-nitrophenyl-α-D-glucopyranoside (≥ 99%) (p-PNG), acarbose (≥ 95%), (+)-catechin hydrate (> 98%), quercetin-3-*O*-glucoside (≥ 90%), theobromine (≥ 98.5%), caffeine (≥ 98.5%), α-amylase from *Bacillus* sp., pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and bile salts were supplied by Sigma-Aldrich (Milan, Italy). Sodium carbonate, potassium phosphate dibasic, potassium phosphate monobasic, potassium chloride, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, hydrochloric acid, and calcium chloride dihydrate were provided by Carlo Erba (Milan, Italy). All chemicals were of high purity. Gallic acid (≥ 98%), ethanol (≥ 99.9%), sodium hydroxide (1M), (–)-epicatechin (> 90%), procyanidin B1 (PCB1) (≥ 98.5%), procyanidin B2 (PCB2) (≥ 98.5%), protocatechuic acid (> 97%), and caffeic acid (≥ 95%) were provided by Fluka (Milan, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

2.2. Samples

2.2.1. Beverage Ingredients

The CBSs were obtained from Colombia roasted CBs (Criollo variety) that were kindly provided by Guido Gobino S.r.l. (Turin, Italy). They were ground with an Ultracentrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) and, according to Rojo-Poveda et al. [13], particle sizes of 500–1000 μm and 250–500 μm were chosen for the capsules and tea bags, respectively. The CBS composition, expressed as g/kg dry matter, was 178.0 protein, 34.0 fat, 106.0 carbohydrates, 545.0 dietary fibre, 68.0 water, and 69.0 ashes.

The CBs from Venezuela (Merida area and Criollo variety) were kindly provided by Venchi S.p.A. (Milan, Italy). The beans were ground and sieved (< 1000 μm) with an Ultracentrifugal Mill ZM 200 (Retsch GmbH).

Turmeric, curry, vanillin, rooibos, coconut, mint, cinnamon, and liquorice, widely used as flavourings in tea and infusions for their intense aromas, were purchased from a local market in powdered form except for the coconut (grated), rooibos (dried leaves), and mint (dried leaves).

2.2.2. Beverage Formulation

Thirteen formulations were prepared for each extraction method (Table 1). For each formulation, only one aromatic ingredient or a mix of two ingredients (at the same concentrations) were added to a mix of CBSs and CBs. The composition of mixes was defined according to other beverages on the market. The capsules were filled with 7 g of product while 3 g of product was used for the tea bags.

2.2.3. Beverage Preparation

Among the extraction techniques tested by Rojo-Poveda et al. [13] (Chapter 2.1), the two most commonly used (capsules and tea bags) were selected to prepare the beverages.

An AEG capsule machine LM 3100 (Milan, Italy) was used for the capsule extraction. The capsules (SelfCap®; Mokitalia, Milan, Italy) were prepared according to Table 1, and 120 mL of beverage was obtained from each formulation in order to have a typical *lungo coffee*, which differs from the *espresso coffee* as it is less concentrated and requires a larger size of the cup (20–40 mL for the *espresso coffee* vs. 100–250 mL for the *lungo coffee*) [296]. The tea bags were produced with Teeli flip filters (Teeli®; Hamburg, Germany), filled according to Table 1,

and infused in 100 mL of boiling water (100 °C) for 4 min. Natural mineral water (Valmora, Luserna San Giovanni, Italy) was used to prepare the beverages. Three independent beverages were prepared from each formulation.

For the analytical determinations, the obtained beverages were cooled in a dark room at 20 °C and centrifuged with a Heraeus Megafuge 11R centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 7,600 × *g* for 5 min and then filtered through 0.45 µm cellulose acetate filter (Carlo Erba). The samples were kept at -20 °C until subsequent analysis.

Table 1. Quantities (%) of CBS, cocoa beans (CB) and aromatic ingredients used for the production of beverages.

Formulation	CBS	CB	Turmeric	Curry	Vanillin	Rooibos	Coconut	Mint	Cinnamon	Licorice
A	97	3								
B1	94	3	1.5	1.5						
B2	91	3	3	3						
C1	96.6	3			0.4					
C2	96.1	3			0.9					
D1	92	3				5				
D2	87	3				10				
E1	87	3					10			
E2	77	3					20			
F1	95.5	3						1.5		
F2	94	3						3		
G1	95.4	3							0.8	0.8
G2	93.8	3							1.6	1.6

2.3. Sensory Evaluation

Two sensory evaluations were performed. The first was a paired comparison test (ISO 5495:2005) used to compare the two concentrations of each tested aromatic ingredient. A two-sided difference test was used with $\alpha = 0.05$, $\beta = 0.5$, and a pd = 40% and carried out by 24 untrained tasters (female = 70%; age range: 21–45 years). Participants received two cups with samples and were asked to rinse their mouths with still water before beginning the evaluation and in between samples. Beverages were served in a randomized and balanced order. The obtained results were evaluated according to ISO 5495:2005. The second test was a consumer acceptance test, which was conducted on 20 untrained tasters (female = 80%; age range: 21–65 years). Participants received individual cups with the samples and still water to rinse their mouths before beginning the evaluation and in between samples. Participants tasted the samples in a randomized and balanced order without any information about the beverage composition to avoid any potential bias on the liking scores. Participants rated their liking for "appearance", "odour", "taste", "flavour", and "overall liking" using a nine-point hedonic scale (1 = extremely dislike to 9 = extremely like [297]). "Purchase interest" was rated on a seven-point scale (1 = absolutely no to 7 = absolutely yes). Consumers took 3–10 minutes to complete the evaluation.

Written informed consent was collected from all participants before the tests. The tests were performed in an air-conditioned room with white light at approximately 21 °C.

2.4. In vitro *Simulated Gastrointestinal Digestion (GID)*

The most appreciated beverages, identified by the consumer acceptance test, underwent *in vitro* GID. The digestion was carried out through a three-phase (oral, gastric, and intestinal) standardized protocol according to Minekus et al. [298]. Briefly, 5 mL of each selected beverage was mixed with simulated digestive fluids (simulated salivary fluid, simulated gastric fluid, and simulated intestinal fluid) consisting of the corresponding electrolyte stock solutions, enzymes, and water. Electrolyte stock solutions were previously heated in a SW-20 water bath (Julabo GmbH, Seelbach, Germany) at 37 °C. The digestion process was replicated three times for each beverage. A control, in which the sample was replaced by ultrapure water, was also prepared in triplicate in order to assess the contribution of digestion enzymes and simulated fluids in the subsequent analysis. Once the digestive phase was completed, the pH was brought down to 5.4 in order to stop the process. The samples were centrifuged at 12,500 × *g* for 10 min at 4 °C, and the supernatants were passed through 0.45 μm cellulose acetate filters (Carlo Erba, Milan, Italy). The filtered samples were stored at 20 °C until subsequent analyses.

In vitro bioaccessibility was calculated according to the following equation:

$$\% \text{ Bioaccessibility} = (C_{POST} / C_{PRE}) \times 100$$

where C_{POST} and C_{PRE} correspond to the concentration after and before the digestion process, respectively.

2.5. Analytical Determinations

2.5.1. Total Phenolic Content (TPC) Assay

The TPC of the beverages was determined according to the Folin-Ciocalteu colorimetric method adapted to a 96-well microplate [57] using a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy), as described in Chapter 1.1. All determinations were performed in triplicate. A calibration curve of standard gallic acid (100–500 μM) was built, and the results were expressed in mg of GAE per litre of beverage.

2.5.2. Radical Scavenging Activity (RSA) Assay

RSA was determined by the $DPPH^{\bullet}$ method as reported by Von Gadow et al [233] with slight modifications and adapted to a 96-well microplate [57], as described in Chapter 1.1.. The

decrease in $DPPH^{\bullet}$ absorbance was measured at 515 nm in a BioTek Synergy HT microplate reader. All determinations were performed in triplicate. A Trolox standard curve (12.5–300 μM) was prepared for scavenging activity quantification, and the results were expressed in mmol of Trolox equivalents (TE) per litre of beverage.

2.5.3. *In vitro* α-Glucosidase Inhibition

The antidiabetic capacity of the beverages, evaluated as their α-glucosidase inhibition capacity, was determined by the α-glucosidase colorimetric assay detailed in Rojo-Poveda et al. [13], using a BioTek Synergy HT microplate reader, as described in Chapter 2.1. All measurements were run in triplicate. The antidiabetic capacity was expressed as percentage of α-glucosidase inhibition, and acarbose 0.5 mM (IC_{50}) was used as the positive control.

2.5.4. Bioactive Compound Analyses by Reverse-Phase Liquid Chromatography

Polyphenols and methylxanthines were determined by reverse-phase high pressure liquid chromatography coupled with photodiode array detector (RP-HPLC-PDA) as described by Rojo-Poveda et al. [13] and as detailed in Chapter 1.1. The samples were preliminarily filtered with PTFE filters (LLG-Labware, Meckenheim, Germany) at 0.20 μm. The correlation coefficients of the external calibration curves obtained under the same chromatographic conditions and used for quantification were as follows: R^2 = 0.9995 for theobromine, R^2 = 0.9996 for caffeine, R^2 = 0.9999 for catechin, R^2 = 0.9998 for epicatechin, R^2 = 0.9997 for protocatechuic acid, R^2 = 0.9999 for caffeic acid, R^2 = 0.9998 for PCB1, R^2 = 0.9999 for PCB2, and R^2 = 0.9996 for quercetin-3-*O*-glucoside. Catechin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside were quantified as catechin and quercetin-3-*O*-glucoside equivalents, respectively.

2.6. Statistical Analysis

The results were statistically analysed with SPSS Statistics 25 software (IBM-SPSS Inc., Chicago, Illinois, USA). An analysis of variance (ANOVA) and Duncan's post hoc test (95% confidence level) for TPC and RSA were used to compare the differences between mean values of the different formulations. The Kruskal-Wallis H-test (95% confidence level) with a multiple comparison test was applied for the consumer acceptance evaluation.

3. Results and Discussion

3.1. Comparison of the Beverages Based on Consumer Tests

A comparison test was done in order to select the best quantity of each aromatic ingredient for the capsules and tea bags. The obtained results (data not shown) highlighted that, for the formulation with turmeric and curry, the B1 mix with a lower quantity of aromatic ingredients was the most appreciated by tasters. The same result was obtained for F1 (mint) and G1 (cinnamon and liquorice mix) while for the other beverages, the higher quantity of ingredients was preferred (C2, D2, and E2). No differences ($p > 0.05$) were highlighted between the capsule and tea bag results, confirming that the selected quantities for each aromatic ingredient were the most appreciated by consumers.

Regarding the consumer test, Table 2 shows the results reported as the sum of ranks calculated for each beverage obtained by the capsule and tea bag methods.

Table 2. Results of Kruskall-Wallis test (reported as sum of ranks) of consumer acceptance evaluation and results of the multiple comparison test.

Extraction technique	Formulation	Appearance	Odor	Taste	Flavor	Overall liking	Purchase interest
Capsule	A	2672	2773 ^{abc}	2777 ^{abc}	2685 ^{abc}	2717 ^{ab}	3078 ^{ab}
	B1	2753	2060 ^{c}	1700 ^{c}	2261 ^{bc}	2021 ^{b}	2050 ^{b}
	C2	2803	3848 ^{a}	2629 ^{abc}	2407 ^{abc}	2740 ^{ab}	2627 ^{ab}
	D2	3309	2577 ^{bc}	3082 ^{ab}	2994 ^{abc}	2921 ^{ab}	3090 ^{ab}
	E2	3170	3533 ^{ab}	3652 ^{a}	3607 ^{a}	3775 ^{a}	3635 ^{a}
	F1	2166	1813 ^{c}	2343 ^{bc}	2126 ^{c}	2305 ^{b}	2153 ^{b}
	G1	2435	2311 ^{c}	3123 ^{ab}	3227 ^{ab}	2829 ^{ab}	2674 ^{ab}
	Sig.	n.s.	***	***	**	***	***
Tea bag	A	2814	3134 ^{ab}	2175 ^{b}	2149 ^{b}	2304 ^{bcd}	2091 ^{b}
	B1	2627	1666 ^{c}	2012 ^{b}	2154 ^{b}	1985 ^{d}	2197 ^{b}
	C2	2027	3866 ^{a}	3278 ^{ab}	3163 ^{ab}	3406 ^{ab}	3689 ^{a}
	D2	2831	1959 ^{bc}	2562 ^{b}	2411 ^{b}	2267 ^{bcd}	2257 ^{b}
	E2	3394	3809 ^{a}	3935 ^{a}	3985 ^{a}	3965 ^{a}	3995 ^{a}
	F1	2802	1707 ^{c}	2260 ^{b}	2256 ^{b}	2098 ^{cd}	2235 ^{b}
	G1	2813	3166 ^{ab}	3085 ^{ab}	2798 ^{b}	3282 ^{abc}	2843 ^{ab}
	Sig.	n.s.	***	***	***	***	***

Means followed by the same letter in the same column are not significantly different at $p < 0.05$ (multiple comparison test).
Significance: ** = $p < 0.01$; *** = $p < 0.001$; n.s. = not significant.

Significant differences ($p < 0.01$) were found between the different formulations for all sensory parameters of both the capsule and tea bag methods with the exception of appearance, meaning that the different flavourings did not influence taster judgement on the look of the beverages, which resulted in equal rankings. This fact could be due to, among other possibilities, the small amount of the flavourings in the formulations compared to the other

two main ingredients, namely CBSs and CBs, thus not having a strong impact on the visual aspect of the drink.

Table 3. Results of comparison with Kruskall-Wallis test between capsule and tea bag for each formulation. Values are reported as sum of ranks.

Formulation		Appearance	Odor	Taste	Flavor	Overall liking	Purchase interest
A	Capsule	2672	2773	2777	2685	2717	3078
	Tea bag	2814	3134	2175	2149	2304	2091
	Sig.	*	n.s.	n.s.	n.s.	n.s.	n.s.
B1	Capsule	2753	2060	1700	2261	2021	2050
	Tea bag	2627	1666	2012	2154	1985	2197
	Sig.	n.s.	n.s.	*	n.s.	n.s.	n.s.
C2	Capsule	2803	3848	2629	2407	2740	2627
	Tea bag	2027	3866	3278	3163	3406	3689
	Sig.	n.s.	n.s.	**	**	**	***
D2	Capsule	3309	2577	3082	2994	2921	3090
	Tea bag	2831	1959	2562	2411	2267	2257
	Sig.	n.s.	*	n.s.	n.s.	n.s.	n.s.
E2	Capsule	3170	3533	3652	3607	3775	3635
	Tea bag	3394	3809	3935	3985	3965	3995
	Sig.	n.s.	n.s.	*	*	n.s.	n.s.
F1	Capsule	2166	1813	2343	2126	2305	2153
	Tea bag	2802	1707	2260	2256	2098	2235
	Sig.	**	n.s.	n.s.	*	n.s.	n.s.
G1	Capsule	2435	2311	3123	3227	2829	2674
	Tea bag	2813	3166	3085	2798	3282	2843
	Sig.	n.s.	n.s.	n.s.	n.s.	**	*

Significance: ** = $p<0.01$; *** = $p<0.001$; n.s. = not significant.

In capsule drinks, the coconut formulation (E2) obtained the highest score for all parameters with the exception of odour and appearance, in both of which it got the second highest score. On the other hand, formulations B1 (turmeric and curry) and F1 (mint) were the least appreciated by tasters with scores for odour, taste, flavour, overall liking, and purchase interest that were almost half of those characterizing formulation E2. Tea bag beverages showed a similar trend for capsule beverages, where the E2 formulation displayed the highest score for all parameters except appearance. Obtaining the highest scores, coconut turned out to be a valid ingredient to cover the acidity and bitterness of CBs and CBSs while also giving a tasteful flavour to the beverages. Coconut proved to be a valid ingredient to be combined with cocoa in other studies as well, gaining high levels of consensus among consumers [299,300]. For both the capsule and tea bag methods, formulation A, which contained only CBSs and CBs with no other flavouring, did not achieve the lowest score for all parameters

with the exceptions of flavour and purchase predisposition in tea bag beverages. Thus, flavouring besides CBs did not always lead to an improvement of the beverage sensory characteristics as observed with formulations B1 and F1.

Comparing the two extraction techniques, capsule and tea bag beverages were not significantly different in most cases ($p > 0.05$) (Table 3). However, where significant differences ($p < 0.05$) were found, tea bags were characterized by a higher score. Hence, tea bags seemed to be more appreciated than capsules.

On the basis of the aforementioned reported results, the coconut formulation (E2) was used for further study of the bioaccessibility of bioactive compounds after *in vitro* GID.

3.2. Total Phenolic Content (TPC) and Radical Scavenging Activity (RSA)

TPC and RSA were evaluated for the seven formulations selected using the paired comparison test. For all capsule and tea bag beverages, significant differences ($p < 0.05$) were found between the different formulations (Table 4).

Table 4. Values (mean ± standard deviation) of total phenolic content (TPC) and radical scavenging activity (RSA) for beverages obtained with capsule and tea bag extraction techniques and results of ANOVA with Duncan's test performed between formulations for each extraction method (columns) and between extraction methods for each formulation (rows).

	Formulation	Capsule	Tea bag	*Sig.*
TPC (mg GAE/L)	A	384.98 ± 13.19 ^{bc}	325.94 ± 26.43 ^{c}	*
	B1	599.64 ± 62.14 ^{a}	306.33 ± 12.86 ^{c}	**
	C2	551.06 ± 50.37 ^{a}	586.20 ± 57.27 ^{a}	n.s.
	D2	455.18 ± 24.43 ^{b}	476.93 ± 79.30 ^{b}	n.s.
	E2	343.57 ± 15.46 ^{c}	343.58 ± 4.77 ^{c}	n.s.
	F1	431.55 ± 65.07 ^{b}	339.39 ± 64.64 ^{c}	n.s.
	G1	600.48 ± 25.22 ^{a}	320.37 ± 28.43 ^{c}	***
	Sig.	***	***	
RSA (mM TE/L)	A	1.97 ± 0.05 ^{bc}	1.78 ± 0.12 ^{b}	n.s.
	B1	2.92 ± 0.30 ^{a}	1.70 ± 0.05 ^{b}	**
	C2	2.01 ± 0.18 ^{bc}	1.88 ± 0.04 ^{b}	n.s.
	D2	2.31 ± 0.11 ^{b}	2.53 ± 0.37 ^{a}	n.s.
	E2	1.75 ± 0.08 ^{c}	1.88 ± 0.09 ^{b}	n.s.
	F1	2.21 ± 0.27 ^{b}	1.96 ± 0.35 ^{b}	n.s.
	G1	2.99 ± 0.25 ^{a}	1.81 ± 0.09 ^{b}	***
	Sig.	***	*	

GAE, gallic acid equivalents; TE, Trolox equivalents.
Means followed by the same letter in the same column are not significantly different at $p < 0.05$.
Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s. = not significant.

For the capsule method, the formulations B1 (turmeric and curry), C2 (vanillin), and G1 (cinnamon and liquorice) displayed the highest content of TPC followed by formulations D2 (rooibos), F1 (mint), A (control), and E2 (coconut), which reported the lowest value. However, it should be highlighted that formulations A, B1, C2, F1, and G1 contained 97–100% CBSs and CBs while formulation E2 contained 80% of these two main ingredients,

suggesting that coconut did not contribute to the TPC. Formulation D2 contained 90% CBSs and CBs but showed a high TPC due to the presence of rooibos. On the other hand, when tea bags were used, the highest TPC value was observed in formulation C2 followed by D2. For all other formulations (A, B1, E2, F1 and G2), no significant differences emerged ($p > 0.05$), thus presenting equivalent values.

Regarding antiradical activity, it is well known that one of the beneficial properties of polyphenols is their ability to react with free radicals as a result of the presence of hydroxyl groups [301]. Thus, the RSA is often correlated to TPC as confirmed by the present study with the exception of formulation C2 where vanillin was used. However, the high reactivity of vanillin with Folin-Ciocalteau's reagent and its lack of response to the DPPH assay have been previously identified [302].

No significant differences ($p > 0.05$) were found between the two extraction methods for each formulation with the exceptions of formulations A, B1, and G1, which showed both higher TPC and RSA in capsule drinks. However, it should be noted that the two techniques used different quantities of ingredients and water (7 g in 120 mL for capsules and 3 g in 100 mL for tea bags). In fact, if the data are normalized on the grams of filling and volume of water used, tea bag beverages will show greater values than capsule drinks in almost all cases (Table 5) as demonstrated by Rojo-Poveda et al. [13]. This fact could be due to the lower solid/liquid ratio and longer time of contact between the ingredients and hot water (4 minutes vs. < 30 seconds in tea bags and capsules, respectively) that occurred in the tea bag method. In fact, Ludwig et al. [303] and Gloess et al. [296] reported that a lower solid/liquid ratio and longer extraction time increased the extraction efficiency of bioactive compounds.

Compared with some of the drinks that can be found on the market, the formulated beverages have TPC values equivalent to or even higher than other infusions, such as mint (315 mg GAE/L), and fresh juice, including apple (339 mg GAE/L), pineapple (358 mg GAE/L), and white grape (519 mg GAE/L) [304]. Moreover, considering the TPC values normalized on the grams of filling obtained in this study (5.89–10.29 mg GAE/g and 10.05–19.54 mg GAE/g for capsules and tea bags, respectively, reported as the range from the lowest to the highest value among the formulations, Table 5), it can be observed that they are greater compared to that reported by Quijano-Aviles et al. [120] in an experimental dairy drink made of milk, CBSs, coffee husks, and orange peel (5.74 mg GAE/g).

Table 5. Values (mean ± standard deviation) after normalization of total phenolic content (TPC) and radical scavenging activity (RSA) for beverages obtained with capsule and tea bag extraction techniques and results of ANOVA with Duncan's test performed between formulations for each extraction method (columns) and between extraction methods for each formulation (rows).

	Formulation	Capsule	Tea bag	*Sig.*
TPC (mg GAE /g of filling)	A	6.60±0.23^{bc}	10.86±0.88^{c}	**
	B1	10.28±1.07^{a}	10.21±0.43^{c}	n.s.
	C2	9.45±0.86^{a}	19.54±1.91^{a}	**
	D2	7.80±0.42^{b}	15.90±2.64^{b}	*
	E2	5.89±0.26^{c}	10.05±1.42^{c}	**
	F1	7.40±1.12^{b}	11.31±2.15^{c}	*
	G1	10.29±0.43^{a}	10.68±0.95^{c}	n.s.
	Sig.	***	***	
RSA (µmol TE /g of filling)	A	33.72±0.86^{bc}	59.25±3.84^{b}	**
	B1	50.12±5.16^{a}	56.78±1.53^{b}	n.s.
	C2	34.50±3.05^{bc}	62.67±1.29^{b}	***
	D2	39.67±1.94^{b}	84.19±12.27^{a}	**
	E2	30.02±1.33^{c}	62.38±3.55^{b}	***
	F1	37.90±4.68^{b}	65.40±11.81^{b}	*
	G1	51.18±4.27^{a}	60.28±3.14^{b}	*
	Sig.	***	**	

GAE, gallic acid equivalents; TE, Trolox equivalents.
Means followed by the same letter in the same column are not significantly different at $p<0.05$.
Significance: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; n.s. = not significant.

3.3. Bioaccessibility of Bioactive Compounds and Functional Characteristics

The beverages with the highest values of overall liking —the E2 formulation in both capsules and tea bags— and therefore the greatest potential to be commercialized as final products underwent *in vitro* GID in order to assess the bioaccessibility of their bioactive compounds, comparing the results obtained before digestion with those obtained after the digestion process. In particular, phenolic content through TPC and HPLC analyses (the latter for methylxanthines as well), RSA, and α-glucosidase inhibition capacity were evaluated.

3.3.1. Determination of Polyphenol and Methylxanthine Composition of Undigested and Digested Beverages through RP-HPLC-PDA.

Table 6 shows the compounds detected in the beverages through liquid chromatography before and after GID (two methylxanthines and nine polyphenols). As far as polyphenols are concerned, phenolic acids (protocatechuic acid and caffeic acid), flavan-3-ols (catechin-3-*O*-glucoside, catechin and epicatechin), B-type procyanidins (PCB isomers), and flavonols (quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside) were identified.

Table 6. Values (mean ± standard deviation) of methylxanthines and identified polyphenols before (PRE) and after (POST) *in vitro* gastrointestinal digestion for capsule and tea bag coconut flavored beverages (E2) and results of ANOVA. Significance is reported for each compound between PRE and POST *in vitro* gastrointestinal digestion within the same extraction technique (column) and for each compound both PRE and POST *in vitro* gastrointestinal digestion between the two extraction methods (row).

Extraction method **Formulation**		**Capsule** **E2**	**Tea bag** **E2**	***Sig.***
Methylxanthines *(mg/L of beverage)*				
Theobromine	PRE	172.09 ± 4.55	144.36 ± 18.83	n.s.
	POST	185.81 ± 27.59	150.70 ± 21.29	n.s.
	Sig.	n.s.	n.s.	
Caffeine	PRE	40.90 ± 2.02	27.57 ± 4.71	*
	POST	38.11 ± 5.62	25.97 ± 4.08	*
	Sig.	n.s.	n.s.	
Polyphenols *(mg/L of beverage)*				
Phenolic acids				
Protocatechuic acid	PRE	2.35 ± 0.14	1.93 ± 0.18	n.s.
	POST	0.90 ± 0.07	0.72 ± 0.08	*
	Sig.	***	***	
Caffeic acid	PRE	1.81 ± 0.14	1.51 ± 0.34	n.s.
	POST	0.40 ± 0.04	0.33 ± 0.02	n.s.
	Sig.	***	**	
Flavan-3-ols				
Catechin-3-*O*-glucoside	PRE	2.37 ± 0.08	1.94 ± 0.18	**
	POST	1.12 ± 0.13	1.08 ± 0.15	*
	Sig.	***	**	
Catechin	PRE	2.04 ± 0.11	1.65 ± 0.15	**
	POST	2.55 ± 0.25	2.71 ± 0.29	n.s.
	Sig.	**	***	
Epicatechin	PRE	4.64 ± 0.30	3.94 ± 1.17	n.s.
	POST	0.48 ± 0.22	0.47 ± 0.24	n.s.
	Sig.	***	**	
Procyanidins type B				
Procyanidin B isomer (PCB)	PRE	4.92 ± 0.56	4.41 ± 0.59	n.s.
	POST	4.21 ± 0.34	3.53 ± 0.49	n.s.
	Sig.	n.s.	n.s.	
Procyanidin B2 (PCB2)	PRE	6.36 ± 0.71	5.21 ± 1.22	n.s.
	POST	2.79 ± 0.38	2.72 ± 0.39	n.s.
	Sig.	**	*	
Flavonols				
Quercetin-3-*O*-glucoside	PRE	0.24 ± 0.03	0.23 ± 0.05	n.s.
	POST	n.d.	n.d.	n/a
	Sig.	n/a	n/a	
Quercetin-3-*O*-rhamnoside	PRE	0.21 ± 0.02	0.18 ± 0.03	n.s.
	POST	n.d.	n.d.	n/a
	Sig.	n/a	n/a	

n.d. = not detected
n/a = not applicable.
Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s. = not significant.

In non-digested beverages (PRE), the most abundant compound was theobromine, a methylxanthine alkaloid that characterizes cocoa (72.34 and 74.89% of the total identified compounds for capsules and tea bags, respectively) followed by caffeine (17.18 and 14.24%

of the total compounds identified for capsules and tea bags, respectively), another biologically active alkaloid. The total methylxanthine content was equal to 212.99 mg/L and 171.93 mg/L for capsules and tea bags, respectively. The ratio between these two alkaloids was approximately 5:1, which is in line with the data published by Rojo-Poveda et al. [13]. Concerning polyphenol composition, capsule and tea bag beverages showed the same profile with PCB2, PCB, and epicatechin as the most abundant compounds. In fact, for the total quantified polyphenols in capsule drinks, PCB2, PCB, and epicatechin were 25.47, 19.75, and 18.60% with respect to the total polyphenols quantified, respectively. Likewise, in tea bag drinks, PCB2, PCB, and epicatechin were 24.59, 21.05, and 18.45%, respectively.

After GID, the content of methylxanthines remained unchanged in both beverages, as they are stable under gastric and intestinal conditions and not degraded by pH and enzymes. Hence, the 5:1 ratio between theobromine and caffeine was maintained after GID. On the contrary, the concentration of polyphenols changed during GID with capsule and tea bag beverages showing the same behaviour. The total content of polyphenols quantified before GID was 24.95 and 20.99 mg/L for capsule and tea bag beverages, respectively. After GID, these values decreased, reaching a total of 12.45 and 11.56 mg/L with bioaccessibility of 50.00% and 55.46% for capsules and tea bags, respectively. These values represent the amount of soluble and accessible polyphenols not only to be absorbed but also to potentially exert their functions at the intestinal level, such as the ability to inhibit the α-glucosidase enzyme and the anti-inflammatory effects demonstrated by Rossin et al. [78]. The phenolic acids in both drinks degraded considerably with bioaccessibility that ranged from 37.43–38.21% for protocatechuic acid and 22.17–22.82% for caffeic acid. With regard to flavan-3-ols, in both capsule and tea bag beverages, catechin-3-*O*-glucoside degraded partially during GID (bioaccessibility of 47.41% and 55.75%, respectively) while epicatechin degraded almost completely (bioaccessibility of 10.55% and 12.50%, respectively). These results agree with the literature, which reports a poor availability of flavan-3-ols due to their instability in the gastrointestinal environment [294,305-307]. In fact, pH plays a key role in the stability of catechins and catechin glucosides, making them very unstable and rapidly subjected to degradation in neutral or alkaline solutions, whereas they are relatively stable in acidic solution [308]. Moreover, the high binding capacity of these compounds to digestive enzymes, which entail the polymerization and thus formation of insoluble aggregates, has been broadly reported [294,309]. The considerable loss of epicatechin during GID could therefore be explained by its high affinity for such enzymes and the mild alkaline milieu that typifies the intestinal phase. On the other hand, after GID, catechin content in capsule and tea

bag beverages increased significantly ($p < 0.01$) with a bioaccessibility exceeding 100%. This boost could be derived from three different pathways. First, catechin-3-*O*-glucoside may have been hydrolysed in the intestinal phase, releasing aglycone. In fact, as reported by Raab et al. [308], catechin-3-*O*-glucoside shows a consistent degradation starting at pH 7 while it remains stable at low pH values. Hence, since glucoside shows a better resistance to degradation compared to aglycone [308], glycosylation could increase the bioaccessibility of catechin, which is delivered intact to the small intestine in an absorbable form. The second hypothesis is the epimerization of epicatechin into catechin under acidic conditions as has been widely reported by many authors [245,310,311]. Lastly, a depolymerization of type B procyanidin with a consequent liberation of free catechin may have occurred, and this result could be confirmed by several studies on polyphenol bioaccessibility after GID [245,294,310,311]. Finally, regarding flavonols, both quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside degraded completely after *in vitro* GID.

Comparing the beverages obtained with the two different extraction techniques, significant differences ($p < 0.05$) were found for protocatechuic acid after GID, catechin before GID, and caffeine and catechin-3-*O*-glucoside both before and after GID, which were found to be higher in capsules drink (Table 6). However, it should be highlighted that, once again, the solid/liquid ratio was different between capsules and tea bags (7 g of preparation in 120 mL of water in capsule beverages and 3 g in 100 mL in tea bag beverages). Thus, the results obtained from HPLC have been normalized based on the grams of preparation contained in the capsules and tea bags (Table 7).

Considering the results, the tea bag method had a greater efficiency in extracting bioactive compounds (the reasons for which have already been discussed above), including 56.96% more methylxanthines, 63.64% more polyphenols, and 57.66% more total bioactive compounds. In fact, all compounds with the exceptions of caffeine and epicatechin were significantly higher in tea bags than capsules ($p < 0.05$).

Table 7. Values (mean ± standard deviation) after normalization of methylxanthines and identified polyphenols before (PRE) and after (POST) *in vitro* gastrointestinal digestion for capsule and tea bag coconut flavored beverages (E2) and results of ANOVA. Significance is reported for each compound between PRE and POST *in vitro* gastrointestinal digestion within the same extraction technique (column) and for each compound both PRE and POST *in vitro* gastrointestinal digestion between the two extraction methods (row).

Extraction method **Formulation**		**Capsule** **E2**	**Tea bag** **E2**	***Sig.***
Methylxanthines (mg/g of filling)				
Theobromine	PRE	2.95 ± 0.08	4.81 ± 0.63	**
	POST	3.19 ± 0.47	5.02 ± 0.71	*
	Sig.	n.s.	n.s.	
Caffeine	PRE	0.70 ± 0.03	0.92 ± 0.16	n.s.
	POST	0.65 ± 0.10	0.87 ± 0.14	n.s.
	Sig.	n.s.	n.s.	
Polyphenols (μg/g of filling)				
Phenolic acids				
Protocatechuic acid	PRE	40.35 ± 2.44	64.44 ± 7.53	**
	POST	15.38 ± 1.20	24.09 ± 2.53	**
	Sig.	***	**	
Caffeic acid	PRE	30.96 ± 2.42	50.25 ± 11.24	*
	POST	6.86 ± 0.64	11.08 ± 0.68	**
	Sig.	***	**	
Flavan-3-ols				
Catechin-3-*O*-glucoside	PRE	40.62 ± 1.30	64.74 ± 5.92	**
	POST	19.27 ± 2.20	36.07 ± 4.98	**
	Sig.	***	**	
Catechin	PRE	34.93 ± 1.86	55.06 ± 4.90	**
	POST	43.70 ± 4.37	90.26 ± 9.55	***
	Sig.	**	***	
Epicatechin	PRE	79.58 ± 5.12	131.18 ± 38.99	n.s.
	POST	8.25 ± 3.83	15.75 ± 8.04	*
	Sig.	***	**	
Procyanidins B				
Type B procyanidin	PRE	84.31 ± 9.55	146.94 ± 19.61	**
	POST	72.10 ± 5.86	117.58 ± 16.40	**
	Sig.	n.s.	n.s.	
Procyanidin B2	PRE	109.08 ± 12.18	173.58 ± 40.67	*
	POST	47.90 ± 6.49	90.58 ± 12.98	**
	Sig.	**	*	
Flavonols				
Quercetin-3-*O*-glucoside	PRE	4.19 ± 0.52	7.62 ± 1.66	*
	POST	n.d.	n.d.	n/a
	Sig.	n/a	n/a	
Quercetin-3-*O*-rhamnoside	PRE	3.60 ± 0.28	5.96 ± 0.86	*
	POST	n.d.	n.d.	n/a
	Sig.	n/a	n/a	

n.d. = not detected
n/a = not applicable.
Significance: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; n.s. = not significant.

Considering the quantity that may be consumed for 1 cup of each beverage (120 mL for capsules and 200 mL for tea bags), the dose intake of theobromine and caffeine would be 20.65 mg and 4.91 mg for capsules and 28.87 mg and 5.51 mg for tea bags, respectively, which also represents the potential amount available to be absorbed into the bloodstream. On the other hand, the dose intake of total polyphenols detected in this study would be 2.99 mg for capsules and 4.20 mg for tea bags with a potential post-GID availability of 1.49 mg and 2.31 mg, respectively. The U.S. Food and Drug Administration (FDA) has established 400 mg of caffeine per day in adults as a dose not generally related to dangerous and negative effects; however, this is contingent on individual sensitivity to the alkaloid and how fast it is metabolized [312]. Likewise, the European Food Safety Authority (EFSA) states that caffeine intake from all sources up to 400 mg per day (about 5.7 mg/kg body weight (bw) per day for a 70 kg adult) does not give rise to safety concerns for healthy adults with the exceptions of pregnant and lactating women, adolescents, and children, for whom the EFSA sets a limit of 200 mg per day (approximately 3 mg/kg bw per day) [313]. As for theobromine, firm conclusions have not been drawn yet. In fact, while in clinical studies of with a 3–4-week duration, dose levels of 150 mg theobromine/day (1.5–2.1 mg/kg bw) were well tolerated and adverse effects (such as nausea, vomiting, headache and diarrhoea) were only observed from doses higher than 500 mg theobromine/day, an actual level of no safety concern in humans has not yet been identified; however, the EFSA suggests that it is probably higher than 150 mg/day [314]. Based on these findings, the EFSA decided to derive its reference dose from the caffeine data since the results of pharmacokinetics studies of caffeine and its metabolites suggest that about 11% of caffeine oral intake is converted into theobromine and the two substances show a similar pharmacological profile [314]. Moreover, although the pharmacological effects of caffeine and theobromine can overlap, the latter shows a much lower potency than caffeine with respect to effects on the central nervous system, kidneys, or heart [314]. Therefore, the EFSA predicts a level of 0.6 mg/kg bw per day for healthy adults and 0.3 mg/kg bw per day for pregnant and lactating women, adolescents, and children to be of no safety concern [314] but also suggests that exceeding these doses would not necessarily result in a health risk. In view of this, considering that their values of caffeine and theobromine are lower than the recommended doses, both capsule and tea bag beverages could be considered safe for human health and are not expected to cause negative effects generally associated with overconsumption of these two methylxanthines, such as insomnia, anxiousness, tachycardia and nausea [312,314].

3.3.2. TPC

The TPC results are reported in Figure 1a. In digested beverages, the TPC was significantly lower compared to non-digested beverages. In fact, polyphenols are soluble in the matrix, and some of them are not very stable in the GID conditions due to degradation by enzymes, salts, and pH. However, the bioaccessibility of polyphenols in the capsule and tea bag beverages was still high at 80.32% and 76.28%, respectively. The bioaccessibility of polyphenols observed with this analytical method is not in line with that observed using HPLC, demonstrating once again that the methodology based on Folin-Ciocalteu's reagent is not precise or reliable and tends to overestimate the result [315,316]. However, it should be noted that only the identified polyphenols were quantified by HPLC. Comparing the two different beverages (capsules vs. tea bags), no significant differences were found before GID. After GID, the TPC in capsule drinks was significantly ($p < 0.01$) higher than in tea bag drinks.

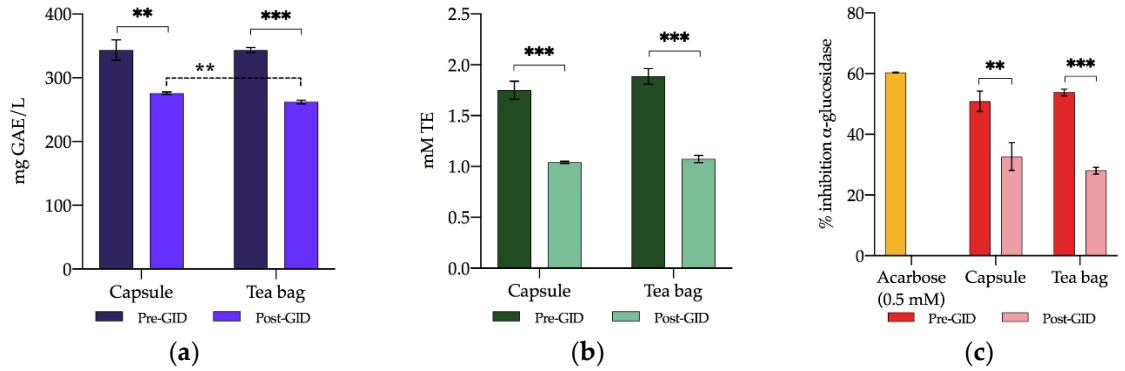

Figure 1. (**a**) TPC (mg GAE/L); (**b**) RSA (mM TE); (**c**) antidiabetic capacity (% α-glucosidase inhibition). For each panel results are reported before (Pre-GID) and after (Post-GID) GID for both capsule and tea bag coconut flavored beverages (E2), as well as results of ANOVA. Significance: ** = $p < 0.01$; *** = $p < 0.001$.

3.3.3. RSA

The RSA significantly decreased ($p < 0.001$) as a result of the digestive process (Figure 1b), and the loss of activity was about 40.50% for capsules and 42.91% for tea bags. The greater loss of RSA relative to TPC can be ascribed to the nature of the phenolic compounds degraded after GID. In fact, it has been reported in the literature that RSA is highly controlled by the number and nature of the hydroxylation pattern on the aromatic ring of phenolic compounds: the more the hydroxyl groups on the aromatic ring, the more the antiradical activity [317]. In this way, each phenolic compound exhibits a higher or lower RSA depending on the redox properties of its hydroxyl groups and its potential for electron

delocalization across the chemical structure [317]. The decrease in RSA could then be due to the almost complete degradation of caffeic acid, a hydroxycinnamic acid that is recognized to be one of the most effective scavengers among phenolic acids, followed by protocatechuic acid, a hydroxybenzoic acid with lower but still remarkable activity [317]. However, after GID, the free RSA remained considerable with a recovery of 59.50% for capsules and 57.09% for tea bags. No significant differences ($p > 0.05$) were found between the extraction methods before and after GID.

3.3.4. α-Glucosidase Inhibition Capacity

In this study, the α-glucosidase inhibition capacity of the beverages was evaluated, and both capsule and tea bag drinks displayed a significant enzyme inhibition capacity (Figure 1c). It is interesting to observe that the concentration of polyphenols in the beverages obtained through the two systems (capsules and tea bags) is able to inhibit the enzyme to almost the same extent as 0.5 mM acarbose. As stated for TPC and RSA, during GID, a loss of activity occurred by 35.85% for capsules and 47.91% for tea bags. Since the α-glucosidase inhibition capacity is linked to the presence of polyphenols, the degradation of the latter during the digestive process led to the loss of enzyme inhibition capacity of the beverages. However, recovery of the inhibition activity was still appreciable (64.15% and 52.08% for capsules and tea bags, respectively). No significant differences emerged between the capsule and tea bag methods both before and after GID ($p > 0.05$). The flavoured beverages, both in the capsule and tea bag forms, before GID displayed almost twice as much enzyme inhibition activity as that found by Rojo-Poveda et al. [13]. This fact could be explained, among other things, with the most caffeic and catechin content in the flavoured beverages. In fact, it was reported that caffeic acid and catechin revealed to efficiently inhibit the α-glucosidase, especially catechin, which is able to induce 99.6% inhibition on the enzyme [318,319]. In recent years, daily consumption of cocoa or chocolate has been recommended in order to ensure an intake of flavanols, for which the role in protection against diabetes mellitus type 2 was proposed [84]. However, most of the cocoa products on the market contain little amounts of flavanols and are rich in fats and sugars, thus frustrating their potential protective effect and even worsening the disease [84]. Considering their composition, both the capsule and tea bag beverages could represent a natural and healthy approach to guarantee the intake of flavanols without the above mentioned negative aspects.

4. Conclusions

The present study highlights the potential for CBSs to be used as a functional ingredient in the preparation of hot beverages obtained using two homemade extraction techniques, capsules and tea bags. The flavoured beverages were sensorially appreciated, especially the coconut-flavoured formulation, which turned out to be the most preferred by consumers. This formulation, in both the capsule and tea bag forms, showed a significant polyphenol content and was able to exert functional properties, such as antiradical and antidiabetic capacities. Moreover, the *in vitro* bioaccessibility of polyphenols exceeded 50% in both capsule and tea bag beverages, highlighting their potential to be absorbed in the gastrointestinal tract or have beneficial effects at the intestinal level. Nonetheless, since *in vitro* assessments often do not reflect what actually happens in the human body, further investigation would be needed to assess whether the bioaccessibility and the functional properties are also found *in vivo*. For these reasons, the present study displayed that CBSs can be turned into a health-promoting food ingredient, a valid alternative to its current uses (e.g., fuel, feed, and fertilizer).

CHAPTER 2.3

Physical Properties and Consumer Evaluation of Cocoa Bean Shell Functionalized Biscuits Adapted for Diabetic Consumers by the Replacement of Sucrose with Tagatose

After the confirmation of the CBS antidiabetic potential by the inhibition of α-glucosidase in the beverages of the previous chapters, Chapter 2.3 proposes the utilization of CBS as a functional ingredient in functional biscuits. In this chapter, different biscuits were elaborated by replacing different percentages of wheat flour with CBS powder and by using different sugars: sucrose or tagatose (a low glycemic index sugar adapted for diabetic consumers). This chapter reports the study of the influence of CBS addition and the utilization of tagatose in the technological qualities of the biscuits. Besides, the addition of CBS allowed for an increase of the dietary fiber content of the biscuits, and this factor was also assessed in the present chapter. Moreover, the present chapter also reports the consumer acceptance evaluation of the different studied biscuits and their perceptible sensorial differences, which were evaluated by performing a Napping® sensory characterization.

This chapter was issued and adapted from a published scientific article:

Physical Properties and Consumer Evaluation of Cocoa Bean Shell Functionalized Biscuits Adapted for Diabetic Consumers by the Replacement of Sucrose with Tagatose

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If the reader desires it, the original paper can be consulted in this link

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1. Introduction

Nutraceuticals and functional foods have received increasing interest among consumers in the last several years. One of the main reasons for this is the introduction of the idea that the diet can promote health benefits, reducing the risk of several diseases, such as cardiovascular affections, diabetes, obesity, cancer, osteoporosis, and arthritis, among others. Furthermore, emerging healthy eating trends and concerns, which are becoming increasingly prevalent in our societies in recent years, have given force to these new concepts. The term nutraceutical, first described in 1989, unites nutrition and pharmaceuticals, and it is defined as any substance that is considered a food or part of one and is able to provide medical or health benefits, including disease prevention and treatment, and that can be presented in different forms, from dietary supplement capsules to different processed foods, consumed mainly for health reasons [320]. On the other hand, functional foods could be considered a type of nutraceutical and are described as fortified foods with dietary components that can provide health benefits beyond basic nutrition [320]. Notably, polyphenols are one of the compound groups most commonly characterized as a nutraceutical. Polyphenols are non-nutritive microconstituents present in vegetable and cereal products, are usually employed in functional foods, providing them with special characteristics such as antioxidant, antidiabetic, or anticarcinogenic activities, among others [219]. Another compound responsible for imparting functional characteristics to foods is dietary fiber, whose consumption has been associated with the reduced incidence of disorders and diseases, such as chronic bowel disorders, obesity, diabetes, cardiovascular diseases, and cancer, among others [321]. Similar to polyphenols, dietary fiber does not have nutritional properties since it cannot be digested by the human organism, but it contributes to proper intestinal transit while retaining compounds and slowing glucose, lipid, sterol, and bile acid absorption [38,77]. For these reasons, dietary fiber plays a crucial role in the diet, and the European Food Safety Authority (EFSA) recommends a daily intake of 25 g of fiber for adults [322].

In particular, great attention has been given to vegetable and fruit by-products as nutraceuticals or ingredients for functional foods because of their content in interesting compounds, combined with the aim to create revalorization strategies for food industry wastes. In the manufacture of cocoa, one of the main by-products is the cocoa bean shell (CBS), which is the external tegument covering the cocoa bean. CBS constitutes 10-17% of the total cocoa bean weight and is normally discarded after its separation from roasted cocoa beans, entailing economic and environmental issues since more than 700 thousand tons of

CBS is estimated to be produced yearly around the world [218]. Nevertheless, CBS has demonstrated to be an interesting food ingredient since it has a similar nutritional composition to that of cocoa nibs (except for the fat content, which is substituted by fiber in the case of CBS) [218] and a similar aromatic profile to that of cocoa powder [29,66]. However, what attracts more attention in scientific research and adds value to this cocoa by-product is its content in dietary fiber, which ranges from 39.3% to 66.3%, and polyphenols, mainly flavanols [218]. Indeed, several researchers have studied the possibilities of its utilization as a fortifying ingredient in different foods, mainly baked goods such as functional cakes [117], muffins [118], bread [115], and biscuits [116]. Both CBS dietary fiber and polyphenols have been shown to give special characteristics to CBS regarding human health, which means that CBS may be considered a potential nutraceutical. In particular, dietary fiber and polyphenols in CBS have demonstrated different biofunctional potentials, such as antibacterial and antiviral properties, benefits to the cardiovascular system, anticarcinogenic effects, antidiabetic properties, anti-inflammatory effects, and even neuroprotective properties [218]. In this work, a special focus is given to the CBS antidiabetic aspect, which, in the case of CBS polyphenols, has been demonstrated to be caused by insulin secretion regulation, pancreatic cell protection, inhibition of glucose degradation enzymes, and insulin sensitivity enhancement, among other effects [218]. Indeed, some researchers have taken advantage of this fact for the development of functional beverages with antidiabetic properties [13,222]. In the case of CBS dietary fiber, some studies have reported that its antidiabetic properties are due to its capacity to absorb glucose during gastrointestinal digestion, retarding its diffusion and absorption through the intestinal wall [218]. This antidiabetic potential of CBS is of great importance, considering that the World Health Organization estimated that 422 million people worldwide aged over 18 years were living with diabetes in 2014 and that this number will be doubled by 2030 [323]. Therefore, increasing interest has appeared in the last several years for alternative ways to treat or prevent diabetes, mainly through the diet, by developing new foods adapted for diabetic consumers.

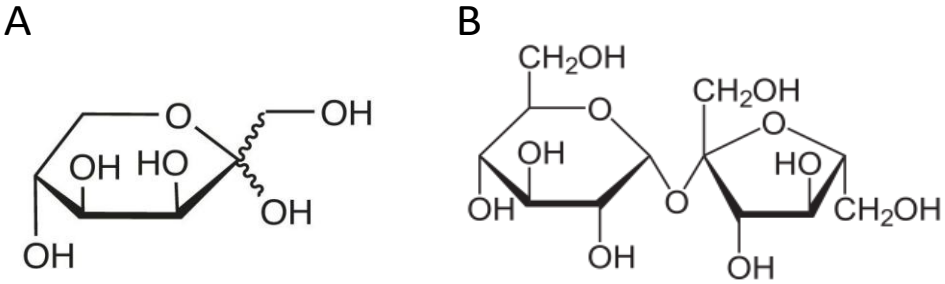

Figure 1. Chemical structures of tagatose (d-tagatopyranoside) (A) [324] and sucrose (α-d-glucopyranosyl-(1→2)-β-D-fructofuranoside) (B) [325].

On the other hand, for similar reasons, increasing interest has appeared for alternative low-glycemic sweeteners in recent years. One of these alternative sweeteners is tagatose, which is a natural hexoketose (a D-galactose isomer) considered a rare sugar because of its limited occurrence in nature (Figure 1A) [326]. It can be obtained by both chemical and biological processes: the former one is based on D-galactose isomerization catalyzed by calcium or sodium hydroxide, and the latter one involves galactitol dehydrogenase, L-arabinose isomerase, different bacteria species capable of acting as a biocatalyst, or production by fungal conversion of D-psicose into D-tagatose, among other biological converters [326,327]. Although it has up to 92% of the common sucrose sweetness and a similar taste without a cooling effect or aftertaste, tagatose presents a low caloric value (1.5-3 kcal/g) and is tooth-friendly, without the laxative effects produced by other reduced-calorie sweeteners such as polyols [324,326-328]. Tagatose has been considered a nutraceutical compound; it shows beneficial effects on obesity control, pregnancy and fetal development (it reduces overeating and the related-health risks to both mother and fetus, among other effects), and blood factor regulation and has anti-aging properties (it reduced cross glycosylation in muscle and brain tissue, which is a major cause of aging), antioxidant properties (it suppresses the formation of iron-catalyzed free radicals in cells), and, especially, prebiotic and antidiabetic properties [326]. Its prebiotic properties are due to the fact that tagatose is a mal-absorbing sugar because of its spatial configuration at the C-4 position, and therefore, only 20−25% of it is absorbed in the small intestine, while the rest reaches the large intestine and is subjected to fermentation by the gut microflora. This fermentation leads to the production of several short-chain fatty acids (SCFA), among which butyrate plays an important role since it is the major fuel for colonic epithelial cells, contributing to their development, acting as an anti-inflammatory agent, and helping in the prevention of colon cancer [326]. On the other hand, its antidiabetic properties are based on the fact that this rare sugar is able to avoid high peaks in the postprandial glycemic index, blunting the rise of blood glucose levels, with no influence on the insulin levels thanks to its particular metabolism in the liver and its inhibition of carbohydrate-digesting enzymes in the small intestine, such as sucrase and maltase [326]. Tagatose was declared a 'generally recognized as safe' (GRAS) product by the United States Food and Drug Administration (FDA) in 2001, which authorized its use at up to 30% *w/w* in baked goods [329], and its consumption as a novel food in the European Union was authorized by EFSA in June 2005 [328]. Indeed, since that moment, several researchers have studied the possibilities of its inclusion in functional foods to replace common sucrose, mainly in baked goods [330,331]. However, when compared with common sucrose (Figure

1B), tagatose has some different properties that could influence foods from a technological and sensory point of view. Among other differences, tagatose is a reducing sugar, while sucrose is not, and tagatose has a lower solubility when compared with sucrose (160 g/100 mL, 58% at 20 °C and 200 g/100 mL, 67% at 21 °C, respectively) [324,332,333], and it is also less hygroscopic than the latter [326,333].

As mentioned above, several researchers have studied the possibilities of using CBS and tagatose separately for the development of functional foods. However, to our knowledge, there are no studies in the literature proposing the use of these two biofunctional ingredients together. The aim of this work is, therefore, to develop CBS functionalized biscuits using tagatose as a sweetener agent and, in this way, getting the advantage of the fiber content and antidiabetic capacities of CBS, together with the sweetener properties and beneficial effects of tagatose. To this end, the combined effects on the physicochemical and sensory properties of the developed biscuits were studied by adding different percentages of CBS flour in place of wheat flour (10% and 20%) and by substituting sucrose with tagatose. For comparison purposes, a sucrose control group and biscuits with no CBS substitution were also produced and tested.

2. Materials and Methods

2.1. Chemicals

Ethanol (99%), acetone (≥99%), sulfuric acid (96%), oxygen peroxide (50%), and Nessler's reagent were obtained from VWR chemicals (Leuven, Belgium). Celite, sodium hydroxide (1N solution), and hydrochloric acid (≥32%) were acquired from Sigma-Aldrich (Steinheim, Germany). MES hydrate (>99%) and ammonium sulfate were provided by Alfa Aesar (Kandel, Germany). TRIS (>99,8%) was obtained from Acros Organics (Geel, Belgium). Petroleum ether (60–80 °C) was supplied by LAB-SCAN analytical sciences (Gliwice, Poland). Amylase thermostable (3000 U/mL), protease (9 tyrosine equivalent units/mg), and amyloglucosidase (3260 U/mL), suitable for AOAC International total dietary fiber and starch analytical procedures, were obtained from Megazyme (Te Huissen, Netherlands). Ultrapure water was prepared in a Simplicity UV water purification system (Millipore, Molsheim, France).

2.2. Samples

Roasted CBS from São Tomé cocoa beans (Forastero variety) was kindly provided by Pastiglie Leone S.r.l. (Turin, Italy). CBS was first ground to a grain size lower than 500 μm using a BA200N vibrating sieve (CISA, Barcelona, Spain). Then, it was micronized in a ball mill until obtaining CBS powder with a particle size below 20 μm, which was confirmed by measurement with a PSA 990 particle size analyzer (Anton-Paar GmbH, Graz, Austria). The characterization of the employed roasted CBS was perform on a previous work [13] with the following results: protein: 20.9 g, fat: 2.3 g, carbohydrates: 7.85 g, dietary fiber: 55.1 g, water: 5.9 g, and ash: 7.9 g. Tagatose was supplied by NuNaturals (Eugene, OR, USA).

2.3. Formulation of the Biscuits

Six different types of biscuits were produced using three percentages of wheat flour substitution by CBS powder (0%, 10%, and 20%) and two different types of sugar (sucrose and tagatose). The six biscuit types were coded: 0S (0% CBS and sucrose), 0T (0% CBS and tagatose), 10S (10% CBS and sucrose), 10T (10% CBS and tagatose), 20S (20% CBS and sucrose), and 20T (20% CBS and tagatose). Biscuits were formulated and baked according to the official method AACC 10-53.01 [334] with slight modifications, which comprises the use of butter instead of all-purpose shortening and the elimination of nonfat dry milk and high-fructose corn syrup as ingredients. The ingredient quantities employed are shown in Table 1.

Table 1. Quantities of ingredients employed for the biscuits' preparation according to the official method AACC 10-53.01 [334], with slight modifications.

Ingredients	Quantities (g)		
	0% (control biscuits)	10% CBS substitution	20% CBS substitution
Wheat flour	225.0	202.5	180.0
Sucrose or tagatose	100.0	100.0	100.0
Butter (>82% fat)	90.0	90.0	90.0
CBS powder	0.0	22.5	45.0
Baking powder	7.0	7.0	7.0
Cocoa powder	3.0	3.0	3.0
NaCl	1.2	1.2	1.2
Water	51.0	55.0	69.0

All ingredients, except CBS and tagatose, were acquired from local markets. Water was added to the dough in variable quantities, depending on the CBS powder quantity that was added in place of wheat flour, in order to counterbalance the lower moisture provided by the former in comparison with the latter. All ingredients were homogeneously mixed in a KitchenAid® dough beater (Whirpool Corporation, Benton Harbor, MI, USA). Butter was

first whisked alone for one minute. After that, sugar or tagatose, salt, baking powder, and cocoa powder were added and mixed for three minutes. Then, water was added and mixed for another minute. Finally, wheat and CBS flours were added, and the dough was further mixed for three minutes. The obtained dough was gauged to obtain a 7 mm thick dough sheet and cut with a circular mold to obtain 6 cm width biscuits that were baked in a ventilated OLIS oven 044-054S (Ali Group, Milan, Italy) at 180 °C for 15 minutes. The different quantities of added CBS in place of wheat flour accounted for 0%, 5.28%, and 10.56% *(w/w)* of the total final biscuit weight for the 0%, 10%, and 20% substitutions, respectively. For the analyses that required a powdered sample, biscuit samples were converted into 500−1000 μm particle size powder by using an Ultracentrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany).

2.4. Analytical Procedures

2.4.1. Total Dietary Fiber Determination

Total dietary fiber content was determined by implementing the gravimetric AOAC official method 991.43 [335] on a Fibertec system E composed of a 1023 filtration module and a 1024 shaking water bath (Foss Tecator, Hilleroed, Denmark).

Before dietary fiber analysis, dried CBS flour and powdered biscuit samples were defatted and desugared as indicated by the official method. For the defatting phase, samples were extracted three times with 25 mL of petroleum ether per gram of sample for five minutes with strong agitation by a Reax 2 overhead shaker (Heidolph Instruments, Schwabach, Germany). Sugar was extracted three times with 85% ethanol for five minutes on the overhead shaker. Samples were dried overnight in a Venti-Line ventilated oven (VWR, Leuven, Belgium) after each phase and before dietary fiber determination.

For sample digestion, 40 mL of 0.05M MES-TRIS buffer pH 8.2 was added to 1.0000 g of dried, defatted, and desugared CBS or biscuit sample, and the mixture was stirred until the sample was dispersed. Analyses were performed in quadruplicate, and blanks were prepared and analyzed together with the samples during the whole process. The solutions were incubated with 50 μL of thermostable amylase, covered with aluminum foil for 15 minutes in a bath at 100 °C, and left to cool to 60 °C. Any ring or gels were scraped before adding 100 μL of protease (50 mg/mL) and incubating while covered with aluminum with agitation at 60 °C for 30 minutes. After that, 5 mL of 0.561N HCl was added while stirring, and pH was adjusted with a PP-15 pH-meter (Sartorius, Goettingen, Germany) at 60 °C to 4.0-4.7 by adding 1N NaOH or 1N HCl. Finally, 30 μL of amyloglucosidase was added, and the solution

was further incubated with agitation for 30 minutes at 60 °C. Two-hundred milliliters of 95% ethanol at 60°C was then added, and the solution was left to precipitate for 1 hour while covered with aluminum foil.

Approximately one gram of celite was previously added and washed with 15 mL of 78% ethanol on previously weighed Fibertec adapted filter crucibles and dried overnight. The resulting digestate was filtered through the crucibles and washed with 78% ethanol. Additional washes were done with 15 mL of 78% ethanol, 95% ethanol, and acetone (two times each), and crucibles were dried overnight to obtain the digestate residue weight. Two replicates of the obtained digestate, composed of fiber, proteins, and inorganic residues, were incinerated for 5 hours at 525 °C on a muffle furnace (Nabertherm, Lilienthal/Bremen, Germany) for ash calculation.

The two remaining replicates were used for protein determination. To do this, a Digesdahl system (Hach Company, Loveland, CO, USA) was employed. The crucible content (celite + digestate residue) was transferred to a beaker and stirred with 5 mL of 50% H_2SO_4 for 30 minutes. The solution was transferred to the Digesdahl digestion flask, washed twice with 500 µL of 50% H_2SO_4, and heated inside the Digesdahl system at 440 °C until water evaporation (release of white smoke). Four minutes after this moment, 10 mL of 50% H_2O_2 was added at a 3 mL/min flux employing a capillary funnel and heating for another two minutes. After complete cooling, ultrapure water was added to the solution up to 100 mL. The ammonium sulfate formed from the protein nitrogen after the complete destruction of the organic matrix was then monitored by UV spectrophotometry with a Genesys 10UV UV–Vis spectrophotometer (Thermo Spectronic, Rochester, NY, USA). The obtained solutions from the protein digestion were further diluted 50 times, and 1 mL of Nessler reagent was added to 25 mL of solution. Absorbance was measured at 420 nm, and a standard curve made with $(NH_4)_2SO_4$ corresponding quantities for 0-80 µg of nitrogen was employed to estimate the nitrogen content. Protein content was obtained by multiplying the nitrogen content by a factor of 6.25.

The total dietary fiber content was calculated as follows:

$$\mathrm{DF\ (g/100g)} = \{[(R_1 + R_2 + R_3 + R_4)/4] - P - A - B]\} / [(S_1 + S_2 + S_3 + S_4)/4] \times 100$$

where R_1–R_4 are the residue weights for sample replicates (mg), P is the average protein weight of the final digestate residue (mg), A is the average ash weight of the final digestate residue (mg), B is the blank residue weight (mg), and S1–S4 are the initial weights for sample replicates (mg). Blank residue weight was calculated as follows:

$$B = [(BR_1 + BR_2)/2] - P_B - A_B$$

where BR_1–BR_2 are the residue weights for blank replicates (mg), P is the protein weight of the blank (mg), and A is the ash weight of the blank (mg).

2.4.2 Physicochemical Analyses

A MAC210/NH thermo-moisture analyzer (Radwag, Radom, Poland) was used for moisture calculations, for which 5 g of powdered biscuits was used. For water activity determination, 2 g of powdered biscuits was used on an AquaLab PRE water activity-meter (Decagon devices, Pullman, WA, USA).

Color analyses were conducted as in [13] (Chapter 2.1), using powdered biscuit samples on a CM-5 spectrocolorimeter (Konica Minolta, Tokyo, Japan) on transmittance and SCE (Specular Component Excluded) mode. The color space parameters *L**, *a**, and *b** (CIELAB values) were used to measure the colorimetric characteristics.

2.4.3. Structural Analyses

Weight loss was determined by weighing the biscuits before and after baking, and it was calculated as the percentage of the initial uncooked biscuit weight that was lost after the baking process. A total of 12 samples per biscuit group were used for the weight loss calculation.

The width and thickness measurements and the spread calculation were taken following the AACC 10-50.05 method [336]. The width and the thickness were measured for 12 baked samples per biscuit group and used to calculate the spread parameter. The spread was calculated as follows: $\text{Spread} = \frac{\text{width}}{\text{thickness}} \times \text{conversion factor} \times 10$, where the conversion factor is a parameter dependent on the atmospheric pressure and the height level above the sea on the day in the laboratory where the analyses were conducted, and it is reported in the mentioned method.

The hardness of the biscuit samples was measured on a TAXT2i Texture Analyzer® (Stable Micro Systems, Godalming, UK) equipped with a 25 kg load cell. An HDP-BS cutting blade was used for the analysis at a speed of 1 mm/s. For each biscuit prototype, a set of 12 biscuits was used for measuring this parameter. Version 2.54 of the Texture Expert Exceed software package for Windows (Stable Micro Systems, UK) was used for data acquisition, and the results were expressed in N as the maximum force needed for breaking the biscuit.

2.5. Sensory Analyses

2.5.1. Consumer Evaluation

A consumer acceptance evaluation was performed as in [13] with a panel of 25 untrained tasters, to whom biscuits were given in random order. Tasters were asked to evaluate different parameters, such as appearance, odor, taste, flavor, texture, overall liking, and purchase predisposition on a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like) [297]. Water was provided for mouth rinsing between sample tastings. Tests were carried out in an adapted air-conditioned room that was equipped with white light and was approximately 21 °C.

2.5.2. Napping® Sensory Analysis

For the Napping holistic sensory analysis [337], projective mapping positioning was performed by 12 tasters during two sensory sessions. The six different biscuits were given simultaneously to the tasters, who were asked to taste them in different random orders. Tasters were then asked to place the samples on a 40 × 60 cm tablecloth according to their similarities (the more similar the biscuits were perceived, the closer they should be positioned), without specific direction for sample positioning. Next, tasters were asked to freely attribute some descriptive terms to the samples and to write them down next to the samples positioned on the tablecloth, according to the Ultra-Flash Profiling (UFP) approach [338].

Measures were taken for each sample positioned in the sensory space, represented by the x- and y-axes, where the left lower corner was considered the axis origin point (0,0). The different UPF terms were then collected, and their frequencies were computed when repeated. Additionally, UPF terms with similar meanings were grouped as shown in table 5. All the terms were collected in a contingency table, which was added as supplementary information to the sensory space position of each sample for further statistical analysis.

2.6. Statistical Analysis

The results of fiber, physicochemical, and structural analyses were subjected to one-way analysis of variance (ANOVA) with Duncan's post hoc test at a 95% confidence level in the SPSS Statistics 25 software (IBM-SPSS Inc., Chicago, IL, USA).

Values obtained by the consumer acceptance test were analyzed by the Kruskal–Wallis test (test H) in the STATISTICA software for Windows, version 13.3 (StatSoft Inc., Tulsa, OK, USA).

Data obtained from the Napping® sensory analysis (positions in the sensory space) were treated through a geometric analysis using SensoGraph in order to obtain proximity graphs from the Napping® data. Python implementation [339] of the SensoGraph method proposed in [340] was used to analyze the (x,y) coordinates provided by the tasters. This method comprises three steps: first, a clustering technique from Computational Geometry called the Gabriel graph is applied to each tablecloth so that only pairs of samples without third samples nearby are connected. Second, a global similarity matrix is created, whose i,j entry is 1 if samples i-j are connected and 0 otherwise. Third, a graph-drawing algorithm uses that matrix to provide the 2D positioning of the samples.

3. Results and Discussion

3.1. Total Dietary Fiber Content

For each type of biscuit, total dietary fiber content (TDF) values are reported in Table 2. The obtained value for the CBS powder was 61.87 ± 1.50 g of TDF per 100 g of dried CBS, which is in accordance with the gravimetrically obtained TDF values reported in the literature [218].

Table 2. Total dietary fiber content, moisture, water activity, and CIELAB color values obtained for each biscuit and ANOVA among different percentages of CBS substitution within each sugar group and among both sugars for the same %CBS group.

Biscuit sample	Sugar	CBS percentage	Fiber (g/100g dried product)	Moisture (%)	a_w	*L**	*a**	*b**
S0		0	2.48 ± 0.23^{cA}	1.69 ± 0.26^{cB}	0.12 ± 0.03^{cB}	50.78 ± 1.93^{aA}	11.70 ± 0.39^{aB}	23.66 ± 1.08^{aB}
S10	Sucrose	10	5.66 ± 0.56^{bA}	3.21 ± 0.14^{bB}	0.25 ± 0.00^{bB}	33.56 ± 0.38^{bB}	12.04 ± 0.29^{aB}	17.10 ± 0.48^{bB}
S20		20	8.70 ± 0.34^{aA}	4.50 ± 1.05^{aB}	0.37 ± 0.09^{aB}	28.54 ± 0.67^{cA}	9.04 ± 2.45^{bA}	11.77 ± 2.90^{cA}
	Significance		***	***	***	**	***	***
T0		0	2.79 ± 0.07^{cA}	4.76 ± 0.11^{cA}	0.37 ± 0.01^{cA}	44.78 ± 0.90^{aB}	16.04 ± 0.34^{aA}	30.90 ± 0.37^{aA}
T10	Tagatose	10	5.66 ± 0.58^{bA}	5.69 ± 0.46^{bA}	0.43 ± 0.02^{bA}	35.49 ± 1.66^{bA}	13.76 ± 0.32^{bA}	21.63 ± 1.20^{bA}
T20		20	8.71 ± 0.38^{aA}	7.36 ± 0.51^{aA}	0.52 ± 0.03^{aA}	28.48 ± 1.50^{cA}	9.54 ± 2.27^{cA}	13.64 ± 3.19^{cA}
	Significance		**	***	***	***	***	***

Means followed by different lowercase superindexes indicate significant difference at $p < 0.05$ among CBS percentages within each sugar group; means followed by different uppercase superindexes indicate significant difference at $p < 0.05$ for different sugars within the same %CBS group; ** $p < 0.01$, *** $p < 0.001$; data are expressed as mean values ($n \geq 3$) ± standard deviation.

Statistically similar values were found when comparing biscuits with the same CBS content but different sugar groups, proving that the employed sugar had absolutely no influence on this parameter. For the control biscuits, values of 2.48 g and 2.79 g TDF/100 g of dried product were found for S0 and T0, respectively. These values are in accordance with the work of Kārkliņa et al. [116], who obtained a TDF value of 2.9 g TDF/100 g for a sugar control biscuit with a similar composition to the ones employed in this study. Increasing CBS content significantly increased the TDF in both sugar biscuit groups to an expected extent when taking into account the CBS quantity added and the fact that this ingredient possesses 61.87% TDF. In this way, we obtained values of 5.66 g TDF/100 g for both S10 and T10, in which the CBS final content of the biscuit was 5.28 g of CBS/100 g of biscuit (10% wheat flour substitution). Again, these values were in accordance with the previously mentioned research work, in which 4.22 g TDF/100 g was found for biscuits with a CBS final content of 4.33 g per 100 g of biscuit (10% wheat flour substitution) [116]. On the other hand, values of 8.70 and 8.71 g TDF/100g were found for S20 and T20, respectively, which are the biscuits in which 20% of the wheat flour was substituted with CBS powder.

In view of these results and according to the European Union regulation for nutrition and health claims for food [341], both the sucrose and tagatose biscuits in which 10% of the wheat flour was substituted with CBS could be claimed as a 'source of fiber' since both S10 and T10 contained more than 3 g of fiber per 100 g of product, which is the minimum necessary for this claim. On the other hand, and according to the same regulation, both S20 and T20 could be commercially claimed as 'high fiber' biscuits since both surpass a quantity of 6 g of fiber per 100 g of product, which is the minimum necessary for this other claim.

3.2. Physicochemical Characterization

Moisture, water activity, and CIELAB parameters for color measurement of the biscuits are compiled in Table 2.

Moisture and water activity (a_w) are two important parameters to be monitored for the storage stability of biscuits since these factors can influence their resistance to microbes and their rheological properties [342]. The results for both parameters were found to be significantly different for all six types of biscuits. Thus, differences in these parameters were found for both variables of 'different CBS percentages within the same sugar group' and 'different sugars within the same CBS percentage'. Moisture and a_w had proportionally correlated results, which was expected since a_w is known to be significantly influenced by moisture. For both sugars, humidity and a_w considerably increased when increasing the

percentage of CBS added in place of the wheat flour. This effect could be explained by the higher dietary fiber content provided by CBS in comparison with that in wheat flour, which provides a higher water retention capacity, as described by Martínez-Cervera et al. [118]. On the other hand, if the comparison is made between the different employed sugars, it can be observed that, for the same CBS percentage, the tagatose biscuits, in all cases, had higher moisture and a_w values than the biscuits made with sucrose. Being less soluble than sucrose, tagatose can allow for greater re-crystallization after baking, which results in water release and, thus, higher moisture and a_w values [330,343]. Nevertheless, the water activity values of all the studied biscuits were below the 0.60 threshold under which they can be considered microbiologically stable [342].

For contrast purposes, Figure 2 shows the six different biscuit types for a visual comparison. The chromatic characteristics of the biscuits were studied through the CIELAB color space, composed of *L**, which measures the lightness of the samples from 0 (black) to 100 (white); *a**, which measures the degree of redness (when positive) or greenness (when negative); and *b**, which indicates the yellowness (when positive) or blueness (when negative) of the sample color [13]. When looking into the obtained CIELAB values for all six biscuit types, the most relevant parameter was *L**. This parameter was first observed to significantly decrease when increasing %CBS within both sugar groups, as the biscuits became darker because of the brown color provided by the CBS ingredient. Besides this, significant differences can be observed in the *L** parameter of both control biscuits S0 and T0, where the CBS had no influence. T0 had a significantly lower *L** value than that of S0, with values of 44.78 and 50.78, respectively. This fact is explained by the reducing nature of tagatose in contrast to sucrose, which is not a reducing sugar. This allows for an extra browning of the tagatose biscuit surface during baking (observed in Figure 2 for T0) since Maillard browning reactions may occur between the reducing sugar (tagatose) and the proteins contained in the biscuit dough, as reported by Struck et al. [344]. Both the *a** and *b** parameters significantly decreased for both sugar groups when increasing the CBS content, meaning that the biscuits became less red and less yellow. In this case, it is important to remark that, regarding *a** and *b**, significant differences were found between sugars in the control biscuits and in those with 10% of the wheat flour substituted by CBS, with these values higher for the tagatose group in both cases. However, both biscuits with the higher percentage of CBS (S20 and T20) had statistically similar values of the three chromatic parameters, meaning that at this percentage of wheat flour substituted with CBS, the employed sugar no longer had an influence on the color.

Figure 2. Visual comparison between the six different biscuit types for color-contrasting purposes. From left to right: S0 (sucrose control biscuit with 0% of wheat flour substituted with CBS), T0 (tagatose control biscuit with 0% of wheat flour substituted with CBS), S10 (biscuit with sucrose and 10% of wheat flour substituted with CBS), T10 (biscuit with tagatose and 10% of wheat flour substituted with CBS), S20 (biscuit with sucrose and 20% of wheat flour substituted with CBS), and T20 (biscuit with tagatose and 20% of wheat flour substituted with CBS).

Concurrently, for comparison purposes in terms of color, the ΔE parameter was calculated using the CIELAB parameters. This parameter is calculated according to the equation $\Delta E_{1-2} = \sqrt{(L*_1 - L*_2)^2 + (a*_1 - a*_2)^2 + (b*_1 - b*_2)^2}$, and it indicates whether the differences in color between two different samples are perceivable by the human eye (perceivable when ΔE ≥ 2.5) [279]. In the case of the studied biscuits, when comparing biscuits with the same CBS percentage, ΔE values were higher than 2.5 for the comparison S0-T0 and S10-T10 ($\Delta E_{S0\text{-}T0}$ = 5.22 and $\Delta E_{S10\text{-}T10}$ = 10.36), while ΔE resulting from the comparison between S20 and T20 was $\Delta E_{S20\text{-}T20}$ = 1.94. This means that, as perceived by the human eye, all the biscuits were different in color except for the ones with the higher percentage of CBS; the similarities between them, without regard to the employed sugar, were previously observed with the significant similarities in the CIELAB parameters, as observed in Figure 2.

3.3. Structural Characterization

Weight loss after baking, width, thickness, spread, and hardness of the biscuits were measured for structural characterization and are reported in Table 3. The influence of the biscuit ingredients' nature on the structural and texture parameters of biscuits is well known. As reported by several authors, these characteristics depend on, among other parameters, the sugar nature and its degree of solubility, the protein content, and the amount of water available for gluten development and the relative extent of the creation of a gluten network [345].

Regarding weight loss, no significant differences were found among the different wheat flour substitutions with CBS for the same sugar group. However, a slight increase in weight loss was observed when increasing the CBS content for sucrose biscuits, and the opposite effect was observed for the tagatose biscuit group. This could be due to the lower solubility of

tagatose during baking, which leaves more free water that is absorbable by the CBS fiber. This phenomenon is also confirmed by the humidity data (see Table 2).

Table 3. Weight loss after baking, width (diameter), thickness (height), spread, and hardness values obtained for each biscuit and ANOVA among different percentages of CBS substitution within each sugar group and among both sugars for the same %CBS group.

Biscuit sample	Sugar	CBS percentage	Weight loss (%)	Width (cm)	Thickness (cm)	Spread	Hardness (N)
S0		0	15.84 ± 1.19^{aA}	6.51 ± 0.26^{aA}	0.86 ± 0.05^{aB}	75.85 ± 6.73^{aA}	68.83 ± 11.95^{aA}
S10	Sucrose	10	16.08 ± 0.48^{aA}	5.93 ± 0.10^{bA}	0.91 ± 0.08^{aA}	65.28 ± 6.53^{bA}	53.92 ± 6.46^{bB}
S20		20	16.44 ± 0.48^{aA}	5.81 ± 0.12^{bA}	0.90 ± 0.01^{aA}	64.13 ± 1.67^{bA}	54.75 ± 12.21^{bB}
	Significance		ns	***	ns	***	**
T0		0	15.33 ± 1.14^{aA}	6.12 ± 0.08^{aB}	0.92 ± 0.07^{aA}	66.52 ± 4.07^{aB}	72.77 ± 29.72^{bA}
T10	Tagatose	10	14.25 ± 0.46^{aB}	5.88 ± 0.07^{bA}	0.89 ± 0.10^{aA}	66.47 ± 7.61^{aA}	88.96 ± 19.39^{abA}
T20		20	14.69 ± 0.75^{aA}	5.81 ± 0.12^{bA}	0.90 ± 0.01^{aA}	64.13 ± 1.67^{aA}	120.34 ± 51.14^{aA}
	Significance		ns	***	ns	ns	*

Means followed by different lowercase superindexes indicate significant difference at $p < 0.05$ among CBS percentages within each sugar group; means followed by different uppercase superindexes indicate significant difference at $p < 0.05$ for different sugars within the same %CBS group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant; data are expressed as mean values ($n \geq 3$) ± standard deviation.

The width parameter or biscuit diameter showed significant differences between both control biscuits without the CBS addition, with values of 6.51 and 6.12 cm in width for S0 and T0, respectively. The significantly lower width of the tagatose control biscuit was probably due to the lower water-binding capacity of tagatose compared with sucrose, which allows less spreading to occur because a lower amount of syrup is produced during baking, as reported by Taylor et al. [330], who obtained similar results when replacing sucrose with tagatose in cookies. However, when considering biscuits in which a percentage of wheat flour has been substituted by CBS, this effect was no longer observed, and no significant differences were observed between either the different sugars or different CBS additions. In these four cases, the effect of the incorporation of the CBS fiber prevailed over the difference between sugars for S10, S20, T10, and T20 biscuits. A similar slight decrease in the biscuit width was therefore observed when compared with the control biscuits as a result of the water absorption by the added CBS fibers [346].

The thickness or biscuit height results of the different biscuits followed a similar trend to that of the width parameter. No significant differences were found between the different CBS substitutions within the sugar groups or between both sugar groups, although a slight increase in this parameter was observed (mostly for the sucrose biscuits) when the CBS content was increased. This slight increase was probably due to higher gluten development because of a higher amount of water available. However, the sucrose control biscuit S0 presented a

thickness value of 0.86 cm, which is significantly lower than the 0.92 cm thickness of the tagatose control biscuit T0. This difference can again be attributed to the lower hygroscopy of tagatose in comparison with sucrose, which, in this case, leaves more water available for the gluten proteins to create a network and to develop in height [330,345].

The biscuit spread parameter is the result of the adjusted ratio of the width and the thickness and, therefore, represents a combination of the different sugar and CBS content effects observed above. When comparing sugar groups, significant differences were only observed between the control biscuits with no CBS fortification. The sucrose control biscuit (S0) showed a 75.85-spread value, significantly higher than the 66.52-spread value of the tagatose biscuit (T0). As mentioned above, this result comes from the combination of the lower solubility of tagatose in comparison with sucrose (lower width) and its greater thickness due to the allowance for more gluten development. When observing the effect of the different CBS percentages on the spread value, significant differences were found within the sucrose biscuit group. The spread of the control biscuit S0 was found to be significantly higher than those of the CBS-fortified biscuits S10 and S20. In this case, a decrease in the spread was observed because the gluten network was diluted by the fiber addition. As described by Ktenioudaki and Gallagher [346], a higher fiber content absorbs some of the water, which is then no longer available for the gluten network development, which translates into a lower biscuit spread. In general, the inclusion of CBS in the biscuit formulation resulted in less spread and more compacted biscuits due to the increase in fiber content, which is in accordance with the results observed by Collar et al. [115] for CBS soluble-fiber-fortified bread.

The hardness parameter represents the force required by the cutting probe to penetrate the biscuit and cut it into halves. It could, therefore, be understood as the necessary force to be employed for biting the biscuit. In the CBS functional biscuits, this parameter will be influenced by the interaction between the different ingredients. The sugar type can also influence this parameter since sugars may crystallize, which increases the biscuit hardness [330]. The obtained results for the hardness parameter showed that sucrose biscuits were significantly crispier than tagatose biscuits. This can be explained by the fact that sucrose possesses a higher solubility compared with tagatose, yielding a weaker gluten network, less sugar crystallization after the cooling of the baked biscuits, and, therefore, crispier biscuits, as described by Garcia-Serna et al. [347] and Mamat et al. [345]. When regarding the CBS content within the same sugar group, an increase in the hardness value was observed for the tagatose biscuits. These results were expected since increased CBS content leads to higher

fiber content, which increases the biscuit hardness. CBS incorporation causes water absorption by the CBS dietary fiber, leaving less water for gluten development and, therefore, increasing hardness and losing crispiness since the biscuit mass does not retain gas (the biscuit surface breaks and cracks during baking, as observed for T20 in Figure 2) and does not expand as the control biscuit does [346]. The obtained tagatose biscuits were thus tough and not crumbly. Similar trends of the hardness parameter were observed in other research works in which CBS was added as a fat replacer for functional cake production [117] or for nutritionally fortified biscuit development [116]. However, in the sucrose biscuit group, the increasing CBS content did not lead to an increase in the hardness values, which were even lower for S10 and S20 than for S0. A hypothesis for these unexpected results might be that, in these biscuits, because sucrose is more soluble than tagatose, the sugar has already used the available water before CBS is added during the dough mixing, which does not leave much water available for its absorption by the CBS dietary fiber.

3.4. Sensory Analyses

3.4.1. Hedonic Consumer Acceptance Evaluation

Results from the hedonic sheet compliance are shown in Table 4. Data are shown as the sums of the ranks obtained for each consumer evaluation parameter for each of the six biscuits. These values were subjected to a Kruskal–Wallis test to highlight differences in consumer acceptance between the six different biscuits.

Table 4. Consumer evaluation of the six biscuit types and results of the Kruskal–Wallis test (test H). Data are expressed as the sum of ranks of the results obtained from 25 tasters who filled out a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like).

Biscuit sample	Sugar	CBS percentage	Appearance	Odor	Taste	Flavor	Texture	Overall liking	Purchase predisposition
S0		0	2602.0^{a}	2890.0^{a}	2843.5^{a}	2877.5^{a}	3057.0^{a}	2961.5^{a}	2992.5^{a}
S10	Sucrose	10	2652.0^{a}	2394.5^{ab}	2737.5^{a}	2716.5^{a}	2754.0^{a}	2773.5^{a}	2823.0^{a}
S20		20	1692.5^{ab}	2175.0^{abc}	2451.5^{a}	2451.5^{a}	2164.0^{ab}	2339.0^{a}	2365.0^{a}
T0		0	1526.0^{b}	994.0^{d}	986.5^{b}	1051.5^{b}	1567.0^{bc}	1146.5^{b}	1009.0^{b}
T10	Tagatose	10	1781.0^{ab}	1502.0^{bcd}	1226.0^{b}	1225.5^{b}	1078.5^{c}	1149.5^{b}	1199.5^{b}
T20		20	921.5^{b}	1369.5^{cd}	1080.0^{b}	1002.5^{b}	704.5^{c}	955.0^{b}	936.0^{b}
	Significance		***	***	***	***	***	***	***

Means followed by different letters are significantly different at $p < 0.05$. Significance: *** $p < 0.001$.

Concerning the biscuit appearance, significant differences were observed between the sucrose and the tagatose groups, although tasters liked S20 and T10 to a similar extent. In general, sucrose biscuits were preferred, and lower values were found for T0 and T20. In the case of T0, this was probably due to the excessive browning color, caused by the Maillard

reactions, that was observable in this biscuit (Figure 2, T0) and that gave a 'burnt aspect' to the biscuit. In the case of T20, the lower appearance preference was probably linked to the cracked aspect of the biscuit (Figure 2, T20), which broke during baking, possibly as a result of the absence of gluten network formation during the mixing process because of the fiber content, which led to greater gas release during the cooking phase.

As far as the odor is concerned, a similar trend was observed in which the sucrose biscuits were preferred, followed by T10, with T0 and T20 as the less liked biscuits. Again, this could be linked to the produced Maillard reactions, which were more prevalent in the tagatose biscuits and caused the development of some aromas that were not highly accepted.

The taste and flavor parameters showed similar results in terms of significant differences. The whole sucrose group was significantly more accepted than the whole tagatose biscuit group. Again, these values could be explained by the Maillard reactions that occurred to a greater extent in the tagatose biscuit group, developing a more bitter taste and undesired flavors.

Regarding texture, sucrose biscuits were again the most liked. Furthermore, a decrease in the acceptance was observed when increasing the CBS percentage in this group, presumably due to the greater moisture and water activity provided by CBS powder that made the biscuits denser to chew, as was also reported by Martínez-Cervera et al. [118]. Similar to the observations above, the rating values for the tagatose biscuits were much lower than the ones obtained for sucrose biscuits, decreasing with the addition of CBS. In this case, the texture parameter for T20 received the lowest acceptance value within the whole consumer evaluation. This fact is surely linked to the already reported high hardness of T20 and its high value of water activity (0.52), which, combined, resulted in tough and non-crumbly biscuits, since it has been reported that biscuits lose their crispiness at a_w values higher than 0.5 [348].

Finally, the overall liking and the purchase predisposition parameters were naturally linked and had similar results, in which the sucrose biscuits were significantly preferred to the tagatose biscuits, with S0 presenting the highest values and T20 the lowest.

Generally, sucrose biscuits were highly preferred over tagatose biscuits for all the different consumer acceptance parameters, although tagatose has been reported to not differ highly from sucrose in terms of taste [324,327]. For the sucrose group, it was observed that the addition of CBS caused a slight decrease in the acceptance values, although this decrease was not significant. However, it is interesting to remark that the opposite happened within the tagatose group, where the addition of CBS resulted in a rise in preference for almost all parameters (excepting texture) when considering T10 and, to a lower extent, T20.

3.4.2. Napping® Sensory Characterization

The Napping® technique consists of a holistic approach (considering each sample as a whole), which has been shown to be very useful for sensory characterization, with advantages such as the possible discrimination among products [337]. In this work, the Napping® results were analyzed through geometric analysis using SensoGraph [339], which allowed for better visualization of the obtained results.

Data from the Napping® sensory characterization were obtained from the different positions that were given to the samples on the tablecloths. These positions were characterized by different UPF terms freely given by the tasters, which served as criteria for positioning samples in the sensory space and were, in some cases, grouped into categories for terms with similar meanings (Table 5).

Table 5. Grouped terms generated from the Ultra-Flash Profiling (UFP) during the Napping® sensory analysis.

Friability	**Hardness**	**Softness**	**Crunchy**	**Astringent**	**Sweet**
Shortbread	Hard on the first try	Damp			Honey
Dry		Tender			Not sweet
Granulose		Undercooked			Jam
		Gummy			
		Soluble			
		Sticky			

Bitter	**Salty**	**Umami**	**Sour**	**Butter**	**Toasted**
Stale		Stock cube		Fermentation	Hazelnut

Chocolate	**Caramel**	**Burned**	**Cooked Corn**	**Milk**	**Vegetal**
Unsweetened cocoa powder	Amaretto biscuit		Straw	Condensed milk	Wet grass
Chocolate milk			Puffed rice		
Sweet cocoa					

The plots obtained from the SensoGraph geometric analysis of the Napping® data are shown in Figure 3. Figure 3A shows the output provided by the SensoGraph method, as introduced in [340]. Samples appear in the diagram as black points labeled with the sample code. Samples that appear close to each other were perceived as more similar by the panel of tasters and vice versa. In addition, the segments between the samples represent the strength of the connections between samples using a two-fold codification: from red (weaker) to green (stronger) and from thin (weaker) to thick (stronger). In addition, Figure 3B shows the global similarity matrix obtained. For samples i and j, the position in the i-th row and the j-th column indicates the number of tasters for whom those samples became connected after the clustering process. The same color code explained above for the consensus plot was used for the matrix.

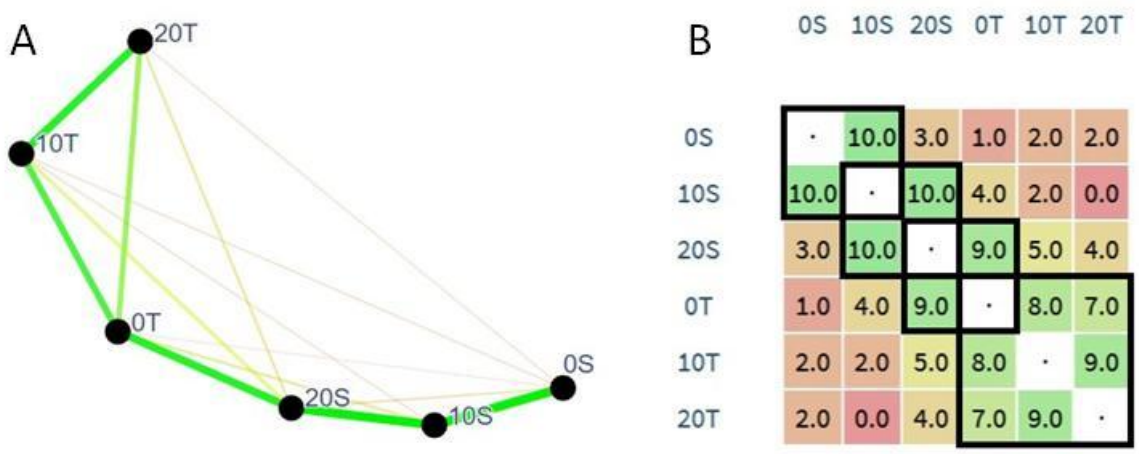

Figure 3. SensoGraph consensus plot of the samples tasted (A) and Global similarity matrix from the SensoGraph method, with groups highlighted as black squares (B).

A clear group of samples 0T-10T-20T appears on the consensus plots in Figure 3A, with strong mutual connections between these three samples (appearing as a triangle of thick green lines). The matrix shows that these three connections have similar and strong values (see the lower-right group in Figure 3B). Both facts show that the tasters identified a strong similarity between samples 0T-10T-20T, which is in accordance with the consumer acceptance evaluation results, in which the tagatose biscuits produced significantly similar results for almost all the hedonic parameters.

On the other hand, samples 0S-10S-20S form a line, with a strong connection for 0S-10S and a strong connection for 10S-20S, but a weak connection for 20S-0S. In other words, the tasters perceived sample 10S to be similar to both samples 0S and 20S, but they did not perceive samples 0S and 20S to be similar (see the remaining groups in Figure 3B).

Finally, Figure 3A shows that the triangle 0T-10T-20T and the path 0S-10S-20S are strongly connected through samples 0T and 20S, providing a kite-like configuration. This shows that the T- and S-samples were not perceived as completely independent, but that the tasters perceived a similarity between two samples of those types, namely, between 0T and 20S.

4. Conclusions

The addition of CBS powder in place of wheat flour led to the production of biscuits that meet the criteria for claiming them a ‘source of fiber’ and ‘high fiber’ biscuits for the 10% and 20% wheat flour substitution, respectively. The various produced biscuits presented considerable differences at both technological and sensory levels. Both factors—the use of tagatose and increasing percentages of CBS—led to water retention, thus affecting the

physical and structural properties of the biscuits. From a sensory point of view, sucrose biscuits were highly preferred over tagatose biscuits, which suggests important sensorial differences between the two sugars and the lack of appreciation by the tasters for the physical differences provided by tagatose utilization. However, it was observed that the addition of CBS enhanced the preferability of the tagatose biscuits, namely, those with 10% of wheat flour substituted with CBS flour (T10). The geometric analysis using SensoGraph of the Napping® results showed a clear association of the biscuits depending on the employed sugar, although these groups were not perceived as completely independent.

The possibility of producing tagatose and CBS-based biscuits destined for diabetic consumers is confirmed by these results. However, an adapted recipe in which some of the tagatose is substituted by polyols is recommended in order to improve the tagatose biscuit characteristics (water adsorption, gluten network formation, taste, and aroma). Additionally, the specific aspects of the CBS-based biscuit functionality need to be assessed, which will be done in a future work.

CHAPTER 2.4

Polyphenolic and Methylxanthine Bioaccessibility of Cocoa Bean Shell Functional Biscuits: Metabolomics Approach and Intestinal Permeability through Caco-2 Cell Models

This last chapter of the CBS-based functional foods (Part 2) was devoted to the study of the biscuits developed in the previous chapter with a nutritional approach. In this case, the model biscuits served to study the bioaccessibility of the polyphenols and methylxanthines contained in CBS in the presence of a more complex matrix when compared to that present in the beverages of Chapter 2.2. In addition, a metabolomic approach was proposed for the bioaccessibility study in this case, and additional intestinal permeability studies were also performed in order to understand how much of the available compounds at the intestinal level after gastrointestinal digestion is able to pass the intestinal barrier into the bloodstream.

This chapter was issued and adapted from a published scientific article:

Polyphenolic and Methylxanthine Bioaccessibility of Cocoa Bean Shell Functional Biscuits: Metabolomics Approach and Intestinal Permeability through Caco-2 Cell Models

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1. Introduction

In recent years, functional foods and ingredients have received increasing interest. It has been claimed that when consumed in the diet, these foods possess the ability to treat, prevent, or reduce the risk of several pathologies, such as cancer, cardiovascular diseases, autoimmune disorders, diabetes, or even the process of aging through the neutralization of reactive oxygen species in the human organism. Polyphenols are one of the main compound groups responsible for the activity of different functional ingredients. These are secondary metabolites found in plants which have been demonstrated to have several health benefits in organisms, such as antioxidant, antidiabetic, and anticarcinogenic activities, among others [219]. In particular, great attention has been given to biofunctional compounds coming from food waste or food by-products as their use in foods would reduce the economic and environmental costs of their disposal, giving them a role in the sustainability and bioeconomical framework of the modern food industry [349]. In the cocoa industry, one of the main by-products is the cocoa bean shell (CBS). This cocoa by-product constitutes around 10–17% of the total cocoa bean weight, and more than 700 thousand tons are produced yearly worldwide. However, CBS contains several nutrients and non-nutritive microconstituents such as proteins, vitamins and minerals, dietary fiber, methylxanthines such as theobromine and caffeine, and polyphenols (mainly flavan-3-ols); thus, they have attracted interest from several researchers [218]. Moreover, it has been found that CBS presents a very similar aromatic profile to that of cocoa powder, which makes it even more attractive for functional food development [29,66]. Polyphenols contained in cocoa and CBS have been demonstrated to have several beneficial effects on the human organism, such as antimicrobial, antiviral, anti-inflammatory, anticarcinogenic, and antidiabetic effects, among others [218]. These effects are largely due to the antioxidative characteristics of this compound group, which cause them to act as chemopreventors of several chronic diseases [169]. Nevertheless, cocoa and CBS polyphenols could also promote beneficial effects such as antidiabetic actions by acting as enzyme inhibitors or modulating insulin secretion in pancreatic cells [84]. However, most of these bioactivities have been demonstrated *in vitro* through direct use of CBS extracts, without taking into account how the bioactive compounds contained in the cocoa by-product would change or what their availability would be during gastrointestinal digestion. The consumed compounds do not all arrive at the target organ in their original form or concentration to exert their biofunction, and sometimes, they may not even be released into the bloodstream [294].

The bioaccessibility of polyphenols during gastrointestinal digestion and their interactions with the food matrix are crucial factors that influence their bioavailability in the human organism and their actions at a local level within the gastrointestinal tract. This factor determines how much of the biofunctional compound fractions actually reach the intestinal tract and are available for absorption and passage into the bloodstream or action at the local level. It depends on different factors, such as compound release from the food matrix and the characteristics of this matrix, their solubilization degree, their molecular size, interactions with food components, hydrophilic and hydrophobic characteristics, or different types of transformation dependent on the pH that could take place [294,350].

Several studies have been carried out to develop functional foods containing CBS in order to revalorize this by-product. Due to its similarity to cocoa powder and its high fiber content, CBS has mainly been used to produce high-fiber baking goods with the ability to provide health benefits while utilizing a low-cost food ingredient [218]. However, little research has been carried out on the bioaccessibility of compounds of interest contained in these CBS functional foods or on how the food matrix could affect this parameter.

In this work, high-fiber CBS-based biscuits containing either sucrose or tagatose (a prebiotic sugar with low glycemic index adapted for diabetic consumers [326]) were studied to investigate the bioaccessibility and bioactivity (antioxidant and α-glucosidase inhibition capacities) of their bioactive compounds (polyphenol content and methylxanthines) after simulation of gastrointestinal digestion. Furthermore, the intestinal permeability of some targeted compounds was assessed. The effects of the biscuit food matrix on both bioaccessibility and intestinal permeability were evaluated. This work has a different approach and is a continuation of our previous work, in which technological aspects linked to the production of the biscuits were assessed [221].

2. Materials and Methods

2.1. Chemicals

Hexane (≥99%), acetone (≥99.5%), formic acid (≥98%), α-amylase from human saliva, pepsin from porcine gastric mucosa, bile salts, pancreatin from porcine pancreas, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer; ≥99.5%), dimethyl sulfoxide (DMSO; 99.9%), Lucifer Yellow CH dipotassium salt, Folin and Ciocalteu's phenol reagent, sodium carbonate (≥99.5%), sodium nitrite (≥99%), aluminum chloride (99%), (+)-catechin hydrate (>98%), methanol (≥99.9%), vanillin (99%), hydrochloric acid (37%), 2,2′-diphenyl-

1-picrylhydrazyl (DPPH; 95%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox; 97%), α-glucosidase from intestinal acetone powder from rats, p-nitrophenyl-α-D-glucopyranoside (p-PNG; ≥99%;), potassium phosphate monobasic, acarbose (≥95%), epicatechin (>98%), caffeine (≥99%), MS quality formic acid, and MS quality methanol were obtained from Sigma-Aldrich (Milan, Italy; Steinheim, Germany). Theobromine (99%) was provided by Alfa Aesar (Kandel, Germany). Procyanidin B1 (PCB1; ≥99%) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Calcium chloride dehydrate, hydrochloric acid, potassium chloride, potassium phosphate dibasic, sodium carbonate, sodium chloride, magnesium chloride hexahydrate, and ammonium carbonate were acquired from Carlo Erba (Milan, Italy). Sodium hydroxide (1M), ethanol (≥99.9%), and gallic acid were supplied by Fluka (Milan, Italy). D-(+)-Glucose was provided by Carl Both (Kalrlsruhe, Germany), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 98%) was acquired from Acros Organics (Geel, Belgium). Minimum essential medium (MEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine (200 mM), penicillin–streptomycin (10,000 U/mL), and sodium pyruvate (100 mM) were provided by Life Technologies (Paisley, UK).

Ultrapure water was prepared using a Milli-Q filter system (Millipore, Milan, Italy).

2.2. Samples and Formulation of the Model Food (Biscuits)

Roasted CBS extracted from Trinitario variety cocoa beans (São Tomé origin) was kindly supplied by Pastiglie Leone S.r.l. (Turin, Italy) and employed for the preparation of biscuits. CBS was micronized before model food preparation to obtain particles smaller than 20μm, as measured with a PSA 990 particle size analyzer (Anton-Paar GmbH, Graz, Austria).

Six biscuit types were prepared following the official method AACC 10-53.01 with slight modifications, as described by Rojo-Poveda et al. [221] (Chapter 2.3). Two biscuit groups containing different types of sugar were developed, one with sucrose and one with the low-glycemic sugar tagatose. For each sugar group, a control biscuit was developed with no CBS added and another two types of biscuit were produced in which 10% and 20% of the wheat flour content was replaced with CBS flour (5.28% and 10.56% w/w for the final product). The obtained model biscuits were coded according to these characteristics: 0S (sucrose control biscuit with 0% CBS), 0T (tagatose control biscuit with 0% CBS), 10S (10% CBS and sucrose), 10T (10% CBS and tagatose), 20S (20% CBS and sucrose), and 20T (20% CBS and tagatose). For comparison purposes, and in order to study the food matrix effects, samples in which only CBS flour was present were prepared. These samples contained CBS quantities

equivalent to those in 10% (CBS10) and 20% CBS (CBS20) biscuits, and water replaced the rest of the food matrix weight.

2.3. Preparation of Polyphenolic Extracts, In Vitro *Digests, and Non-Digested Extracts*

The biscuit and CBS ingredient alone samples were subjected to three different types of extraction: polyphenolic extraction with organic solvents (Org), simulated *in vitro* gastrointestinal digestion (Dig), and aqueous extraction (No_Dig).

For the polyphenolic extraction with organic solvents, 1 g of the sample was extracted following the procedure described by Mota-Gutierrez and co-workers [351]. Briefly, samples were washed with hexane for defatting, and the precipitate was dried and extracted by means of an acetone–water–formic acid solution (70:29.5:0.5). After centrifugation and filtration through 0.45µm PTFE filters (LLG-Labware, CA, USA), the extract was evaporated and resuspended in 5 mL of ultrapure water.

Five grams of each sample underwent *in vitro* gastrointestinal digestion. The simulated digestion was performed following the three-stage model (oral, gastric, and intestinal phases) proposed by the INFOGEST consortium and described by Minekus et al. [298] with the slight modifications described in [222] (Chapter 2.2). Briefly, during the oral phase (2 min), samples were mixed at 37 °C in a SW-20 water bath (Julabo GmbH, Seelbach, Germany) with an electrolyte solution to simulate salivary fluid and with amylase enzyme. Samples then underwent a gastric phase (2 h) in which they were mixed with simulated gastric fluid and pepsin enzyme. Finally, during the intestinal phase (2 h), samples from the gastric phase were mixed with simulated intestinal fluid, bile salts, and pancreatin. Solutions were adjusted to pH 3 and 7 for the gastric and intestinal phases, respectively, with a MICROpH 2002 pH-meter (CRISON, Carpi, Italy), and the digestion process was stopped by bringing the pH down to 5.4. Samples were then centrifuged at 12,500 ×*g* and 0 °C for 10 min in a Heraeus megafuge 11R centrifuge (THERMO electron, Chateau-Gontier (France), and the supernatants were filtered through 0.45 µm CA syringe filters and immediately stored at −20 °C until further analysis. The digestion process was replicated four times for each sample. Digestion blanks in which the whole sample was replaced with 5 mL of water were also prepared in order to monitor the possible contributions of the enzymes and salts employed during *in vitro* digestion in the subsequent analytic determinations.

Non-digestion extraction was performed to allow a comparison with the samples digested *in vitro*. This employed the same temperature and time conditions and the same weight and volume ratios. Samples were extracted at 37 °C for 4 h and 2 min (the whole time for the

simulated gastrointestinal digestion) with just Milli-Q water and without enzymes or simulated fluids in order to avoid food matrix degradation due to those components. After this, non-digested samples were centrifuged and filtered in the same way as the digested samples.

2.4. Analytical Determinations

2.4.1. Total Phenolic, Flavonoid, and Tannin Contents

The total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) were assessed by the Folin–Ciocalteu, aluminum chloride, and vanillin methods, respectively. Methods were adapted as described in [57] for analysis in 96-well microplates, and a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy) was employed as detailed in Chapter 1.1. For TPC determination, a gallic acid standard curve was prepared (20–100 mg/L), and the results are expressed as milligrams of gallic acid equivalents (GAE) per gram of sample. For TFC and TPC, quantification was performed by using a calibration curve of catechin (5–500 mg/L), and the results are expressed as milligrams of catechin equivalents (CE) per gram of sample. All measurements were performed in triplicate for all of the different sample extracts (Org, Dig, and No_Dig), and the values obtained for the control biscuits S0 and T0 were used as blanks and subtracted from S10 and S20 and T10 and T20, respectively, in order to adjust the final results and avoid food matrix interference, as indicated in [63].

2.4.2. Radical Scavenging Activity

The radical scavenging activity (RSA) or antioxidant capacity of the samples was determined by the DPPH radical-scavenging method described by [57] in 96-well plates using the BioTek Synergy HT spectrophotometric reader (BioTek Instruments). The RSA was calculated as the inhibition percentage (IP) of the DPPH radical according to the following equation:

$$\text{IP}\ (\%) = (A_0 - A_{30})/A_0 \times 100$$

where A_0 is the absorbance at the initial time, and A_{30} is the final absorbance after 30 min.

A calibration curve of Trolox was employed (12.5–300 μM) for the quantification of radical scavenging activity, and the results are expressed as micromoles of Trolox equivalents (TE) per gram of sample. All measurements were performed in triplicate for all of the different sample extracts (Org, Dig, and No_Dig), and the values obtained for the control

biscuits S0 and T0 were used as blanks and subtracted from S10 and S20 and T10 and T20, respectively, in order to avoid food matrix interference.

2.4.3. *In vitro* α-Glucosidase Inhibition Capacity

The α-glucosidase inhibition capacity of the samples was determined by the α-glucosidase colorimetric assay described in [13] by employing 96-well plates and reading results with the BioTek Synergy HT spectrophotometric reader (BioTek Instruments). The enzyme inhibition capacity of the samples was calculated as their inhibition percentage and expressed as micromoles of acarbose equivalents (AcE) per gram of sample by using an acarbose calibration curve (1–25,000 μM). All measurements were performed in triplicate for all of the different sample extracts (Org, Dig, and No_Dig) and the values obtained for the control biscuits S0 and T0 were used as blanks and subtracted from S10 and S20 and T10 and T20, respectively, in order to avoid food matrix interference.

2.4.4. Liquid Chromatography–High-Resolution Mass Spectrometry (LC–HRMS) Analysis

Characterization of polyphenols and methylxanthines from all the extract types (Org, Dig, and No_Dig) of the samples was performed with an untargeted metabolomics approach by using a rapid-resolution LC system (RRLC) 1200 series (Agilent Technologies, Waldbronn, Germany), including a binary pump, thermostatized autosampler, column oven, and a DAD detector. The RRLC system was coupled to a 6520 series electrospray ionization (ESI) source with a quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies). Compound separation was performed using an InfinityLab Poroshell 120 EC-C18 column (2.1 × 100 mm, particle size of 2.7 μm) with a guard column (2.1 × 5 mm) (Agilent Technologies). The column temperature was kept at 35 °C. Water acidified with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B) served as mobile phases. A gradient elution at a flow rate of 0.45 mL/min was conducted as follows: minutes 0–1, 99% A and 1% B; minutes 1–15, linear gradient from 1% to 80% B, minutes 15–17, 80% B; minutes 17–17.5, linear gradient from 80% to 90% B; minutes 17.5–20, 90% B; minutes 20–21, linear gradient from 90% to 1% B; minutes 21–28, 1% B. The sample injection volume was 5 μL. The ESI–QTOF settings for metabolomics analysis were as follows: positive mode, 2 GHz resolution, MS scan range and rate of 100–1500 m/z at 1 spectra/s, drying gas temperature of 325 °C, drying gas flow of 10 L/min, nebulizer pressure of 45 psi, capillary voltage of 4000 V, and fragmentor voltage of 150 V. Reference ions of m/z 121.050873 and 922.009798 were infused and used as continuous ions for mass calibration during analysis. Nitrogen was used

as a nebulizer and drying gas. Data acquisition and analysis were carried out by MassHunter Acquisition® software for QTOF (Version B.04 SP3) and MassHunter Qualitative Analysis® (Version B.08) software (Agilent Technologies). Samples were randomly analyzed in one single batch. The same quality control (QC = pool) sample for all samples (mix of all samples) was injected throughout the run after approximately every ten samples as a control, and blanks (water) were also injected throughout the run.

For the samples from the permeability study through Caco-2 cells, the quantification of five targeted compounds identified by MS was performed using calibration curves of caffeine, theobromine, epicatechin, catechin, and procyanidin B1 (PCB1) standards drawn from 0.01 to 10 ppm. LC-HRMS analysis was performed as mentioned above with slight modifications: 20 µL of sample was injected and analyses were performed in positive (for methylxanthines) and negative (for epicatechin, catechin and PCB1) modes. MassHunter Quantitative Analysis® (Version B.08) software (Agilent Technologies) was employed for concentration determination of the samples.

2.4.4.1. LC–HRMS Data Processing and Statistical Analysis (Metabolomics Study)

Agilent format .d data were converted to .mzXML format using the Peak Picking filter option in ProteoWizard MSConvert tools software (Version 3.03.9393, 64-bit). Preprocessing (filtration, peak identification, peak grouping and smoothing, retention time correction, integration, and annotation), normalization, quality control (metabolites correlation analysis and determination of batch correction), and statistical analysis (multivariate modeling and univariate testing) were conducted using the Galaxy workflow4metabolomics W4M online and freely available platform (http://workflow4metabolomics.usegalaxy.fr [352], version 3.3). The parameters used for processing and statistics were based on a previous study [353], with slight modifications, and can be viewed in Table 1.

Table 1. Steps and parameters employed for the metabolomics analysis in the W4M platform (https://workflow4metabolomics.usegalaxy.fr/). Adapted from the work of Souard et al. [353]

xcms.xcmsSet Filtration and Peak Identification using xcmsSet function from xcms R package to preprocess LC/MS data for relative quantification and statistical analysis (Galaxy Version 3.6.1+galaxy1)	
Scan range option	*hide*
Extraction method for peaks detection	*centWave*
Max tolerated ppm *m/z* deviation in consecutive scans in ppm	*15*
Min,Max peak width in seconds	*20,60*
Signal/Noise threshold	*10*
Min *m/z* difference	*0.01*
Peak limits method	*Peak limits are found through descent on the mexican hat filtered data*
Prefilter step for the first phase	*3,1500*
Noise filter	*500*

xcms.group Group peaks together across samples using overlapping *m/z* bins and calculation of smoothed peak distributions in chromatographic time. (Galaxy Version 3.6.1+galaxy1)	
Method to use for grouping	*density*
Bandwidth	*10*
Minimum fraction of samples necessary	*0.1*
Width of overlapping *m/z* slices	*0.025*
Maximum number of groups to identify in a single *m/z* slice	*50*

xcms.retcor Retention Time Correction using retcor function from xcms R package (Galaxy Version 3.6.1+galaxy1)	
Method to use for retention time correction	*peakgroups*
Smooth method	*loess*
Number of extra peaks to allow in retention time correction correction groups	*1*
Number of missing samples to allow in retention time correction groups	*100*
Degree of smoothing for local polynomial regression fitting	*0.2*
Family	*gaussian*
Plottype	*deviation*

xcms.group Group peaks together across samples using overlapping *m/z* bins and calculation of smoothed peak distributions in chromatographic time. (Galaxy Version 3.6.1+galaxy1)	
Method to use for grouping	*density*
Bandwidth	*5*
Minimum fraction of samples necessary	*0.1*
Width of overlapping *m/z* slices	*0.025*
Maximum number of groups to identify in a single m/z slice	*50*

xcms.fillPeaks Integrate the signal in the region of that peak group not represented and create a new peak (Galaxy Version 3.6.1+galaxy0)	
Filling method	*chrom*

Intensity Check Calculates, among other metrics, the relative intensity of some sample group features with respect to the remaining samples (Galaxy Version 1.2.8)	
Computation method	*Between one class and all the remaining samples*
Class column	*Subclass1_SampleSubGroup*
Selected class	*blank*
Calculate the mean fold change	*Yes*
Where should the class be placed for the mean fold change calculation?	*Denominator*

Generic_Filter Removes elements according to numerical or qualitative values (Galaxy Version 2020.01)	
Deleting samples and/or variables according to Numerical values	*Yes*
Identify the parameter to filter	*Variable metadata*
Name of the column to filter	*fold_Other_VS_blank*
Interval of values to remove	*lower*
Remove all values lower than	*2.5*
Deleting samples and/or variables according to Qualitative values	*Yes*
Removing a level in factor	*Sample metadata*
Name of the column to filter	*sampleType*
Remove factor when	*blank*

Normalization of preprocessed data (Galaxy Version 1.0.7)	
Normalization method	*Quantitative Variable*
Name of the column	*WeightProportion*

Intensity Check Calculates, among other metrics, the relative intensity of some sample group features with respect to the remaining samples (Galaxy Version 1.2.8)	
Computation method	*Between one class and all the remaining samples*
Class column	*Subclass1_SampleSubGroup*
Selected class	*H2O (Digestion blank)*
Calculate the mean fold change	*Yes*
Where should the class be placed for the mean fold change calculation?	*Denominator*

Generic_Filter Removes elements according to numerical or qualitative values (Galaxy Version 2020.01)	
Deleting samples and/or variables according to Numerical values	*Yes*
Identify the parameter to filter	*Variable metadata*
Name of the column to filter	*fold_Other_VS_H2O*
Interval of values to remove	*lower*
Remove all values lower than	*1.0*
Deleting samples and/or variables according to Qualitative values	*Yes*
Removing a level in factor	*Sample metadata*
Name of the column to filter	*Subclass1_SampleSubGroup*
Remove factor when	*blank*

Batch_correction Corrects intensities for signal drift and batch-effects (Galaxy Version 2.1.2)	
Type of regression model	*linear*

Transformation Transforms the dataMatrix intensity values (Galaxy Version 2.2.0)	
Method	*Log10*

Quality Metrics Metrics and graphics to check the quality of the data (Galaxy Version 2.2.6)	
Coefficient of Variation	*Yes*
Which type of CV calculation should be done	*Only pool CV*
Threshold	*0.3*
Advanced parameters	*Use default*

Generic_Filter Removes elements according to numerical or qualitative values (Galaxy Version 2020.01)	
Deleting samples and/or variables according to Numerical values	*Yes*
Identify the parameter to filter	*Variable metadata*

Name of the column to filter	*Pool_CV*
Interval of values to remove	*upper*
Remove all values upper than	*0.3*
Deleting samples and/or variables according to Qualitative values	*Yes*
Removing a level in factor	*Sample metadata*
Name of the column to filter	*sampleType*
Remove factor when	*pool*

Multivariate PCA, PLS and OPLS (Galaxy Version 2.3.10)	
Y Response (for (O)PLS(-DA) only)	*1) PCA: keep the default (none); 2) (O)PLS(-DA): indicate the name of the column of the sample table to be modeled* ***(➔ "Subclass2_ExtractionType")***
Number of predictive components	*1) PCA and PLS(-DA): 4*
Number of orthogonal components (for OPLS(-DA) only)	*Notes: 1) PCA and PLS(-DA): 0*
Samples to be tested	*no*
Advanced graphical parameters	*Full parameter list*
Graphic type	*summary*
Ellipses	***"Subclass2_ExtractionType"***
Sample labels	***"Subclass2_ExtractionType"***
Component to be displayed as abscissa	***1***
Component to be displayed as ordinate	***2***
Amount by which plotting text should be magnified relative to the default	*0.4*
Advanced computational parameters	*Full parameter list*
Scaling	*Standard*
Permutation testing for (O)PLS(-DA): Number of permutations	*20*
Log10 transformation	*No (➔ already done in the transformation step)*
Algorithm	*Default*
Number of cross-validation segments	*7*

Univariate Univariate statistics (Galaxy Version 2.2.4)	
Factor of interest	***"Subclass2_ExtractionType"***
Test	*Analysis of variance (qualitative, more than 2 levels)*
Method for multiple testing correction	*fdr*
(Corrected) p-value significance threshold	*0.05*

2.5. Cell Culture and Assays

Human colon adenocarcinoma Caco-2 cell lines obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used between passages 10 and 42 for cell viability assays and permeability studies with the digested extracts. Cells were cultured in MEM supplemented with L-glutamine (2 mM), penicillin–streptomycin (100 U/mL–100μg/mL), pyruvate (1 mM), and fetal bovine serum (10% *v/v*) in a 5% CO_2 atmosphere at 37 °C inside a Binder incubator (Tullingen, Germany).

2.5.1. Cell Viability Assay

The absence of cytotoxicity from digested extracts was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This is a standard colorimetric assay used for measuring the activity of enzymes able to reduce MTT (yellow) to formazan (purple). This raction occurs mainly in the mitochondria and it can be used to determine the cell viability. Indeed, the mitochondrial succinate dehydrogenase enzyme, which is believed to be involved in this reaction, is active only in living cells, and is responsible for the opening of the tetrazolium ring in the MTT with the consequent formation of formazan Figure 1 [354].

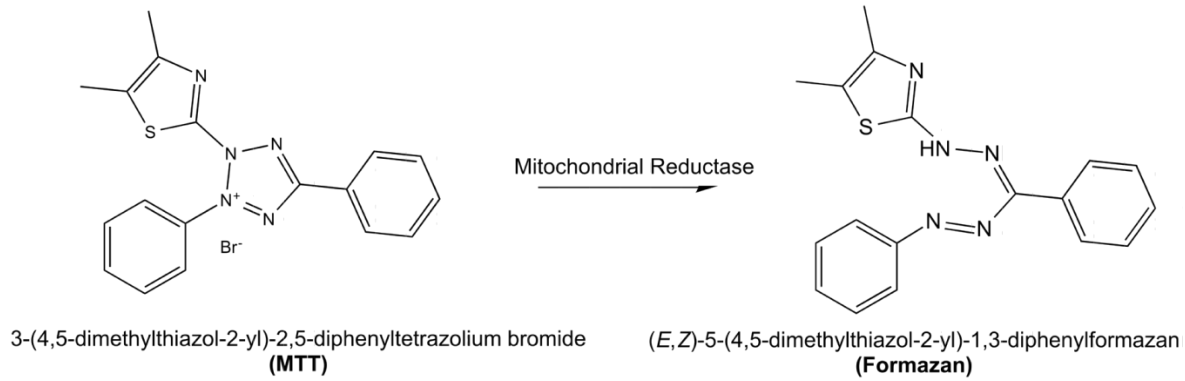

Figure 1. Reduction of the MTT into Formazan by means of the mitochondrial reductase present in living cells.

For the assay, Caco-2 cells were seeded into 96-well plates at a density of around 20,000 cells/well and left to grow for 48 h at 37 °C and 5% CO_2. After incubation, culture medium was removed by aspiration, replaced by 100 µL of digested extract (1/4, diluted in culture medium), and left for incubation for either 2 or 4 h. Then, the extracts were removed, and 100 µL of MTT (0.5 mg/mL) was added. After 3 h of incubation in the dark in a humidified atmosphere (5% CO_2, 37 °C), plates were centrifuged at 560 ×*g* for 20 min, the supernatant was removed by returning the plate, 100 µL of DMSO was added to each well, and the plates were shaken for 5 min in order to dissolve the reduced intracellular formazan created. The absorbance was then read at 540 nm in a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Winooski, VT, USA). The percentage of cell viability was assessed in relation to the value given by the culture medium control, and values higher than 80% were considered acceptable. Experiments were performed in quadruplicate with at least five replicates.

2.5.2. Permeability Study through Caco-2 Cell Monolayers

Permeability assays were performed following the protocol described by Hubatsch et al. [355] with slight modifications. Caco-2 cells were seeded into the apical side (AP) of hanging inserts with a pore size of 0.4 μm and a diameter of 1.12 cm placed on 12-well plates (Greiner Bio-One, Vilvoorde, Belgium). Culture medium (0.5 mL) containing approximately 300,000 cells was added in the apical side (AP) of each insert to give a final cell density of around 260,000 cells/cm^2, and 1.5 mL of culture medium was added to the basolateral side (BL). Cells were left to grow for 21–29 days to differentiate and form a monolayer, with the medium being changed every 2–3 days. For this, aspiration was carried out in the BL and inserts were returned for the AP emptying in order to maintain the integrity of the monolayer. After this period, the transepithelial electrical resistance (TEER) of the monolayers was monitored using a STX2 chopstick electrode coupled to an EVOM2 epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA), and TEER values were calculated in $\Omega \cdot cm^2$ after subtraction of the blank value given by an empty insert without cells. Cells were considered to have reached confluence when TEER values were higher than 260 $\Omega \cdot cm^2$ [355].

The culture medium was changed 24 h prior to the transport study day. Before the permeability assay, the culture medium was removed and 0.5 and 1.5 mL of modified HBSS (with 25 mM D-glucose, 20 mM HEPES, 1.25 mM $CaCl_2$, and 0.5 mM $MgCl_2$) were added to the AP and to the BL, respectively, and incubated for 30 min before TEER measurement (TEER values before the test). Modified HBSS was then removed and 0.4 mL of digested extract (diluted 1:4 with modified HBSS) was introduced into the AP, while 1.2 mL of modified HBSS was added to the BL. Plates were placed under agitation at 37 °C, and after 60 or 120 min (T1 and T2, respectively), 0.5 mL of the sample was taken from the BL, followed by the addition of the same quantity of modified HBSS. Samples were stored at −20 °C until further analysis by LC–HRMS. TEER measurement was carried out at the end of the assay in order to confirm the integrity of the monolayer by comparing it with the values obtained before analysis. Permeability analyses were performed in triplicate, and the apparent permeability coefficient (P_{app}) was calculated as follows [356,357]:

$$P_{app} = dC/dt \times V/(A \times C_0)$$

where dC/dt is the change in the concentration of the compound on the basolateral side (receiving chamber) over time (or slope of the cumulative concentration), V is the volume in the basolateral chamber, A is the membrane surface area, and C_0 is the compound's initial concentration on the apical side (donor chamber).

2.5.3. Study of the Caco-2 Cell Monolayer Integrity by Means of the Lucifer Yellow Assay

In addition to TEER measurements taken before and after the permeability assay, the integrity of the monolayer after the permeability test was also confirmed using Lucifer Yellow, which can serve as a marker for monolayer imperfections, since it is a molecule that is unable to permeate through lipophilic barriers. To do so, the employed inserts were emptied and introduced into a new plate. Then, 1 mL of modified HBSS was added into the BL, and 0.5 mL of Lucifer Yellow (0.1 mg/mL of modified HBSS) was added into the AP. Plates were left for incubation for 60 min at 37 °C without agitation. After this, quadruplicate 150 µL samples of the BL were placed into 96-well plates, and fluorescence was measured with excitation at 485 nm and emission at 530 nm. The percentage of Yellow Lucifer permeability (with a maximum acceptance of 3%) was calculated as follows:

$$\%\ \text{permeabilty}\ (\text{maximum acceptable} = 3\%) = \frac{\text{sample} - \text{blank}_{\text{HBSS}}}{\text{LY} - \text{blank}_{\text{HBSS}}} \times 100$$

where $\text{blank}_{\text{HBSS}}$ is the fluorescence value given by a blank of modified HBSS, and LY is the fluorescence value given by a 0.1 mg/mL solution of Lucifer Yellow.

2.6. Statistical Analyses

The results obtained from spectrophotometric assays (TPC, TTC, TFC, radical scavenging activity, and α-glucosidase inhibition capacity) were analyzed through one-way analysis of variance (ANOVA) with Duncan's post-hoc test at a confidence level of 95% in the 26.0.0.1 version of SPSS Statistics software (IBM-SPSS Inc., Chicago, IL, USA).

Data obtained from the LC–HRMS analysis for metabolomics study were subjected to univariate testing and multivariate modeling (principal component analysis, PCA; and partial least squares discriminant analysis, PLS-DA) using the above-mentioned W4M platform.

3. Results

3.1. Total Phenolic, Tannin, and Flavonoid Contents

The results for the TPC, TFC, and TTC analyses performed for the three extraction types (organic extraction, *in vitro* gastrointestinal digestion, and no digestion) of all samples (biscuits containing CBS and samples with equivalent quantities of the CBS ingredient alone) are shown in Figure 2A–C, respectively, and their numerical values are available in Table 2.

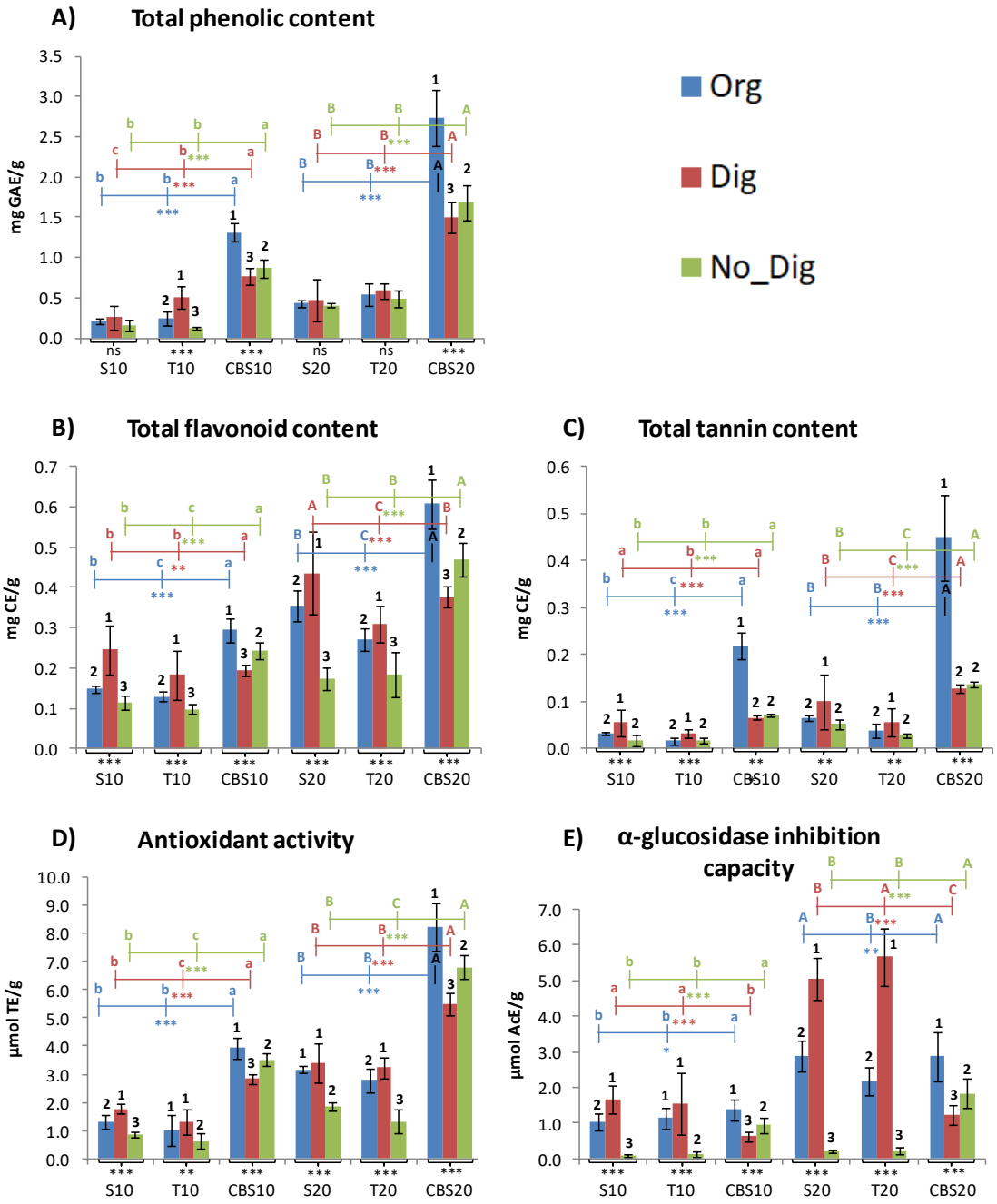

Figure 2. Total phenolic content (**A**), total flavonoid content (**B**), total tannin content (**C**), antioxidant capacity (**D**), and α-glucosidase inhibition capacity (**E**) for the three extraction types (Org, polyphenolic extraction with organic solvents; Dig, *in vitro* digestion; and No_Dig, no digestion) performed for the cocoa bean shell (CBS) biscuits (S10, T10, S20 and T20) and equivalent quantities of CBS powder alone without the food matrix (CBS10 and CBS20). The results are presented as the mean ± standard deviation (n = 6). Statistical comparisons were made using one-way ANOVA and Duncan's post-hoc tests at 95% confidence. For each parameter, the different numbers above the bars indicate significant differences at $p < 0.05$ for the three different types of extraction within the same sample. Different letters indicate significant differences at $p < 0.05$ for the same extraction within samples with equivalent CBS percentage (lowercase = 10% CBS and uppercase = 20% CBS). Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant. S10 (biscuit with sucrose and 10% of wheat flour substituted with CBS), T10 (biscuit with tagatose and 10% of wheat flour substituted with CBS), CBS10 (CBS powder alone without food matrix in equivalent quantity to that in 10% biscuits), S20 (biscuit with sucrose and 20% of wheat flour substituted with CBS), T20 (biscuit with tagatose and 20% of wheat flour substituted with CBS), and CBS20 (CBS powder alone without food matrix in equivalent quantity to that in 20% biscuits).

Table 2. Total phenolic content, total flavonoid content, total tannin content, antioxidant capacity, and α-glucosidase inhibition capacity for the three extraction types (Org, Dig, and No_Dig) performed for the CBS biscuits (S10, T10, S20 and T20) and CBS powder alone without food matrix (CBS10 and CBS20). Results are presented as mean ± standard deviation (n=6).

TPC (mg GAE/g)	Organic extraction	*In vitro* digestion	No-digestion
S10	0.21 ± 0.04	0.26 ± 0.15	0.16 ± 0.07
T10	0.25 ± 0.09	0.50 ± 0.14	0.13 ± 0.02
CBS10	1.32 ± 0.12	0.77 ± 0.10	0.87 ± 0.11
S20	0.43 ± 0.05	0.47 ± 0.26	0.41 ± 0.03
T20	0.54 ± 0.13	0.59 ± 0.09	0.49 ± 0.11
CBS20	2.73 ± 0.35	1.50 ± 0.19	1.68 ± 0.22
TFC (mg CE/g)	Organic extraction	*In vitro* digestion	No-digestion
S10	0.15 ± 0.01	0.25 ± 0.06	0.11 ± 0.02
T10	0.13 ± 0.01	0.18 ± 0.06	0.10 ± 0.01
CBS10	0.29 ± 0.03	0.19 ± 0.01	0.24 ± 0.02
S20	0.35 ± 0.04	0.44 ± 0.10	0.17 ± 0.03
T20	0.27 ± 0.03	0.31 ± 0.04	0.18 ± 0.06
CBS20	0.61 ± 0.06	0.38 ± 0.03	0.47 ± 0.04
TTC (mg CE/g)	Organic extraction	*In vitro* digestion	No-digestion
S10	0.03 ± 0.00	0.05 ± 0.03	0.02 ± 0.01
T10	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
CBS10	0.22 ± 0.03	0.07 ± 0.00	0.07 ± 0.00
S20	0.06 ± 0.01	0.10 ± 0.06	0.05 ± 0.01
T20	0.04 ± 0.02	0.06 ± 0.03	0.03 ± 0.01
CBS20	0.45 ± 0.09	0.13 ± 0.01	0.14 ± 0.01
Antioxidant capacity (µmol TE/g)	Organic extraction	*In vitro* digestion	No-digestion
S10	1.31 ± 0.25	1.78 ± 0.16	0.86 ± 0.09
T10	1.02 ± 0.56	1.30 ± 0.46	0.64 ± 0.29
CBS10	3.93 ± 0.37	2.83 ± 0.20	3.51 ± 0.23
S20	3.17 ± 0.13	3.40 ± 0.70	1.86 ± 0.15
T20	2.78 ± 0.43	3.24 ± 0.38	1.33 ± 0.43
CBS20	8.23 ± 0.84	5.49 ± 0.38	6.80 ± 0.44
α-glu inhibition capacity (µmol AcE/g)	Organic extraction	*In vitro* digestion	No-digestion
S10	1.05 ± 0.24	1.66 ± 0.39	0.10 ± 0.03
T10	1.15 ± 0.30	1.56 ± 0.86	0.15 ± 0.08
CBS10	1.39 ± 0.30	0.63 ± 0.14	0.95 ± 0.21
S20	2.88 ± 0.44	5.04 ± 0.60	0.20 ± 0.05
T20	2.18 ± 0.38	5.65 ± 0.80	0.22 ± 0.09
CBS20	2.87 ± 0.68	1.22 ± 0.27	1.84 ± 0.41

Considering all of the different extractions, TPC values were between 0.13 and 0.50 mg GAE per gram of biscuit for the biscuits in which 10% of wheat flour was substituted by CBS, while the values for the equivalent quantity of CBS without the food matrix were 0.77–

1.32 mg GAE/g. For the S20 and T20 biscuits, TPC values ranged from 0.41 to 0.59 mg GAE/g, while samples with the equivalent quantity of CBS alone gave TPC values of 1.50–2.73 mg GAE/g. These values are in accordance with those obtained by different researchers for other functional foods containing similar recovered ingredients, such as biscuits containing 4.2% coffee fiber from spent coffee grounds (up to 8.98 mg GAE/g of biscuit digest) [358], drinking yogurt containing CBS extract (1.31–2.01 mg GAE/mL) [123], and different CBS-based beverages (0.3–1.8 mg GAE/mL) [13,222].

Generally, no differences associated with the sugar employed for the biscuits were observed. However, significant differences were found when comparing the CBS biscuits with samples with an equivalent quantity of CBS without a food matrix. These differences might be justified by the fact that the CBS ingredient alone underwent a single roasting process—the one undergone by cocoa beans before separating them from the CBS during their manufacture—while the phenolic compounds contained in the biscuits may have undergone extra degradation due to the second high-temperature step during biscuit baking. It has already been demonstrated that a similar baking process to that used in this work could cause a loss in TPC value of up to 50% [63]. In addition, some losses in TPC and biofunctionality (RSA and α-glucosidase inhibition capacity) were expected for the biscuits in comparison with the CBS ingredient alone, since the phenolic contents responsible for these activities have been reported to easily link to the food matrix provided by the ingredients of the biscuits, losing some of their activity. Polyphenols can combine with other components of the food matrix by both hydrogen and covalent bonds. Mostly, they combine with the cell wall components that constitute dietary fiber [359,360]. Dietary fiber in the biscuits with zero CBS added (control biscuits) has been reported to account for 2.48–2.79% of the dried product [221].

TFC values were between 0.10 and 0.25 mg CE/g of biscuit for S10 and T10 and between 0.17 and 0.44 mg CE/g of biscuit for S20 and T20, while values for the CBS ingredient without a food matrix ranged from 0.19 to 0.29 mg CE/g for CBS10 and between 0.38 and 0.61 mg CE/g for CBS20. These values were all in accordance with those previously reported for other types of foods based on CBS, such as drinking yogurt (0.20 mg CE/mL) [123] and CBS beverages (0.01–0.55 mg CE/mL) [13]. In the case of flavonoids, the values obtained for the biscuits accounted for at least half of the TPC values, while values of the CBS ingredients alone were proportionally lower in comparison with the former. This could be due to higher stabilization of this kind of polyphenol in the presence of a food matrix, which would probably reduce its degradation during the biscuit baking process. TFC values corresponding

to organic extraction and non-digestion were proportionally well correlated with those of TPC (r = 0.8985 and r = 0.9943, respectively) and followed a similar variation trend, while the TFC values of the sample digested *in vitro* showed a much lower correlation with the TPC results (r = 0.3044). Indeed, the TPC values obtained for the samples of CBS10 and CBS20 digested in vitro were almost double those of their biscuit counterparts, while the TFC values of the CBS ingredients alone were in line with those of their biscuit counterparts or were even significantly lower (e.g., TFC of digested CBS20 was lower than TFC S20). A hypothetic explanation for this could be that, for the biscuits, a higher concentration of flavonoids was present after gastrointestinal digestion thanks to the liberation of these compounds, which were previously linked to the food matrix. On the other hand, it is also possible that these values increased because of the degradation of polymeric flavonoids during digestion (e.g., condensed tannins) into their monomeric flavonoid compounds.

Values of TTC for the three extractions followed a similar pattern to TPC, where higher values were found for CBS10 and CBS20 (0.07–0.22 mg CE/g and 0.13–0.45 mg CE/g, respectively) than for their biscuit counterparts (0.02–0.05 mg CE/g for the 10%-substitution biscuits and 0.03–0.10 mg CE/g for the 20%-substitution biscuits). In the case of the tannin content, values accounted for 5.72–21.09% of the TPC, which is in accordance with the 3.4–19.2% previously found for beverages in a previous study using the same CBS powder [13]. Strong correlations of the TTC and TPC values were again found for the organic and non-digestion extractions (r = 0.9940 and r = 0.9789, respectively) and, contrary to the TFC, the TTC–TPC correlation for the samples digested *in vitro* was also high (r = 0.7288).

3.2. Biofunctional Characteristics—Radical Scavenging Activity and α-Glucosidase Inhibition Capacity

In this study, two parameters related to the biofunctional properties provided by CBS were studied in the biscuits and in the CBS ingredient alone: the radical scavenging activity (RSA) or antioxidant capacity, and the α-glucosidase inhibition capacity, which can, in part, contribute to the antidiabetic capacity of the products by inhibiting the enzyme involved in the degradation of glucose polymers into glucose, therefore decreasing glucose absorption and postprandial glycemia.

The radical scavenging activity of the CBS containing biscuits and the samples with an equivalent quantity of the CBS ingredient alone was determined for the three different extractions (polyphenolic organic extraction, in vitro gastrointestinal digestion, and no digestion). The results are presented in Figure 2D, and the numerical values are available in

Table 2. RSA values were between 0.64 and 1.78 μmol TE/g of biscuit for S10 and T10, between 1.33 and 3.40 μmol TE/g of biscuit for S20 and T20, and between 2.83 and 3.93 and 5.49 and 8.23 μmol TE/g for the equivalent quantities of the CBS ingredient alone (CBS10 and CBS20, respectively). As reported previously in other works on CBS functional compound extraction [57], the RSA was highly correlated with TPC, TFC, and TTC, and varied proportionally to those. Again, the RSA results of our samples were in line with the values previously obtained for other functional foods developed with CBS, such as different CBS-based beverages (1–7.29 μmol TE/mL) [13,222], drinking yogurt with added CBS extract (up to 1.92 μmol TE/mL) [123], or functional cakes where CBS was used as a fat replacement (1–4.8 μmol TE/g) [117].

Values for the α-glucosidase inhibition capacity measured for all the samples and extractions are shown in Figure 2E, and their numerical values are available in Table 2. These results are expressed as micromoles of acarbose equivalents per gram (μmol AcE/g). Acarbose is an α-glucosidase inhibition drug that is used to treat diabetes by decreasing polysaccharide digestion in the digestive tract and, therefore, producing a decrease in glucose absorption and avoiding arise in the glycemic index. Values between 0.10 and 1.66 μmol AcE/g were found in the different extractions of both S10 and T10, and a similar range was obtained for the CBS ingredient alone (CBS10; 0.63–1.39 μmol AcE/g). For the 20% samples, however, higher values were found for the biscuits (0.20–5.65 μmol AcE/g biscuit for S20 and T20) in comparison with CBS20 (1.22–2.87 μmol AcE/g), contrary to what was observed for the other parameters measured previously. This is mainly due to the significantly higher levels of activity found for the digested biscuit samples, which suggests that significant liberation or transformation of the compounds responsible for the enzyme inhibition occurred during simulated gastrointestinal digestion. These values were, again, in line with the previously reported α-glucosidase inhibition capacities of different foods based on CBS or other biofunctional by-products, such as the coffee fiber cookies studied by Martinez-Saez et al. [358], for which up to 2.04 μmol AcE/g of digested biscuit was obtained, or the different CBS beverage and flavored CBS beverage preparation methods studied by Rojo-Poveda et al. [13] and Cantele et al. [222], which showed enzyme inhibition of up to 0.5 μmol AcE/mL. The obtained values are very interesting if we take into account that when consuming and digesting a CBS-containing biscuit (approximately 15 g), up to 84.75 μmol of AcE could be available and could act by inhibiting the α-glucosidase enzyme at the intestinal level. Considering this dose, an intake of a few biscuits would provide acarbose equivalent values very close to the daily dose of acarbose used for postprandial glycemic control (150–200 mg,

which is equal to 232–310 μmol) [361]. In this way, it would be possible to obtain some of the desired enzyme inhibition through the diet, while avoiding the unpleasant side effects of acarbose, including gastrointestinal symptoms such as diarrhea, flatulence, abdominal discomfort, and even abnormal liver function in some rare cases [362].

3.3. In Vitro *Bioaccessibility of Bioactive Compounds and Functional Characteristics through Spectrophotometric Analysis Results*

For all parameters obtained through spectrophotometric assays (Figure 2), a general trend was repeated, probably because of the interdependence of these parameters. Generally, the No_Dig samples gave the lowest values when comparing them to the other extractions, while the Dig samples presented the highest values for the biscuit samples and the lowest for the CBS ingredient samples without a food matrix. In contrast, organic extraction (Org) led to the highest values for the CBS ingredient samples. Additionally, significant differences were generally found within the same CBS percentage between the biscuit and CBS ingredient samples, since the latter gave higher values than the biscuits in almost all cases. This was probably caused by the two reasons already explained in previous sections: the biscuits underwent an extra high-temperature process that the CBS ingredient alone samples did not, and some bonds between the food matrix of the biscuits (mostly fibers and proteins) and the bioactive compounds could have been created, preventing their liberation from the matrix, as was already reported by Hilary et al. [360] for date-seed-functionalized breads in contrast with date seed extract alone. It is worth mentioning that little or even no significant differences were found when comparing biscuits made with the same quantity of CBS but with different sugars, which suggests that the sugar type does not influence the bioaccessibility of the bioactive compounds at this level. Nevertheless, after observing these repeated differences, two effects were hypothesized: a compound liberation effect and a food matrix protection effect.

The compound liberation effect can be justified by the higher values found for digested CBS biscuits in comparison with their non-digested counterparts. Thus, it is suggested that the destruction of the food matrix during gastrointestinal digestion could lead to the liberation of polyphenolic compounds that were initially bound to this food matrix. This effect has been previously reported in other works for polyphenols that were included in a food matrix. Bertolino et al. [363] found a 12% increase in TPC and a 61% increase in RSA after the digestion of coffee silverskin yogurt, which was attributed the hydrolysis of polyphenolic compounds from polysaccharides and proteins by digestive enzymes. Barbosa-Pereira et al.

observed slight increases in TFC and TTC and up to 50% and 25% increases in TPC and RSA, respectively, after simulated digestion of CBS-based ice cream [364]. Kan et al. [365] observed greater bioaccessibility after intestinal digestion of anthocyanins contained in blueberry-fortified breads, which was not observed when a non-fortified bread was codigested with the equivalent quantity of blueberry extract. Paz-Yépez et al. [366] found that the bioaccessibility of polyphenols contained in dark, milk, and white chocolate considerably increased after gastrointestinal digestion by up to several times the initial values for non-digested chocolate.

On the other hand, the opposite effect was found for the digested samples of the CBS ingredient alone, which led to the food matrix protection effect hypothesis. Indeed, when observing the different values obtained for CBS10 and CBS20, it was noted that the digested samples gave the significantly lowest values, followed by the non-digested samples and those that underwent organic extraction, which gave the significantly highest values in almost all cases for the five parameters measured through spectrophotometric techniques. This was probably due to the absence of the food matrix protection effect, which led to the degradation of polyphenolic compounds caused by the attack of digestive enzymes and acids and to pH changes during the *in vitro* simulated gastrointestinal digestion. This was illustrated by the work of Cantele et al. [222] in which the TPC, RSA, and α-glucosidase inhibition capacity values decreased after gastrointestinal digestion by up to 76%, 57%, and 52%, respectively, for CBS-based beverages, since no food matrix was present in these food samples to protect the polyphenolic compounds. Tarko et al. [367] demonstrated that polyphenols linked to the food matrix are less bioavailable, although they are less easily degraded during digestion when contained in matrices such as whole fruits than when found in extracts. Moreover, Pešić et al. [309] found that a meat- and cereal-based food matrix mixed with grape seed extract increased the total recovery of polyphenols after simulated digestion in comparison to the extracts digested without the presence of the food matrix. Indeed, several authors have stated that the fat content in food matrices can significantly increase polyphenol bioaccessibility and stability during gastrointestinal digestion by interacting with these bioactive compounds by micellarization [359,368], which would be justified in the case of the butter-containing biscuits presented in this work, in contrast to the samples of CBS ingredient alone. Ortega et al. [368] found that the higher fat content in cocoa liquor (approximately 50%) in comparison with cocoa powder (approximately 15% fat content) enhances the digestibility of some of its phenolic compounds thanks to a protective effect during digestion. This effect was also observed in another study in which the bioaccessibility of three different types of chocolate

increased after gastrointestinal digestion and the greater the fat content was, the more the bioaccessibility percentage increased (dark chocolate < milk chocolate < white chocolate) [366].

3.4. LC–HRMS and Metabolomics Analysis for Single Compound Bioaccessibility Evaluation

The samples of biscuits with different sugars and different CBS percentages as well as the samples with an equivalent quantity of the CBS ingredient alone underwent three different types of extraction (Org, Dig, and No_Dig) and were analyzed through reverse-phase LC–HRMS. The W4M platform was used to conduct an untargeted metabolomics study on the obtained data, and a total of 1585 features were obtained after data processing and filtering, which were then subjected to multivariate and univariate analyses.

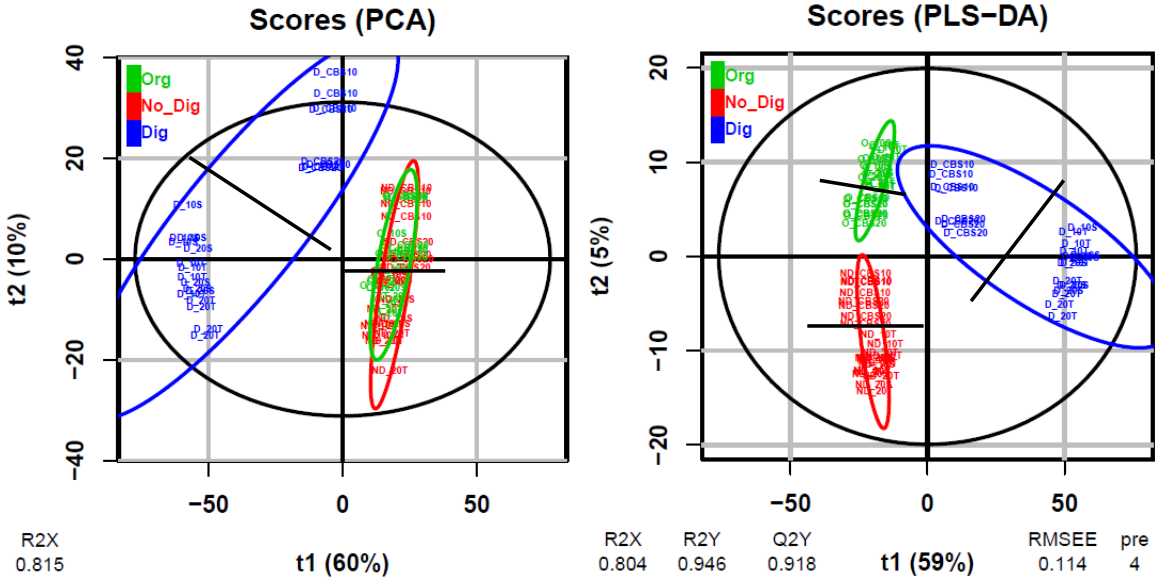

Figure 3. Score plots for the multivariate modeling of variations using PCA (principal component analysis) or PLS-DA (partial least squares discriminant analysis) of all samples (biscuits and equivalent quantities of CBS alone without a food matrix) which were subjected to three different types of extraction (Org, No_Dig, and Dig). For each plot, the percentages of total variation explained by components 1 and 2 (t1 and t2, respectively) are indicated in parentheses. Black ellipses include 95% of the multivariate normal distribution for all samples (subgrouped in colored ellipses according to the extraction type).

In order to show the general differences within the whole sample set, Figure 3 shows the PCA and PLS-DA plots obtained from the multivariate untargeted metabolomics study performed on all samples together. Supplementary information on these plots is available in Figure 4. Both plots allowed for clear separation of the digested samples from the other two extraction types. This separation was mainly driven by the horizontal axis, which corresponds to the first component t1, which explained 60% and 59% of the total variation in the PCA and PLS-DA plots, respectively. The observed separation may suggest and confirm the changes in

polyphenolic compositions during the digestion process due to the attack of enzymes and acids. Additionally, PLS-DA also allowed for separation of the organic and non-digestion extractions. On the other hand, the second component, which explained 10% and 5% of the total variation in PCA and PLS-DA, respectively, may reflect another separation that was observed for the vertical axis in both plots. For all extraction types, this second separation allowed the CBS ingredient alone samples to be separated from the biscuit samples (separation indicated by black lines in each colored ellipse) and, in some cases, it was also possible to visualize the vertical separation of the 20%-substitution samples from the 10%-substitution samples. Again, no differences associated with the employed sweetener were observed between biscuits. All of the obtained and selected features from the untargeted approach then underwent a univariate analysis (fdr < 0.05) to understand how the different compound concentrations (log_{10}-relative intensities) changed depending on the extraction types.

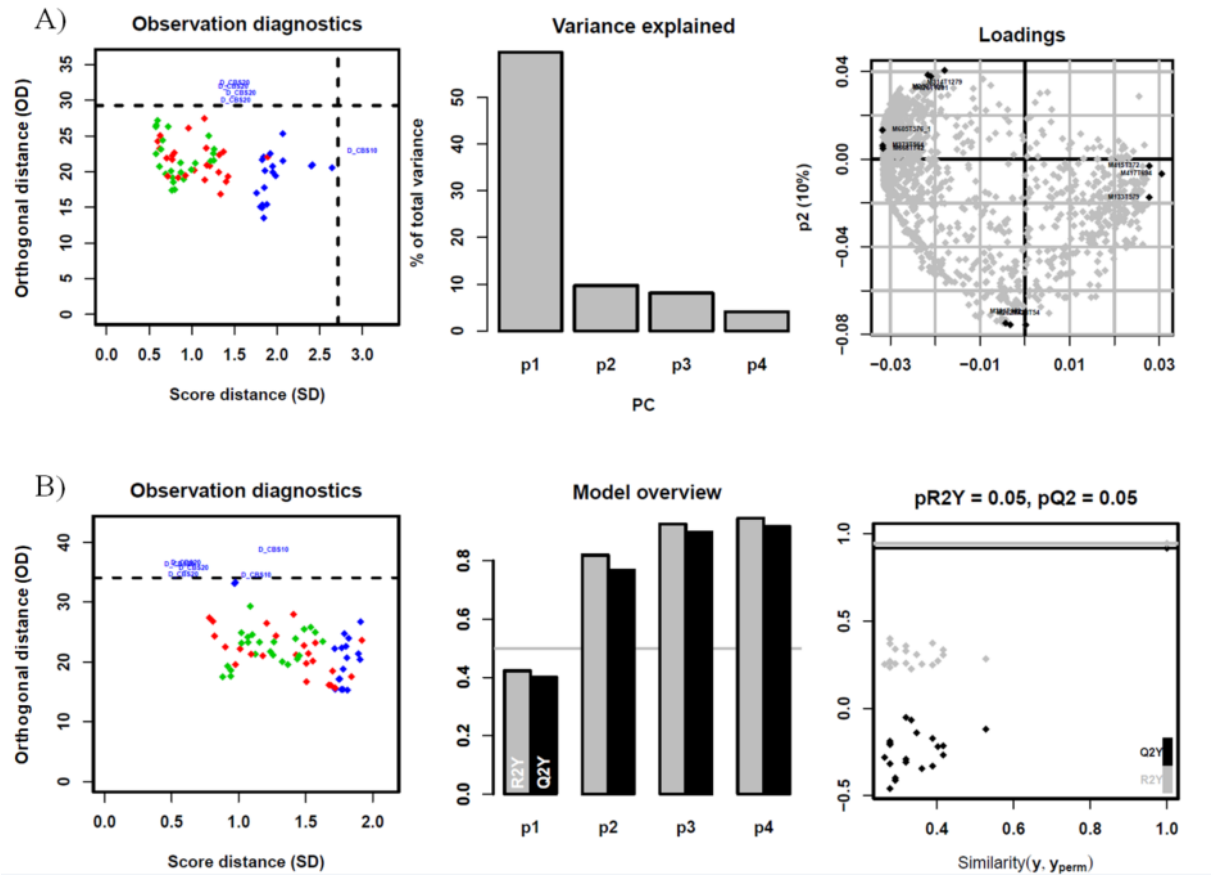

Figure 4. Supplementary information to the score plots for the multivariate modeling using PCA (**A**) or PLS-DA (**B**). The observation diagnostic plot shows the distances within and orthogonal to the selected score plane. For PCA, the variance explained by the first four principal components is shown and, on the loading plots, the names of the 6 variables with most extreme values in each direction, is indicated. For PLS modeling, an additional diagnostic plot shows the Q2Y (and R2Y) values from the model (horizontal lines) compared to the values from the models obtained after random permutations of the y response (dots) [19].

Because of their occurrence and importance as polyphenols and methylxanthines contained in CBS, five specific identified compounds were selected among all of the obtained ion boxplots: catechin, epicatechin, procyanidin B1 (PCB1), and the methylxanthines theobromine and caffeine (Figure 5).

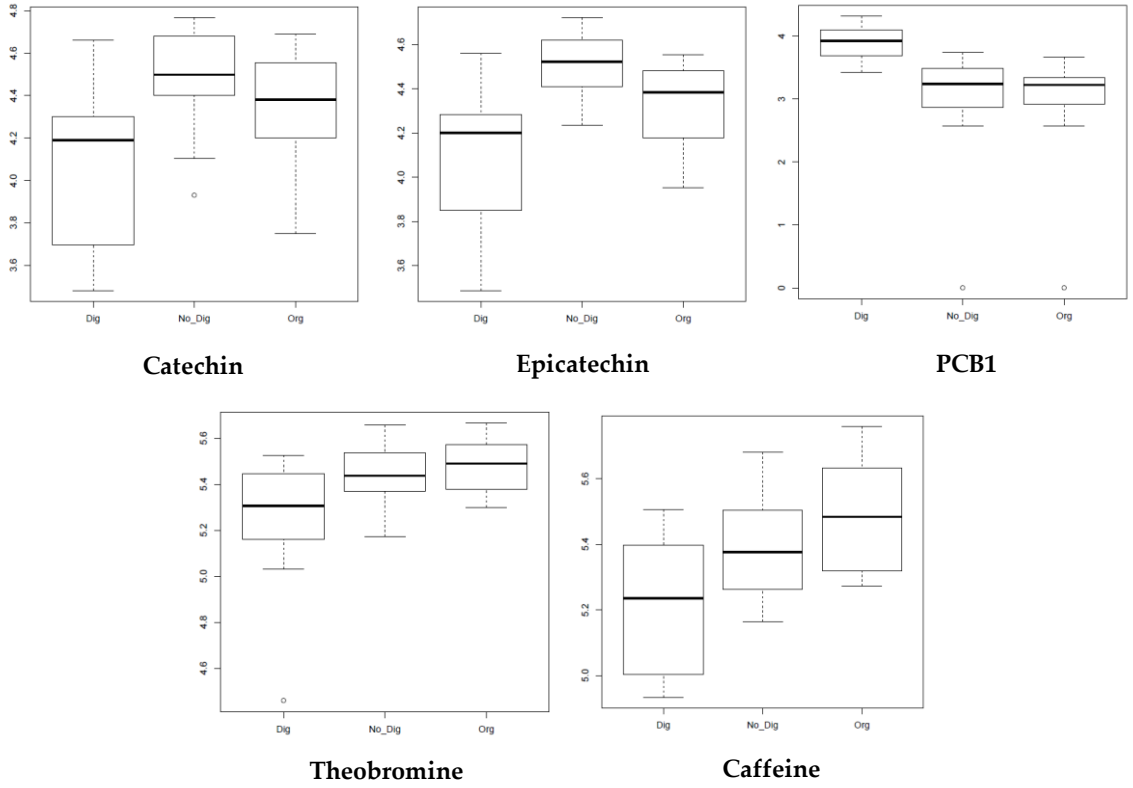

Figure 5. Boxplot of the $\log_{10}$-intensities of catechin (*m/z* = 291.1070, t = 497 s), epicatechin (*m/z* = 291.0969, t = 585 s), PCB1 (*m/z* = 579.2428, t = 378 s), theobromine (*m/z* = 181.3165, t = 371 s), and caffeine (*m/z* = 195.2854, t = 491 s) for the three types of extraction for the whole sample set (ANOVA, $p < 0.05$).

From these plots, it can be observed that the concentration of both flavan-3-ols (catechin and epicatechin) decreased after gastrointestinal digestion in comparison with that of the other extraction types. This has been previously reported by several authors and is mainly explained by the instability of these compounds in neutral or mildly alkaline environments that typify the intestinal phase of digestion. Moreover, these compounds are known to strongly interact with digestive enzymes, which leads to polymerization and aggregate formation of the flavan-3-ol monomers [309]. In different types of chocolate and cocoa beverages, the bioaccessibility of catechin and epicatechin was found to be between 77 and 79% and between 73 and 91%, respectively [369], which is in accordance with the results observed in the plots. However, in the absence of a food matrix, the degradation of these compounds could be even more notable. This was the case for different CBS-based beverages [222] and different types of tea [370] in which flavan-3-ols were degraded by up to 90% after gastrointestinal digestion. This

was explained as being due to the autooxidation of these compounds under alkaline conditions. In guarana seeds, which have been reported to have a very similar flavonoid composition to that of cocoa, the bioaccessibility of catechin monomers was found to be between 65 and 95% while that of dimers (procyanidins) ranged from 50% to up to 140% [305], similar to the values obtained in this work. According to our results, the PCB1 concentration was in fact higher for all samples after gastrointestinal digestion in comparison with the samples that underwent organic solvent extraction or non-digestion extraction, which is also in accordance with the TTC results obtained for the biscuit samples. This could be explained by the previously mentioned phenomenon in which catechin monomers suffer from polymerization during gastrointestinal digestion, increasing the concentrations of procyanidin dimers, such as PCB1, and polymers. Furthermore, this increase in PCB1 bioaccessibility could have been boosted by the presence of fats in biscuits which, according to Ortega et al. [368], would enhance the digestibility of some cocoa compounds, especially procyanidins, under the alkaline conditions of intestinal digestion. However, the lower intensities of the digested samples in the catechin and epicatechin boxplots contradict the results for the total flavonoid content, in which values increased considerably for the digested biscuit samples, while they decreased for the samples containing the CBS ingredient alone. A similar scenario was found by Pešić et al. [309], who justified the higher-than-expected TFC values by the influence of phenolic acids on the overall determination of this parameter via the employed methodology. For the two methylxanthines, theobromine and caffeine, a similar trend was found in which their intensities in the digested samples slightly decreased in comparison with the other two extractions, which had very similar intensities. Methylxanthines have been reported to be stable under gastric and intestinal conditions since they are not degraded by the pH conditions or enzymes [222].

3.5. In Vitro *Intestinal Permeability of Bioactive Compounds through Caco-2 Cell Monolayers*

After conducting the bioaccessibility studies, besides understanding how much of the bioactive compounds contained in the different samples were available for absorption after gastrointestinal digestion, we also determined the concentration that was actually able to permeate through the intestinal wall and pass into the bloodstream. This factor is of great importance since several types of biofunctionality have been attributed to the cocoa bean shell *in vitro* [218], but in order to allow for these processes to really happen in the human

organism, it is mandatory for the compounds of interest to reach the bloodstream and, therefore, the target organs.

Transport through the small intestinal epithelium cells can occur through four different pathways—transporter-mediated, transcytosis, transcellular diffusion, and paracellular transport—and can be affected by different food components and substances. In this study, we employed Caco-2 cell monolayers to study intestinal absorption. Although these cells originated in colon tissue, they are known to create epithelial monolayers and mimic several functions characteristic of the small intestine, including differentiation with microvilli and formation of junctional structures, and the expression of different enzymes [371]. To increase the accuracy and mimic the natural process, only the digested extracts were tested for their absorption through Caco-2 cells. Prior to transport studies and in order to assure the integrity of the Caco-2 cell monolayer, the cell viability was tested after 2 and 4 h of cultivation in the presence of the digested extracts. The results showed cell viability values higher than 80% for all extracts (Figure 6); therefore, it was considered that the incubation conditions employed in the absorption studies were safe for Caco-2 cells. Additionally, the TEER measurements and the Lucifer Yellow assay confirmed the integrity of the monolayer after the transport experiments.

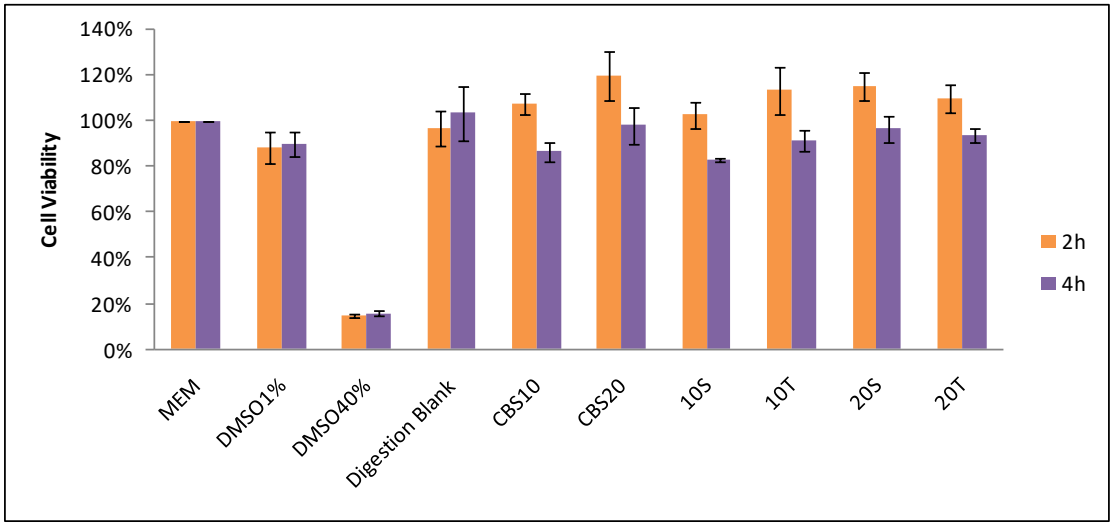

Figure 6. Cell viability in Caco-2 cells of the culture medium used as positive control (MEM), the DMSO percentage used for sample solubilization (DMSO 1%), high-DMSO concentration used as negative control (DMSO 40%), the digestion cocktail (Digestion blank), the digested CBS ingredient alone at different concentrations (CBS10 and CBS20), and the biscuits developed with sugar or tagatose and different percentages of CBS (S10, T10, S20, and T20). Results are presented as mean ± standard error of the mean (SEM) ($n \geq 5$).

Then, the transport of five representative target compounds contained in the samples (catechin, epicatechin, PCB1, caffeine, and theobromine) from the apical to the basolateral compartment of Caco-2 monolayers cultivated onto porous membranes was monitorized at T_0 (initial concentration at the AP), T_1 (concentration at the BL after 60 min), and T_2

(concentration at the BL after 120 min). The apparent permeability coefficients (P_{app}) resulting from the obtained data are shown in Table 3. In addition, the concentrations that were used for the P_{app} calculation and the absorption percentages at different times are presented in Tables 4 and 5, respectively.

Table 3. Apparent permeability coefficients (P_{app}) of the five target compounds found for the digested samples of CBS biscuits and CBS ingredient alone at different concentrations. Statistical comparisons were made using one-way ANOVA and Duncan's post-hoc test at 95% confidence.

	$P_{app} \times 10^6$ (cm/s)				
	Catechin	**Epicatechin**	**PCB1**	**Caffeine**	**Theobromine**
CBS10	30.47 ± 3.64 ^{a}	8.89 ± 0.39 ^{c}	n.d.	42.22 ± 0.91 ^{c}	41.89 ± 0.61 ^{d}
CBS20	22.83 ± 1.07 ^{bc}	4.68 ± 0.13 ^{d}	n.d.	45.00 ± 2.13 ^{c}	49.14 ± 0.48 ^{c}
S10	25.35 ± 2.84 ^{b}	15.92 ± 0.69 ^{a}	n.d.	69.53 ± 0.42 ^{a}	58.84 ± 0.58 ^{b}
S20	9.05 ± 0.60 ^{d}	3.28 ± 0.15 ^{e}	n.d.	62.46 ± 0.36 ^{b}	61.02 ± 1.21 ^{b}
T10	19.73 ± 2.10 ^{c}	11.33 ± 0.32 ^{b}	n.d.	66.66 ± 4.47 ^{a}	58.80 ± 2.35 ^{b}
T20	20.88 ± 3.10 ^{bc}	4.44 ± 0.36 ^{d}	n.d.	61.99 ± 2.67 ^{b}	65.80 ± 1.80 ^{a}

Means followed by different superindexes indicate significant differences at $p < 0.05$ for the same compound among the different samples; data are expressed as mean values (n = 3) ± standard deviations.

Concerning flavan-3-ols, the apparent permeability coefficients for catechin and epicatechin ranged between 3.28 and 30.47 × 10^{-6} cm/s. These values are slightly high compared with other coefficients found in the literature. Deprez et al. [372] found an apparent permeability for catechin of 0.8 × 10^{-6} cm/s, while Fang et al. [373] found variable apparent permeabilities ranging from 0.29 to 33.90 × 10^{-6} cm/s for different flavonoids. Kosińska et al. found that, in purified extracts, catechin and epicatechin showed P_{app} values of 4.19 × 10^{-6} cm/s and 3.38 × 10^{-6} cm/s, respectively. Nevertheless, as stated by Mendes et al. [305], the considerable variability of catechin P_{app} coefficients reported in the literature can be explained by the complex phenotype of Caco-2 cells, which features different TEER values and levels of efflux transporter expression depending on the source and culture conditions. Furthermore, the paracellular permeability can vary significantly depending on the calcium concentration in the digestion cocktail, since it is believed that calcium ions contribute to the integrity of tight junctions and that their removal could lead to the enlargement of the intercellular spaces, with a consequent increase in P_{app} [372]. Indeed, several authors have hypothesized that paracellular transport is the preferential mechanism of intestinal absorption for polyphenols because of their high hydrophilicity and abundance of hydroxyl groups [356,374]. However, in studies where P_{app} values were lower, compound standards or purified extracts were tested. Indeed, it has been demonstrated that the different substances and compounds included in the food matrices can significantly increase the apparent permeability of flavan-3-ols. Some food components, such as proteins, are believed to protect catechins against oxidation by the culture medium during incubation on the apical side [374], while other components, such as capric or lauric acid, which have been reported to be present in CBS [218], could contribute to

increased paracellular intestinal permeability due to tight junction opening [374]. In addition, it has been suggested that the presence of other cocoa polyphenols that may not be absorbed could affect tight junction functioning, enlarging the paracellular space [356], or inhibit intestinal ABC transporters (p-glycoprotein, MRP2, BCRP) that are responsible for bioactive compound efflux from the basolateral to the apical side [375]. Indeed, authors who tested flavanol standard solution absorption stated that the apparent permeability was low because basolateral-apical reflux was considerable [376]. Furthermore, several studies support the idea that co-transport of catechins with other components such as glycine and serine (both present in CBS as free aminoacids at percentages of 0.00973% and 0.01050%, respectively [25]) can enhance catechin absorption by regulating efflux transporters [377]. Moreover, theobromine and caffeine, which were present in considerable concentrations in our samples, have been demonstrated to increase the *in vivo* absorption of epicatechins since they are phosphodiesterase inhibitors that can prevent the inactivation of cAMP and cGMP, which are supposed to regulate MRP2 sorting [378]. All of these reasons could explain the uncommonly high values of P_{app} for catechin and epicatechin in our samples when compared with values reported by other studies where the target compounds were studied alone.

Considering the obtained catechin and epicatechin P_{app} coefficients, significant differences were found between samples, even between those with similar CBS percentages. This could be because, while the initial concentrations at T_0 (Table 4) were in accordance with what was observed previously (generally higher for 20%-substituted samples and higher in digested biscuits compared with samples of the digested CBS ingredient alone), concentrations at T_1 and T_2 were similar for all samples. This led to very different P_{app} values and, unexpectedly, the samples with the highest CBS percentages gave the lowest P_{app} values. It was then hypothesized that, for these compounds, a maximum absorption rate in Caco-2 cells might occur, leading to an absorption plateau in which higher concentrations at T_0 were not correlated with higher absorption rates. Thus, there was a limit to the extent to which these two flavan-3-ols could be absorbed. This absorption limit has been previously reported *in vivo* by other researchers for other flavan-3-ols such as (epi)gallocatechin [379]. Similarly, Gómez-Juaristi et al. [380] found that the availability of flavanols after the intake of different cocoa products was limited to 35% regardless of the initial dose. Nevertheless, the percentages of permeability obtained (Table 5) were in accordance with 2–15% catechin absorption for different types of tea [370], and those observed after 120 min were close to 25.3–29.8% absorption of epicatechin from chocolate and cocoa in human subjects, as reported by Baba et al. [381].

Table 4. Concentrations at the initial time (T_0, apical side) and after 60 and 120 minutes (T_1 and T_2, basolateral side) of the five target compounds for the digested samples of the CBS ingredient alone and biscuits with different CBS-added concentrations.

	Concentration T_0 (μg/g)				
	Catechin	Epicatechin	PCB1	Caffeine	Theobromine
CBS10	0.39 ± 0.03	0.89 ± 0.04	0.46 ± 0.10	48.10 ± 0.95	129.69 ± 3.48
CBS20	0.54 ± 0.03	1.72 ± 0.05	0.68 ± 0.14	82.84 ± 2.73	198.53 ± 2.79
S10	0.48 ± 0.09	0.50 ± 0.05	0.34 ± 0.06	35.67 ± 0.88	121.81 ± 2.58
S20	1.26 ± 0.09	2.23 ± 0.09	0.89 ± 0.07	65.76 ± 0.40	180.92 ± 3.80
T10	0.56 ± 0.05	0.63 ± 0.02	0.38 ± 0.15	40.83 ± 1.14	147.18 ± 1.34
T20	0.57 ± 0.07	1.72 ± 0.06	0.38 ± 0.09	83.14 ± 1.16	203.16 ± 1.21
	Concentration T_1 (μg/g)				
	Catechin	Epicatechin	PCB1	Caffeine	Theobromine
CBS10	0.08 ± 0.00	0.05 ± 0.00	n.d.	15.59 ± 0.37	31.64 ± 2.61
CBS20	0.08 ± 0.01	0.06 ± 0.00	n.d.	29.36 ± 1.13	58.24 ± 2.40
S10	0.09 ± 0.01	0.06 ± 0.01	n.d.	17.45 ± 0.57	38.84 ± 2.42
S20	0.08 ± 0.00	0.05 ± 0.00	n.d.	30.10 ± 0.90	61.41 ± 4.83
T10	0.08 ± 0.00	0.05 ± 0.00	n.d.	19.61 ± 1.27	47.41 ± 5.36
T20	0.08 ± 0.01	0.05 ± 0.01	n.d.	38.00 ± 0.50	74.85 ± 5.03
	Concentration T_2 (μg/g)				
	Catechin	Epicatechin	PCB1	Caffeine	Theobromine
CBS10	0.14 ± 0.00	0.08 ± 0.00	n.d.	26.73 ± 0.23	54.24 ± 1.03
CBS20	0.14 ± 0.00	0.10 ± 0.00	n.d.	50.33 ± 0.61	99.84 ± 2.23
S10	0.14 ± 0.01	0.10 ± 0.01	n.d.	29.91 ± 0.55	66.58 ± 2.53
S20	0.13 ± 0.00	0.09 ± 0.00	n.d.	51.60 ± 0.53	105.27 ± 0.71
T10	0.13 ± 0.00	0.08 ± 0.00	n.d.	33.61 ± 1.43	81.27 ± 4.36
T20	0.14 ± 0.01	0.09 ± 0.00	n.d.	65.14 ± 2.10	128.31 ± 4.50

PCB1 was not detected on the basolateral side during our experiments, although it was present in detectable concentrations in the apical side. It is, therefore, concluded that this catechin–epicatechin dimer was not able to pass through the intestinal barrier, probably due to its bigger size and higher hydrophilicity. This has been frequently reported in the literature, although some authors have detected procyanidins at the BL after 5 h of incubation [305]. However, it is well known that procyanidins can be absorbed and accumulate in Caco-2 cells thanks to their affinity with the intestinal mucosa and luminal proteins. These findings were also observed for catechin monomers, although to a lesser extent [372]. Nevertheless, their accumulation and presence in the intestinal tract is of great importance, since these compounds can act at a local level to protect the intestinal mucosa against different types of damage by acting as antioxidant agents and exerting anti-inflammatory activities, as already reported by Rossin et al. [78]. In this way, they can decrease cancer risk, control cholesterol uptake, and inhibit glucose degradation enzymes [218].

Table 5. Percentage of permeated compound on the basolateral side after 60 and 120 minutes (T1 and T2, basolateral side) in relation to the concentration at T0 on the apical side of the five target compounds for the digested samples of the CBS ingredient alone and biscuits with different CBS–added concentrations.

		Permeability (%)				
		Catechin	Epicatechin	PCB1	Caffeine	Theobromine
CBS10	T1	19.82 ± 1.86	5.54 ± 0.29	n.d.	32.43 ± 1.27	24.37 ± 1.38
	T2	35.45 ± 4.23	10.34 ± 0.45	n.d.	49.12 ± 1.06	48.73 ± 0.71
CBS20	T1	15.35 ± 0.60	3.24 ± 0.09	n.d.	35.48 ± 2.12	29.33 ± 0.87
	T2	26.55 ± 1.25	5.44 ± 0.15	n.d.	52.35 ± 2.48	57.17 ± 0.56
S10	T1	18.08 ± 4.12	11.35 ± 1.80	n.d.	48.91 ± 0.90	31.87 ± 1.31
	T2	29.49 ± 3.30	18.52 ± 0.81	n.d.	80.89 ± 0.49	68.45 ± 0.68
S20	T1	6.33 ± 0.54	2.43 ± 0.28	n.d.	45.77 ± 1.34	33.92 ± 1.97
	T2	10.53 ± 0.70	3.81 ± 0.18	n.d.	72.66 ± 0.41	70.98 ± 1.41
T10	T1	13.70 ± 1.20	7.81 ± 0.28	n.d.	48.08 ± 3.91	32.21 ± 3.61
	T2	22.96 ± 2.44	13.18 ± 0.38	n.d.	77.54 ± 5.20	68.40 ± 2.73
T20	T1	14.52 ± 1.09	3.13 ± 0.44	n.d.	45.72 ± 1.25	36.83 ± 2.26
	T2	24.29 ± 3.60	5.16 ± 0.42	n.d.	72.11 ± 3.11	76.55 ± 2.09

Contrary to polyphenols, methylxanthines are believed to be absorbed by transcellular transport mediated through passive diffusion since their transport is linear over time, independent of transport direction and pH, and they have non-saturable (first-order) kinetics and high P_{app} coefficients [382]. In our study, both methylxanthines showed high P_{app} coefficients, which correlated well with the different samples. Significantly higher values were found for the P_{app} values of both caffeine and theobromine in the biscuit samples when compared with the samples containing the CBS ingredient alone. There was, therefore, an effect of the biscuit food matrix that led to increases in the absorption of both caffeine and theobromine through the Caco-2 cell monolayer. Methylxanthines could have bound to some biscuit food matrix proteins, which facilitated their passage through the cells. Monente et al. [383] found that 36.5% of the caffeine from digested spent coffee ground extracts was absorbed through Caco-2 cells after one hour of incubation, which is in accordance with the values of 32.43% and 35.48% obtained for CBS10 and CBS20 (Table 5) but lower than the values obtained for the biscuits after 1 h of incubation (45.77–48.91%) where the biscuit food matrix was present, in contrast with samples containing the CBS ingredient alone or the spent coffee ground extract. Regarding the P_{app} values obtained for caffeine (42.22–69.53 × 10^6 cm/s), these are in accordance with those reported in other research articles, such as 46.3–53.5 × 10^6 cm/s found by Smetanova et al. [382] for caffeine standards or 44.8 × 10^6 cm/s found for guarana seeds [305]. Taking into account the fact that the U.S. Food and Drug Administration (FDA) has suggested that caffeine is a model drug for *in vitro* permeability

assay validation [377], these values could also serve as evidence for the validation of the P_{app} values obtained for the other target compounds.

Concerning methylxanthines in cocoa, theobromine in particular, the information in the literature about *in vitro* absorption is limited. However, *in vivo* studies have demonstrated that cocoa methylxanthines are highly bioavailable, with maximum absorption after 30–60 min for caffeine and 2–2.5 h for theobromine [384,385]. Our results agree with these data, since higher permeability percentages were found for caffeine in comparison with theobromine at times 1 and 2, suggesting a faster absorption rate. On the other hand, theobromine absorption percentage values seemed to be still increasing after 2 h of incubation, which suggests that the absorption peak had not been reached. Indeed, theobromine was the only studied compound where values at T_2 were found to be double those at T_1 (T_2 values were 1.94–2.12-fold greater than T_1 values for theobromine, while they were 1.63–1.78, 1.56–1.86, and 1.47–1.65-fold greater for catechin, epicatechin, and caffeine, respectively).

4. Conclusions

The present study, which is a continuation of a previous one [221], highlights the potential of using CBS as a functional ingredient for high-fiber functional biscuits, with the possibility of adapting this product for diabetic consumers through the addition of tagatose. It was demonstrated that the substitution of sucrose with tagatose as a sweetener did not affect the bioavailability of bioactive compounds contained in the CBS-based biscuits. The results highlight the importance of considering the food matrix when studying polyphenol bioaccessibility. In this study, it was observed that, although the food matrix could retain bioactive compounds by binding them with the food components, it also has a protection effect, preventing bioactive compounds from degradation during gastrointestinal digestion. Furthermore, a compound liberation effect was observed during *in vitro* digestion of the biscuits, which increased the bioaccessibility of the bioactive compound and gave a considerable α-glucosidase inhibition capacity, which confirms their anti-diabetic potential. CBS bioactive compounds have been shown to permeate the intestinal barrier after *in vitro* digestion of functional biscuits. The exception is the PCB1, which can still exert different functionalities at the local intestine level. The absorption of CBS methylxanthines was high, it correlated well with the results of previously reported *in vivo* studies, and it seemed to be boosted by the food matrix presence. To our knowledge, this is the first study to report the *in vitro* absorption of CBS methylxanthines. CBS has been proven to be an optimal revalorized ingredient for functional biscuits, and its biofunctional potential has been demonstrated.

PART 3 – CBS APPLICATIONS WITH PHARMACEUTICAL APPROACH

Part 3 is composed of one single chapter (**Chapter 3.1**) issued from one scientific article. This part of the results was conceived in order to give CBS revalorization a different approach in which the by-product is not used as a food ingredient, but proposed as a possible food additive or as a pharmaceutical product. The previous obtained knowledge about CBS composition led to propose its use as an antimicrobial agent. The antimicrobial potential of CBS was assessed against different bacteria and fungi strains, and a metabolomic strategy was applied in order to individualize the putative active compounds against the *Streptococcus mutans* proliferation.

CHAPTER 3.1

Evaluation of Cocoa Bean Shell Antimicrobial Activity with a Metabolomic Approach for Tentative Active Compound Identification

Chapter 3.1 is devoted to the study of CBS with a more pharmaceutical approach. In the present chapter, different CBS extracts are studied for their antimicrobial potential, and a metabolomic strategy is applied in order to individualize the putative active compounds against the *Streptococcus mutans* proliferation.

This chapter was issued and adapted from a scientific article:

Evaluation of Cocoa Bean Shell Antimicrobial Activity with a Metabolomic Approach for Tentative Active Compound Identification

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1. Introduction

Plants have been used for their healing potential since ancient times. They have been used in folk medicine even before humans were aware of the existence of microbes and their disease-causing potential. Today, it is known that this antimicrobial potential is derived from secondary metabolites contained in the plants [386,387]. Recently, antibacterial resistance has become a major problem leading to the focus of research on new molecules with antimicrobial potential [388]. To this end, compounds from plant materials have attracted the attention of most researchers in the last several years, and novel vegetal molecules such as polyphenols or essential oils with strong antimicrobial potential have been identified [171,389]. Special attention has been paid to different plant-based extracts. The complexity of these mixtures of molecules and their ability to act synergistically provides them with antibacterial properties that are, in many cases, close to those of the active isolated molecules [175]. This, together with the increasing issue of food spoilage in modern societies, increases the importance of the study and revalorisation of vegetal by-products as high-value-added sources of bioactive compounds [175,390].

In the cocoa industry, one of the main by-products of the chocolate manufacturing process is the cocoa bean shell (CBS), which represents approximately 10%–17% of the total cocoa bean weight and is normally discarded after roasting and husking of the beans [218]. CBS has been reported to be rich in bioactive compounds such as polyphenols and methylxanthines. Hence, it has been largely proposed as a nutraceutical for the development of functional foods and beverages [13,125,222]. Moreover, the possibility of pharmacological application of CBS has been explored. Several biofunctionalities, such as antidiabetic, anticarcinogenic, antiviral, anti-inflammatory, and most importantly, antibacterial properties, have been demonstrated for this by-product. Indeed, the antimicrobial activity of CBS extracts against different pathogens, mostly oral pathogens, has been reported recently [39,176,179]. However, the mechanism of action and the molecular components in the CBS extracts responsible for these activities remain unclear. To this day, polyphenols have been proposed to be the compounds responsible for this activity [171,391].

As CBS has already been proposed to be a low-cost functional ingredient, this study aimed to test different unrelated CBS sample extracts with known polyphenolic contents against various food-borne bacterial and fungal strains. The microdilution method was employed to determine the minimum inhibitory concentration (MIC), and an untargeted metabolomic approach was used to identify the possible active molecules in the CBS extracts

showing MICs lower than a pharmacologically established interesting threshold (≤500 µg/mL) [386,391]. For the first time, different types of CBS sample extracts were tested for their antimicrobial activity, and their differential compositions were analysed by metabolomics. This approach allowed us to consider the chemical variability presented by the CBS samples due to different biotic and abiotic factors such as plant variety or geographical origin.

2. Materials and Methods

2.1. Chemicals, media, and microorganisms

Dimethyl sulfoxide (DMSO; 99.9%), bacterial and yeast growth media (tryptic soy, brain heart infusion, and yeast extract-peptone-dextrose agars and broths), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (+)-catechin hydrate (>98%), epicatechin (>98%), caffeine (≥99%), 7-methylxanthine (≥98%), MS-quality formic acid, and MS-quality methanol were obtained from Sigma-Aldrich.

The 0.85% NaCl physiological solution (2 mL) was obtained from BioMérieux.

Amoxicillin, penicillin V, penicillin G, and theobromine (99%) were provided by Alfa Aesar.

Ethanol (99%) was purchased from VWR chemicals.

Ultrapure water was prepared in a Simplicity UV water purification system (Millipore).

Bacterial and fungal strains were purchased from the Belgian Coordinated Collection of Microorganisms (BCCM, Ghent, Belgium). Details concerning the employed strains, the culture media used, and antibiotics used as positive controls are shown in Table 1.

Table 1. Microorganism used for the MIC assays with CBS extracts.

Microorganism type	Strain	BCCM code	ATCC code	Antibiotic resistance	Culture medium	Antibiotic for positive control
Bacteria gram +	*Staphylococcus aureus* (MSSA)	LMG 8064	6538	Sensitive	TS broth/agar	AMX, PENG, PENV
	Staphylococcus aureus (MRSA)	LMG 15975	43300	Resistant	TS broth/agar	AMX, PENG, PENV
	Streptococcus mutans	LMG 14558	25175	n/a	BHI broth/agar	AMX, PENG, PENV
Bacteria gram-	*Escherichia coli*	LMG 8223	25922	Sensitive	TS broth/agar	AMX, PENG, PENV
	Escherichia coli	LMG 15862	35218	Resistant	TS broth/agar	AMX, PENG, PENV
	Pseudomonas aeruginosa	LMG 6395	27853	Resistant	TS broth/agar	AMX, PENG, PENV
Yeasts	*Candida albicans*	IHEM 9559	90028	n/a	YPD broth/agar	FLC, NYS
	Saccharomyces cerevisiae	MUCL 53497	n/a	n/a	YPD broth/agar	/

2.2. Plant material

Twelve different CBS samples procured from fermented, dried, and roasted cocoa beans of different geographical origins and varieties, purchased from several local cocoa companies, were selected from the 44-sample set employed by Barbosa-Pereira et al. [29,66] (detailed in Chapter 1.1). The sample selection was based on the polyphenolic content previously determined by RP-HPLC-PDA analysis (Chapter 1.1). The total quantities obtained for the sum of all the detected and quantified polyphenols as well as their origins and varieties are shown in Table 2. Ten samples (samples 1–4, 6–10, and 12) were chosen as they contained the highest determined polyphenol contents in the sample set, while the other two (numbers 5 and 11) were chosen for possessing the lowest ones.

Table 2. Geographical origin, variety and polyphenolic content (sum of the total quantified polyphenols, µg/g CBS) of the cocoa bean shells (yielded from fermented and dried cocoa beans) used for the extractions.

Sample code	Variety	Continent	Country	∑ Total polyphenols (µg/g CBS)
1	*Forastero*	America	Colombia	1726.04
2	*Trinitario*	America	Colombia	3199.42
3	*Trinitario*	America	Dominican Republic	3200.76
4	*Forastero*	America	Ecuador	2772.67
5	*Forastero*	Africa	Ivory Coast	423.75
6	*Forastero*	Africa	Madagascar	2926.69
7	*Trinitario*	America	Mexico	3395.40
8	*Forastero*	America	Peru	1828.34
9	*Trinitario*	America	Peru	2131.81
10	*Forastero*	Africa	São Tomé	2879.56
11	*Forastero*	Africa	Uganda	475.11
12	*Trinitario*	America	Venezuela	2101.42

2.3. CBS extract preparation

Extractions were performed by adding CBS powder (0.5 g) to 10 mL of an ethanol-water mixture (50:50, v/v). The extraction was performed at room temperature (25 °C) with constant rotatory oscillation for 2h in an SM 25B-SWIP laboratory shaker (Edmund Buhler, Germany). Samples were then centrifuged at 4200 ×*g* for 10 min in a 4226 ROTOFIX Centrifuge (Hettich, Germany), and the supernatants were collected and filtered through a 0.22-µm PTFE filter. The ethanol from the extracts was evaporated for 10 min in a rotavapor (Pleuger Büchi, Germany), and water was eliminated using an Epsilon 1–6 freeze dryer (Christ, Germany).

2.4. MIC assays

MIC studies were performed following the M07-A10 standardised protocol described in the Clinical and Laboratory Standards Institute guidelines [392] by employing the broth microdilution technique in 96-well plates, adapted as described previously [389]. Briefly, CBS extract stock solutions of 1 mg/mL were prepared in DMSO (3%) and the corresponding broth medium. The stock solutions were serially diluted two-fold to obtain a final concentration range of 500–3.91 μg/mL. Antibiotic stock solutions were used as positive controls and were prepared similarly with an initial concentration of 128 μg/mL, and a final concentration gradient ranging between 64 μg/mL and 1 μg/mL was obtained. For the compound standards (catechin, epicatechin, theobromine, caffeine, and 7-methylxanthine), an initial stock concentration of 8 mg/mL was used, and the concentration gradient was 4000–31.25 μg/mL. A bacterial suspension of 24-h-old colonies prepared in 0.85% NaCl physiological solution with 0.5 McFarland-equivalent turbidity was placed in each well with a final concentration of approximately 5×10^4 CFU/well and then incubated overnight at 37°C. Additionally, growth and non-growth controls were prepared. After 24h of incubation, the MIC was observed with the naked eye and 30 μL of MTT (0.8 mg/mL) was used for staining when needed. As described in Chapter 2.4, the MTT allows to observe viable cells after the analyses. All experiments were performed in triplicates. MIC values were taken as the lowest extract concentrations that produced no visible bacterial growth after incubation.

2.5. LC-HRMS and metabolomic study

A methanolic resuspension of the sample extracts (1 mg/mL) was used for the LC-HRMS analyses. Analyses for the untargeted metabolomic study were performed using a 1200 series Rapid Resolution LC system (RRLC, Agilent Technologies, USA) coupled to a 6520 series electrospray ionization (ESI) source with a quadrupole time-of-flight (Q-TOF) mass spectrometer from Agilent Technologies. The separation was performed using an InfinityLab Poroshell 120 EC-C18 column (2.1 × 100 mm, particle size 2.7 μm, Agilent Technologies) with a guard column (2.1 × 5 mm; Agilent Technologies). Water acidified with 0.1% formic acid (solvent A) and methanol acidified with 0.1% formic acid (solvent B) served as the mobile phases. The separation method and ESI-QTOF settings for data acquisition (positive mode) were performed as described previously [246]. Data acquisition and analysis were carried out using the B.04 SP3 version of the MassHunter Acquisition® software for QTOF and the B.08 version of MassHunter Qualitative Analysis® software (Agilent Technologies),

respectively. Samples were randomly analysed in triplicates in a single batch. The same quality control (QC = pool) sample for all samples (mix of all samples) was injected throughout the run after every 10 samples for control; blanks (methanol) were also injected throughout the run.

The obtained Agilent .d format data were converted into .mzXML format using the ProteoWizard MSConvert software (version 3.03.9393, 64-bit) with the Peak Picking filter option. Pre-processing (peak detection, identification, grouping and smoothing, retention time correction, filtration, and integration) and quality control (metabolite correlation analysis) were conducted using the freely available Galaxy workflow4metabolomics W4M platform (http://workflow4metabolomics.org, [352]). The parameters employed during the workflow were identical to those used in a previous study [353]. The W4M platform was used for statistical analysis. The pre-processed data were subjected to multivariate modelling (PCA and PLS-DA) and univariate testing. Feature selection was performed by using the Biosigner algorithm [393], which is a molecule signature discovery tool implemented in the W4M platform.

3. Results and Discussion

3.1. MICs of the CBS extracts against different microbial strains

Twelve extracts of CBS yielded from different cocoa beans of diverse origins and varieties and with variable and known polyphenolic content (Table 2) were tested against different bacterial and fungal strains (Table 1). The MICs of the different antibiotics used as positive controls against the eight fungal and bacterial strains are shown in Table 3. CBS extracts were tested at concentrations between 3.91 and 500 µg/mL; the latter (500 µg/mL) was the threshold concentration for considering a plant extract as 'pharmacologically active' [386,391]. Consequently, CBS extract concentrations higher than 500 µg/mL were not interesting from a pharmacological point of view and were not considered.

Table 3. MIC values (µg/mL) for all the tested microorganisms against the different antibiotics used as positive controls (n = 6).

Antibiotic	*Staphylococcus aureus* LMG 8064	*Staphylococcus aureus* LMG 15975	*Streptococcus mutans* LMG 14558	*Escherichia coli* LMG 8223	*Escherichia coli* LMG 15862	*Pseudomonas aeruginosa* LMG 6395	*Candida albicans* IHEM 9559	*Saccharomyces cerevisiae* MUCL 53497
	Sensitive strain	Resistant strain	n/a	Sensitive strain	Resistant strain	Resistant strain	n/a	n/a
AMX	0.03	8	<0.5	8	>64	>64	/	/
PEN V	0.015	4	<0.5	>64	>64	>64	/	/
PEN G	0.007	4	<0.5	32	> 64	> 64	/	/
FLC	/	/	/	/	/	/	2	/
NYS	/	/	/	/	/	/	2	/

For most strains tested in this study, no inhibition was found below 500 µg/mL (MSSA, MRSA, resistant and sensitive *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Candida albicans*). Some researchers have previously reported activity from few cocoa-related products such as cocoa powder, cocoa pods, or even CBS extracts against these strains. However, in most cases, this activity was found at higher concentrations than those considered in this study. Pectin extracts from cocoa pods showed MICs of 1.0, 1.0, and 2.0 mg/mL against *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively [394], while other researchers found a MIC of 5.0 mg/mL of fermented cocoa pod extracts against *P. aeruginosa* [395]. However, Todorovic et al. [396] found a MIC of 5 mg/mL against *C. albicans* and MICs between 12 and 20 mg/mL against *E. coli*, *S. aureus*, and *P. aeruginosa* for cocoa powder extracts. These concentrations decreased significantly (up to 8.5–11.5 mg/mL) when alkalised cocoa powder samples were used. Therefore, they associated this activity with some electronegative flavour-related compounds such as aldehydes and ketones whose concentrations increased after alkalisation. Using CBS acetone extracts, Nsor-Atindana et al. [39] found MICs of 0.468 and 0.937 mg/mL against *E. coli* and *S. aureus*, respectively. However, CBS ethanol extracts similar to those prepared in our studies showed MICs of 1.875 mg/mL for both bacteria. Nonetheless, it was stated that those activities were not correlated to the phenolic levels found in the CBS extracts. In all cases, active concentrations were higher than the threshold of interest (0.5 mg/mL) considered in this study.

In *S. cerevisiae*, inhibition of this desirable microorganism was not detected at the tested concentrations. Nonetheless, an increased growth was observed with increasing CBS concentrations (Figure 1). Thus, CBS extracts were not harmful to the yeast, but rather the opposite was true. These results were consistent with the presence of *S. cerevisiae* in the naturally occurring fermentation of cocoa beans [397]. Indeed, during the CBS extraction process with an ethanol/water mixture, the sugars contained in CBS could also have been extracted. These sugars may serve as nutrients for the yeast. Moreover, the confirmation that this yeast was not inhibited by the CBS components was important from a food technology point of view since the valorisation of this by-product as a cocoa powder substitute in baked goods employing yeast has already been reported [115,221].

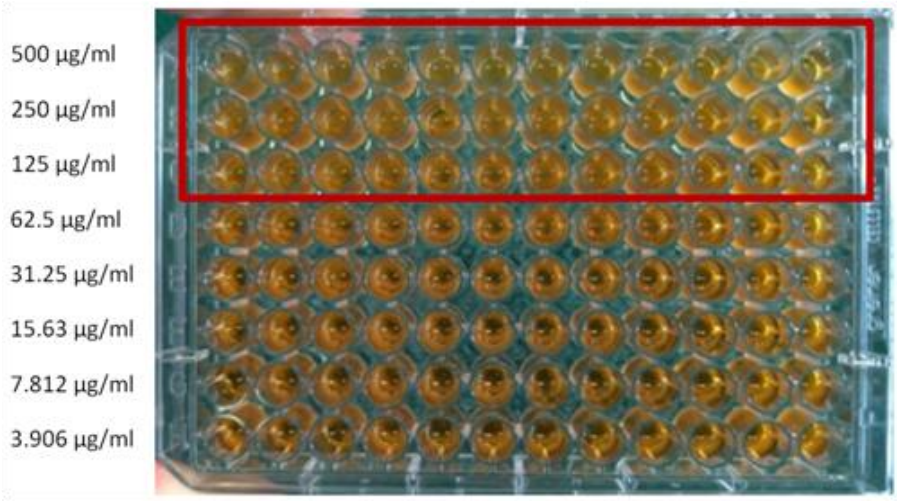


Figure 1. MIC assay of *S. cerevisiae* showing more fungal growth (identified as turbidity, highlighted by the red square) when more CBS extract is present (gradient from top to bottom). Dark areas can be distinguished in less concentrated wells showing less yeast growth.

Regarding the gram-positive bacteria *Streptococcus mutans*, 11 out of the 12 different CBS sample extracts displayed MICs of 500 µg/mL or less (Table 4). Therefore, they were considered of pharmacological interest and further studied for the tentative identification of

Table 4. MIC values (µg/mL) for *S. mutans* against the different CBS extracts and five standards of CBS-contained compounds (catechin, epicatechin, theobromine, caffeine, and 7-methylxanthine) (n = 6). In bold, the most promising results for the extracts considered as 'active' in the metabolomic analysis.

Sample code	*Streptococcus mutans* LMG 14558
1	500
2	500
3	500
4	500
5	>500
6	500
7	500
8	500
9	**125**
10	500
11	**62.5**
12	**125**
Catechin	4000
Epicatechin	>4000
Theobromine	>4000
Caffeine	4000
7-methylxanthine	>4000

their active components. *Streptococcus mutans* is a well-known bacterial strain associated with dental caries [398]. Several studies have already reported the anticariogenic effects of CBS at concentrations of 1 mg/mL [176]. Some researchers have even proposed its use in oral care as a mouthwash for caries prevention with an efficacy significantly similar to that of the chlorhexidine mouthwashes [218]. The MICs of extracts from fermented and unfermented cocoa beans and cocoa liquor against *S. mutans* have already been reported (8, 4, and 8 mg/mL, respectively) [399]. However, although the cariostatic effects of CBS have been reported, there is no specific information in literature about its *in vitro* MIC. To our knowledge, this is the first study in which these particular values for this cocoa by-product have been reported.

3.2 Identification of potentially active compounds in CBS against S. mutans using liquid chromatography–high-resolution mass spectrometry (LC-HRMS) and metabolomic analysis

Amongst all the microbial strains tested, considerable activity was found only against the cariogenic bacterial strain *S. mutans*. Indeed, all the tested CBS samples, except for extract number 5, had MICs ≤500 µg/mL (Table 4). Notably, three extracts were more effective than the others were. Extracts 9, 11, and 12 showed MICs of 125, 62.5, and 125 µg/mL, respectively (Figure 2). Thus, these three extracts were compared to evaluate the differences in their composition and that of the remaining samples for identification of the active compounds. To achieve this, all CBS extracts were analysed through LC-HRMS, and the obtained data were treated in the Workflow4Metabolomics (W4M) platform using an untargeted metabolomic approach. Extracts numbered 9, 11, and 12 were considered ‘active’ and the remaining samples were considered ‘not active’ while performing different statistical analyses. Two types of multivariate analyses were applied to the data: principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (Figure 3). Supplementary information on these plots is provided in Figure 4. PCA, which is an unsupervised analysis, did not allow for sample separation depending on the different activities against *S. mutans*. The PLS-DA, which is a supervised analysis that builds the model with the help of the provided data, allowed instead for a clear separation of the two groups of samples. Besides, univariate analyses (FDR < 0.05) were performed to understand how the concentration of some molecules of interest varied depending on the different CBS samples.

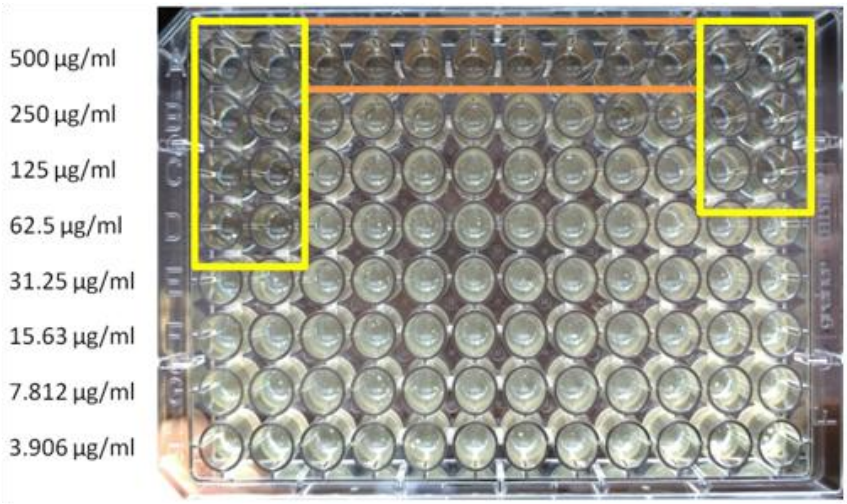


Figure 2. MIC assay conducted with S. mutans. Clear wells, marked in yellow and showing no turbidity, indicate the MICs obtained for extracts 11 (at left) and 12 (at right) that were considered 'active' during the metabolomic analysis (62.5 and 125 µg/mL, respectively). The upper clear wells, highlighted in orange, display MICs of 500 µg/mL for the other CBS extracts considered as 'not active' in the metabolomic analysis.

Independent studies describing the antibacterial activity of CBS against different strains have attributed it to the polyphenolic content [171,390,400]. It has been claimed that some polyphenols, such as those found in tea, can inhibit acid production by *S. mutans*, which is the main cariogenic cause of this strain when adhered to dental surfaces [401]. Additionally, several authors have reported the possible antibacterial activity of catechin and epicatechin, contained in cocoa and CBS, by interaction with the bacterial cell membranes. The negative charge makes them bind strongly to the positively charged lipid bilayer of the gram-positive bacteria [396]. Moreover, these molecules are believed to undergo oxidative polymerisation and form molecules that could further polymerise with proteins and sugars. This produces high molecular weight compounds that have been reported to inhibit the glucosyltransferases (GTFs) of *S. mutans* [179,402,403]. Indeed, GTFs are the most significant virulence factors of *S. mutans* and are highly involved in its pathogenicity. They are responsible for the sucrose-dependent synthesis of water-insoluble glucans that ultimately lead to bacterial adhesion on the tooth surface. They form a cariogenic dental plaque biofilm and further cause the demineralisation of the surface by the adhered bacteria metabolic acid production [398,404,405]. However, in this study, we found that the antibacterial activity of CBS against *S. mutans* was not directly related to the total content of polyphenols. Indeed, CBS extract number 11, which showed the highest activity against *S. mutans*, was one of the samples chosen for the comparative analysis because it had the lowest amount of polyphenols amongst the total samples (Table 2). Sample 5 chosen under the same criterion showed instead the highest MIC (lowest activity). Surprisingly, while performing univariate tests in our study,

both catechin and epicatechin concentrations were found to be significantly lower in the most active extracts (Figure 5a and 5b). Further, standards were tested against *S. mutans* using concentrations (31.25–4000 µg/mL) greater than those of pharmacological interest to assess their possible contribution to the antibacterial activity. Catechin and epicatechin showed MICs against *S. mutans* at concentrations of 4 and > 4 mg/mL, respectively (Table 4). Thus, they were both not active enough to be considered as the compounds responsible for the antibacterial activity of CBS.

Furthermore, Ferrazzano et al. [403] reported that caffeine could interfere with the adsorption of *S. mutans*. However, this factor was also excluded in this study because the differences in the main methylxanthines (theobromine and caffeine) contained in CBS were not significant amongst the different extracts (FDR > 0.05; Figures 5c and 5d, respectively). Additionally, caffeine and theobromine standards were tested for their MICs, and the values were found to be 4 and > 4 mg/mL, respectively (Table 4). Therefore, they were not considered pharmacologically active factors against *S. mutans*.

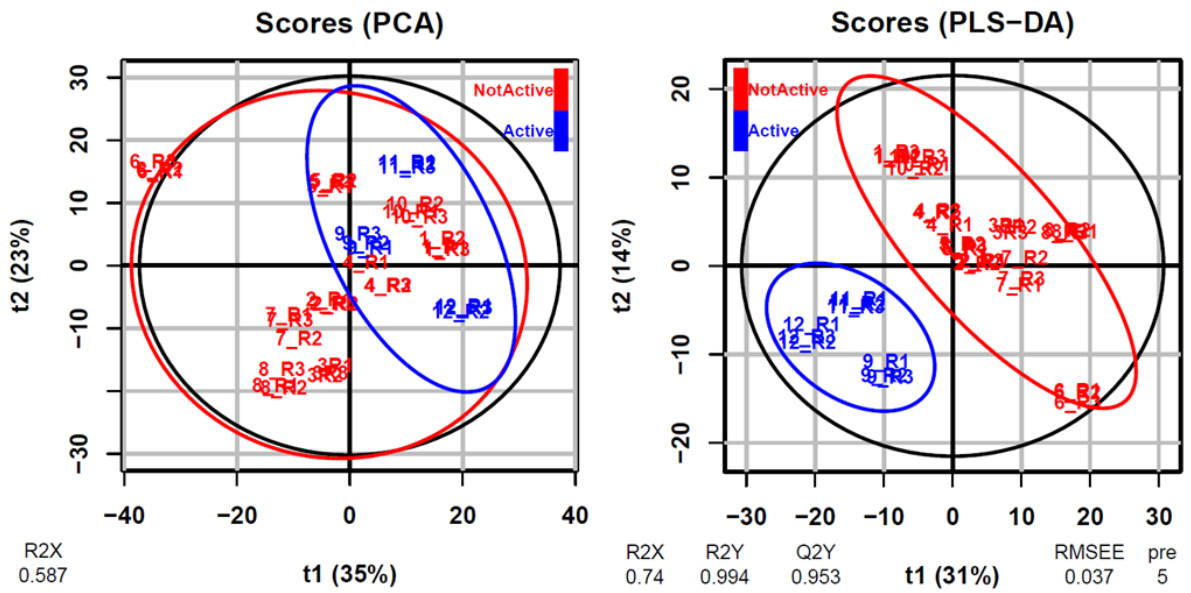
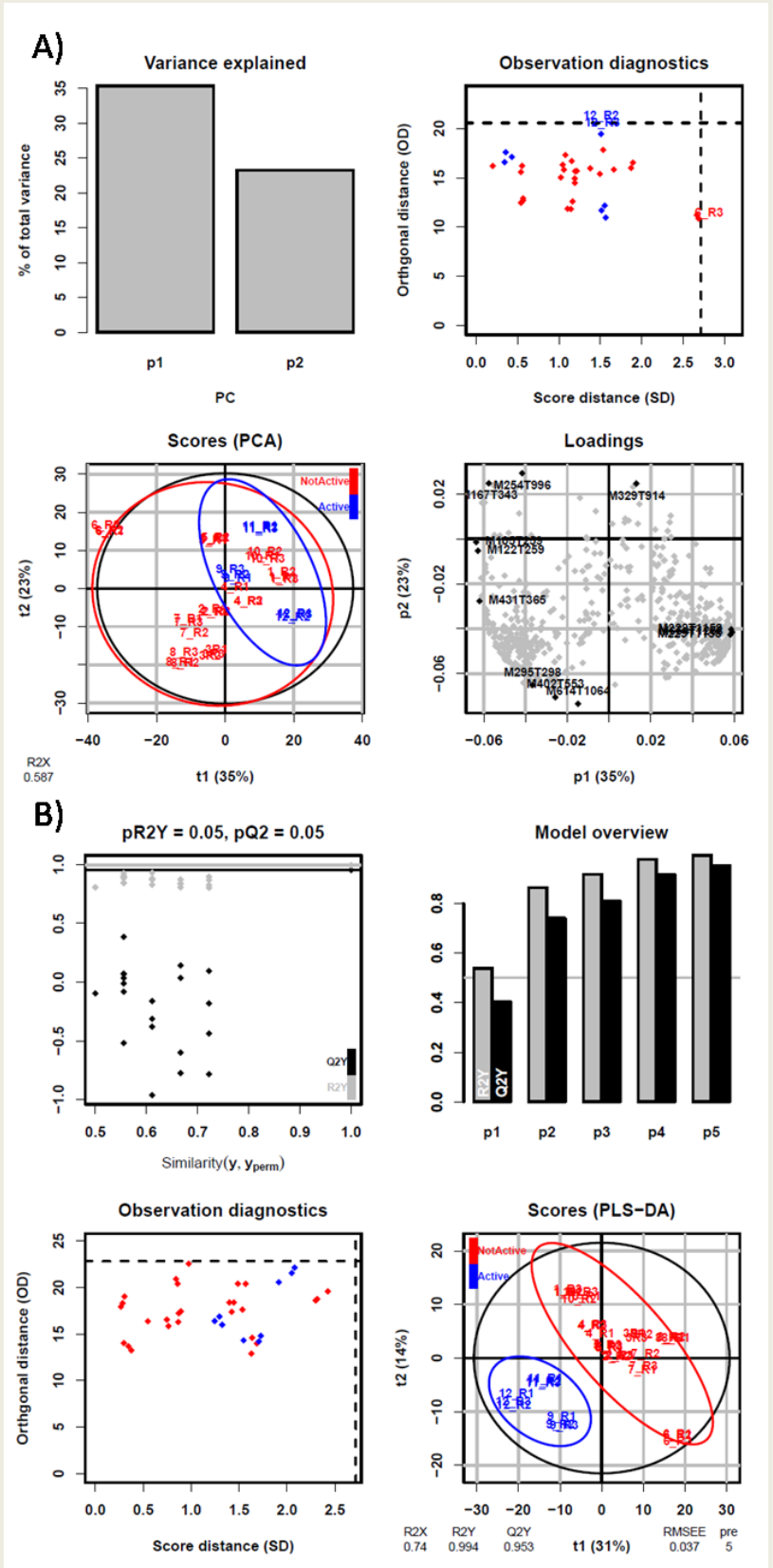


Figure 3. Score plots for the multivariate modelling of variations using principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA) of all CBS extracts (n = 3) according to their activity level against *S. mutans*. Extracts number 9, 11, and 12 were considered 'active' while the remaining extracts were considered 'not active' during both multivariate analyses. For each plot, the percentages of total variation explained by components 1 and 2 (t1 and t2, respectively) are indicated in parentheses. Black ellipses include 95% of the multivariate normal distribution for all samples (sub-grouped in coloured ellipses according to the antibacterial activity level against *S. mutans*). Supplementary information for these plots is given in Figure 4.

Figure 4. Supplementary information on the score plots for multivariate modelling using PCA (**A**) or PLS-DA (**B**). The observation diagnostic plot shows the distances within and orthogonal to the selected score plane. For PCA, the variance explained by the first two principal components is shown, and on the loading plot, the names of the six variables with most extreme values in each direction are indicated. For PLS-DA modelling, an additional diagnostic plot shows the Q2Y and R2Y values from the model (horizontal lines) compared with the values from the models obtained after random permutations of the y response (dots).



As indicated before, a separation by PLS-DA was achieved with the employed metabolomic approach. This separation proved to be driven by some principal features. Herein, two specific molecules presented the highest variable importance in the projection (VIP) coefficients of the model with all components. These were then considered responsible for the separation of the most active CBS extracts from the less active ones: *m/z* = 167.0557, t = 203 s and *m/z* = 369.2042, t = 1344 s (Figures 5e and 5f, respectively). These molecules were indeed present in significantly higher concentrations in the CBS extracts that were considered 'active' against the *S. mutans* strain.

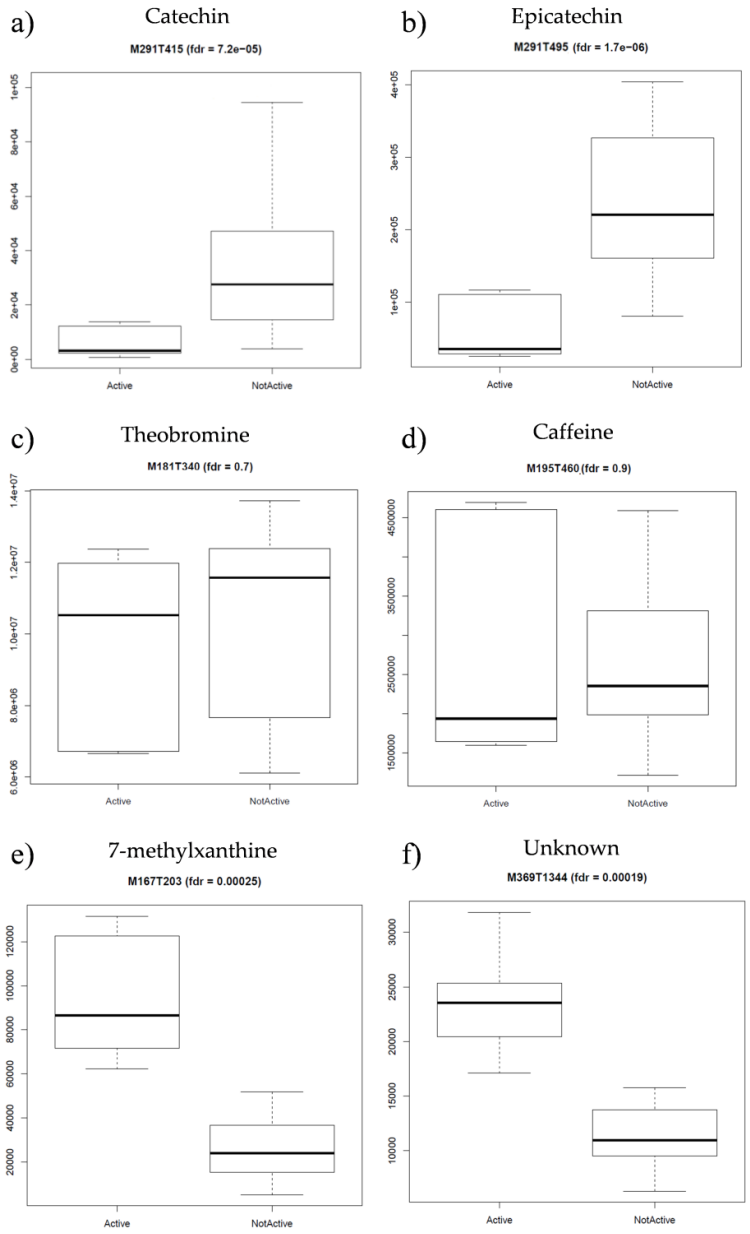


Figure 5. Univariate analysis boxplots of the $\log_{10}$-intensities of (**a**) catechin (m/z=291.0856, t=415 s), (**b**) epicatechin (m/z=291.0859, t = 495 s), (**c**) theobromine (m/z = 181.0713, t = 340 s), (**d**) caffeine (m/z = 195.0669, t = 460 s), (**e**) 7-methylxanthine (m/z = 167.0557, t = 203 s), and (**f**) the putative active molecule (m/z = 369.2042, t = 1344 s) for the two groups of the CBS extracts considered either active (9, 11, and 12) or not active (1–8 and 10) (ANOVA, $p < 0.05$).

The first feature (M167T203) was further confirmed to correspond to 7-methylxanthine (Figure 5e), a theobromine and caffeine precursor that was identified in CBS for the first time. Unfortunately, when testing the 7-methylxanthine alone against *S. mutans*, no direct inhibition was observed below 4 mg/mL (Table 4). However, a possible synergistic effect of this compound along with other bioactive compounds in CBS (mainly polyphenols) was not disregarded because of the higher presence of 7-methylxanthine within the active CBS extracts. Unfortunately, we were unable to provide a specific identification for the second feature (M369T1344, Figure 5f). Nevertheless, four different hypotheses for the identity of the molecule have been proposed from the data found in the European MassBank database (https://massbank.eu/MassBank). According to its mass, this possibly active molecule could be curcumin ($C_{21}H_{20}O_6$), a polyphenol that has already been reported to possess short-term and long-term antibacterial effects against *S. mutans* and display no visible bacterial growth at an MIC of 125 µM (approximately 46 µg/mL) [406]. However, curcumin has never been reported in cocoa or cocoa-similar products. Another possibility is feruloylquinic acid ($C_{17}H_{20}O_9$). This molecule is mainly present in coffee but, similar to curcumin, has never been detected in cocoa products. However, it has been reported that ferulic acid does not show any activity against *S. mutans*. Thus, it could be implied that feruloylquinic acid does not exert such activity [407]. The third hypothesis points to a prenylated flavone ($C_{21}H_{20}O_6$). This could be a very reasonable option since prenylated flavonoids have been reported to strongly inhibit bacterial and fungal activities. Prenylation increases the lipophilicity of flavonoids and consequently increases their affinity for biological membranes. This facilitates their attachment and improves interactions with target proteins [408]. Additionally, it has been proposed that flavones could form a complex with bacterial cell wall components, which would inhibit further adhesion and growth [409]. The fourth option for the molecule conducting the PLS-DA separation according to the feature mass would be lignoceric acid ($C_{24}H_{48}O_2$). This unsaturated fatty acid has already been detected in CBS [73]. Similar to the prenylated flavone, this is a reasonable hypothesis because many researchers have reported the ability of several fatty acids to inhibit bacterial growth and specifically *S. mutans* [410]. Antibacterial properties of free fatty acids are well recognised. They are manifested through different pathways such as the disruption of the electron transport chain and oxidative phosphorylation in the cell membrane, inhibition of certain enzymes, inhibition of nutrient uptake, formation of peroxidation or auto-oxidation products, or direct or induced lysis of bacterial cells [411].

4. Conclusions

CBS extracts did not show a pharmacologically interesting activity (MIC ≤ 500 μg/mL) against most bacterial and fungal strains tested in this study (MSSA, MRSA, resistant and sensitive *E. coli*, *P.aeruginosa*, *C. albicans*, and *S. cerevisiae*). However, interestingly, when tested against *S. cerevisiae*, higher CBS extract concentrations promoted fungal growth. This could be important in the application of this by-product in food product development wherein the yeast action is desired. *Streptococcus mutans* was the only strain against which CBS extracts showed inhibition at pharmacologically interesting concentrations. However, significant differences in the MIC values were observed amongst the extracts tested. In contrast to what was expected, the most active extracts did not contain the highest amounts of polyphenols. Therefore, this factor was not directly related to the antibacterial activity of the CBS extracts. Untargeted metabolomic analysis was performed based on the data obtained by the LC-HRMS analyses of all the extracts and served to elucidate the possible active molecules responsible for the separation of the most active CBS extracts against the cariogenic bacterial strain *S. mutans* from the less active CBS extracts depending on the differences in MIC values. Two molecules were found to be responsible for this separation and were significantly present in the active CBS extracts. The first of these molecules was identified as 7-methylxanthine, a precursor of theobromine and caffeine. Although no direct antibacterial activity was found when testing this molecule alone against *S. mutans*, a possible synergistic effect of 7-methylxanthine with other CBS extract components and polyphenols can be hypothesised. The structure of the second and putative active molecule driving the separation was not completely elucidated. However, according to its mass, four different hypotheses were proposed: curcumin, feruloylquinic acid, prenylated flavone, and lignoceric acid. The last two hypotheses were more plausible according to the known composition of CBS.

GENERAL CONCLUSION AND PERSPECTIVES

The cocoa bean shell (CBS), which represents about 10 – 17% of the total cocoa bean weight, has demonstrated to be a very versatile by-product, which presents the possibility of being valorized and used in several fields such as the food and pharmaceutical domains.

In view of the results obtained during this PhD work, it can be concluded that the CBS is a rich source of cocoa-similar polyphenols and methylxanthines (theobromine and caffeine). Although the concentration levels of these compounds do not reach those found in the cocoa bean, they are not far from the latter. At the same time, the characterization of the volatile organic compounds (aromas) contained in CBS allowed to conclude that the by-product has a cocoa-similar organoleptic profile, although less intense. Considering that CBS is a by-product that has been normally discarded or underused up to now, these discoveries are of great importance for its valorization as a food ingredient or as a pharmaceutical raw material.

Besides, the results obtained for the polyphenolic and VOC characterizations through both chromatographic techniques (RP-HPLC-PDA and HS-SPME/GC-qMS, respectively) and screening methods (spectrophotometric assays and E-nose, respectively) allowed for the classification of CBS with authentication purposes. On the one hand, both aromatic profile characterization techniques allowed for the discrimination of the Criollo (fine aroma variety) CBS samples from the rest of CBS varieties, and to identify several markers for the different cocoa varieties. On the other hand, the polyphenolic study allowed for a different classification of the CBS, based on the geographic origin of samples and not on the cocoa variety as observed with the VOCs. This finding makes sense if we take into account the fact that polyphenolic compounds are created as a response of the plant to its environment. Besides, their concentration variations are highly dependent on the different processing techniques linked to the different cultivation tradition, contrary to the CBS aromas, which are highly linked to the cocoa variety. However, CBS variety differentiation and classification was possible to be achieved by taking into account the methylxanthines ratio, which was a new and very promising finding for the cocoa by-product.

In fact, these classification strategies by means of the identification of common trends among the CBS samples remain highly useful from an industrial point of view, in the frame of food safety and traceability. Moreover, the feasibility of screening methods giving similar results to those of the more sophisticated chromatographic techniques is a great, advantageous and promising discovery for the application of the chemometrical classification of CBS with rapid, easy-to-handle and simpler routine techniques. Still, the confirmation of these authentication strategies with larger and more varied sample matrices could be envisaged as future perspectives of the present work.

When used as a food ingredient, CBS can give cocoa-similar aroma to functional foods. CBS can also provide food products with a high content of dietary fiber. Moreover, the model foods developed in the present work were accepted among consumers in terms of appearance and taste and, in some cases, CBS addition increased the liking of some foods which had been developed with unpleasant ingredients such as tagatose. Besides, CBS has demonstrated to provide functional foods with interesting antioxidants and antidiabetic properties, mainly as a result of its polyphenolic content. However, it has been proved that the availability of these polyphenols during gastrointestinal digestion could be highly influenced by the food matrix in which CBS is comprised. This fact is of great importance since CBS polyphenols need to reach the different organs in which they are supposed to act in order to obtain the several health benefits that have been demonstrated for these compounds. Complex food matrices can retain a higher amount of polyphenols, decreasing their bioavailability. However, complex food matrices such as biscuits can also protect CBS polyphenols while liberating them during gastrointestinal digestion. On the contrary, CBS polyphenols are degraded to a higher extent when included into more simple matrices such as the beverages presented in this work. Nevertheless, *in vivo* studies confirming these facts are of great importance and remain as future perspectives for the present work. It is worth mentioning that safety assays were not performed in the present study when using CBS as a food ingredient because those were already performed by the suppliers. However, mycotoxin presence in cocoa by-products remains a major concern and new strategies for their rapid and safe analysis could be envisaged as future perspectives of this work.

CBS antibacterial activity against *S. mutans*, a bacterial strain involved in dental caries, was confirmed in this work and was demonstrated to be independent of the CBS total polyphenolic content, contrary to what was previously believed. Several molecules contained in pharmacologically active CBS extracts were identified as possible actors of this activity. The future confirmation of these active compounds would contribute to the identification of active CBS extracts against *S. mutans*, which could be used for pharmaceutical applications such as dental care. In this context, CBS uses in the cosmetic field as well as the use of encapsulated biofunctional compounds from CBS could also be proposed.

The present work intends to be contribution for the valorization of a high add-value product such as the cocoa bean shell, and a step towards a zero-waste cocoa industry within the frame of a sustainable circular economy.

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