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Comparative study of different cocoa (*Theobroma cacao* L.) clones in terms of their phytoprostanes and phytofurans contents

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Cocoa has been widely discussed as a bioactive food rich in sensory stimulation and health benefits. However, no information has been provided concerning phytoprostanes (PhytoPs) and phytofurans (PhytoFs) in cocoa. These compounds are of interest because they play a role in the regulation of immune function. The present study included 31 cocoa clones. The PhytoPs and PhytoFs were quantified by UHPLC-QqQ-MS/MS. The total PhytoPs and PhytoFs contents ranged from 221.46 to 1589.83 ng g⁻¹ and from 1.18 to 13.13 ng g⁻¹, respectively. The profiles of the PhytoPs and PhytoFs identified in the cocoa beans showed significant differences among the clones analysed. The results indicate that dry fermented cocoa beans are rich in PhytoPs and PhytoFs, which may represent an additional benefit of the consumption of foods derived from cocoa.

1. Introduction

The interest in the consumption of foods that provide health benefits and possess attractive sensorial characteristics has always stimulated research, much of which has focused on bioactive compounds that promote health or provide sensory stimulation (Marseglia, Sforza et al., 2014). Accordingly, cocoa and its derivatives have been well studied with this approach. Cocoa has been recognized as an important source of polyphenols (Demidchik, 2015), flavonoids (Patras, Milev, Vrancken, & Kuhnert, 2014), catechins, procyanidins (Cádiz-Gurrea et al., 2014), amino acids (Marseglia, Palla, & Caligiani, 2014), polypeptides, and oligopeptides (Marseglia, Sforza et al., 2014). To this list may include

compounds that add sensory properties to the product, known as “single-origin” (Torres-Moreno, Tarrega, Costell, & Blanch, 2012), which are of interest to both researchers and consumers. Transversally, these studies may repeatedly denote homogeneity, but the increased understanding of food chemistry that they have provided has underpinned new guidelines and motivated consumption. In addition, nowadays, the focus on food characteristics related to geographic origin is often prioritized. Furthermore, the study of compounds or metabolites not reported so far (“unknown”) is facilitated by the use of analytical tools with higher resolution and sensitivity.

According to these perspectives, the study of novel compounds like phytoprostanes (PhytoPs) and phytofurans (PhytoFs) is of interest

Abbreviations: BHA, Butylated hydroxyanisole; ALA, α -linolenic acid; FAMES, fatty acids methyl esters; GC-MS, Gas chromatography-mass spectrometry; IsoPs, isoprostanes; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; Nfr2, nuclear factor erythroid-2; OS, oxidative stress; PCA, principal components analysis; PhytoFs, phytofurans; PhytoPs, phytoprostanes; PUFA, polyunsaturated fatty acids; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RSD, relative standard deviation; SD, standard deviation; SPE, solid-phase extraction; UHPLC-ESI-QqQ-MS/MS, ultra-high performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry

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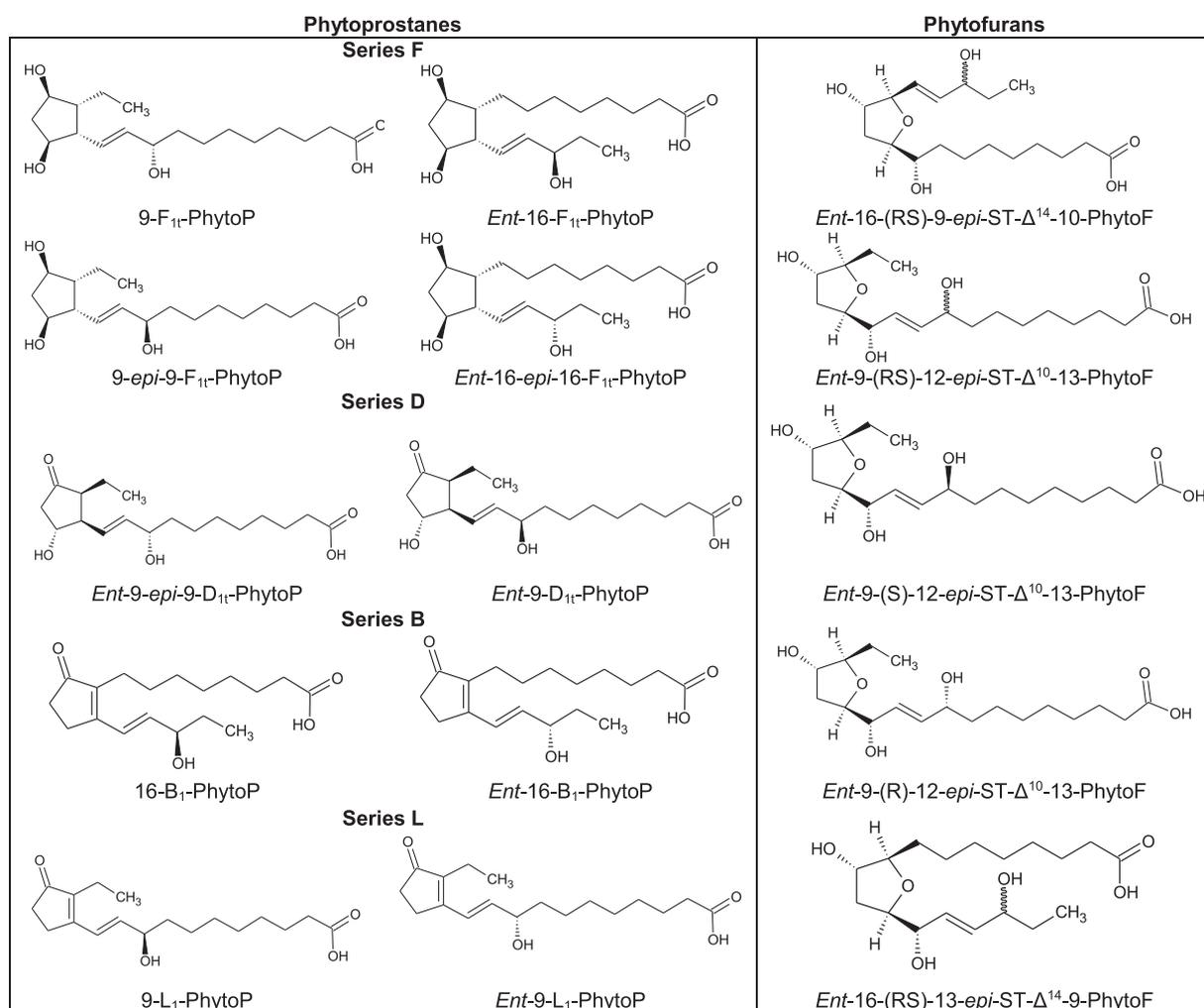


Fig. 1. Chemical structures of phytosteranes and phytofurans (named according to the ((Collado-González et al., 2016) Taber/Roberts nomenclature).

because it is known that they play a role in the regulation of immune function (Barden et al., 2009) and display anti-inflammatory and apoptosis-inducing activities, among others, similar to other prostanooids (Durand et al., 2011).

These metabolites are prostaglandin-like compounds (plant oxylipins) derived from the lipid peroxidation of polyunsaturated fatty acids (PUFAs), such as α -linolenic acid (ALA, C18:3n-3), via a nonenzymatic free-radical-catalyzed pathway (Jahn, Galano, & Durand, 2010). In the biosynthesis of IsoPs which are oxidative stress (OS) markers in mammals, a carbon radical undergoes a 5-exo-trig cyclization (Baldwin rules (Baldwin, 1976; Baldwin, Thomas, Kruse, & Silberman, 1977)) to form the cyclopentane ring of isofurans (IsoFs) (Jahn, Galano, & Durand, 2008; Morrow et al., 1990). Meanwhile, the PhytoPs - like PhytoFs - are oxylipins which may be formed from ALA, but they require a high oxygen tension (higher than 21%) to attack the same carbon radical as PhytoPs and form compounds containing a tetrahydrofuran ring. So, the IsoFs are characterized by a tetrahydrofuran ring and two side chains. These bioactive compounds act as endogenous mediators capable of protecting cells from damage under various conditions related to OS (Loeffler et al., 2005).

In the scientific literature, PhytoPs and PhytoFs have been reported in numerous natural or unprocessed products, as also in processed products (as shown Supplementary Table S1), but, so far, these compounds have not been reported in cocoa beans. For this reason, the objective of the present work was to assess, for the first time, the concentrations of PhytoPs and PhytoFs in cocoa bean from different clones. Hence, a comparative study of the content of PhytoPs and

PhytoFs by UHPLC-MS/MS was developed in cocoa beans obtained from the same farm, the same period of flowering and ripeness of the fruits. In addition, the same process of fermentation and drying was assured. Only the variety of the genetic material of the cocoas was varied. The profile of these metabolites was studied in conjunction with the fatty acids levels. The value of these matrices as a source of these largely unknown oxylipins needs to be addressed in order to plan nutritional trials devoted to shed some light on their biological activity.

2. Materials and methods

2.1. Chemicals and reagents

The PhytoPs - 9-F_{1t}-PhytoP, 9-epi-9-F_{1t}-PhytoP, ent-16-F_{1t}-PhytoP, ent-16-epi-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-epi-9-D_{1t}-PhytoP, ent-16-B₁-PhytoP, 16-B₁-PhytoP, ent-9-L₁-PhytoP, and 9-L₁-PhytoP - were synthesized according to our published procedures (El Fangour et al., 2004; El Fangour, Guy, Vidal, Rossi, & Durand, 2005; Pinot et al., 2008). The PhytoFs - ent-16-(RS)-9-epi-ST-Δ¹⁴-10-PhytoF, ent-9-(RS)-12-epi-ST-Δ¹⁰-13-PhytoF, ent-9-(S)-12-epi-ST-Δ¹⁰-13-PhytoF, ent-9-(R)-12-epi-ST-Δ¹⁰-13-PhytoF, and ent-16-(RS)-13-epi-ST-Δ¹⁴-9-PhytoF - were synthesized according to our previous reports (Cuyamendous et al., 2015) (Fig. 1). Hexane, sodium hydroxide (NaOH), anhydrous sodium sulfate, and water for LC-MS were obtained from Panreac (Castellar Del Vallés, Barcelona, Spain). Fatty acid methyl ester (FAME) Mix Supelco GLC-50, GLC-100, CLA isomers (c9 t11 CLA and t10 c12 CLA), nonanoic acid (C9:0), heptadecanoic acid (C17:0), boron trifluoride (BF₃), and

Table 1

Cacao clones evaluated and their respective characteristics. Small bean size: (< 1.4 g/grain); middle (1.4–1.6 g/ grain); big (\geq 1.6 g/grain).

Samples	Variety	Grain size
Criollo moderno - 1	Criollo moderno	Big
Criollo moderno - 2	Criollo moderno	Big
Criollo moderno - 3	Criollo moderno	Middle
Criollo - 1	Criollo	Big
Criollo - 2	Criollo	Big
Criollo - 3	Criollo	Big
Regional - 1	Regional	Middle
Regional - 2	Regional	Small
Regional - 3	Regional	Middle
Regional - 4	Regional	Big
Regional - 5	Regional	Big
Regional - 6	Regional	Middle
Regional - 7	Regional	Small
Regional - 8	Regional	Middle
Regional - 9	Trinitario	Big
Regional - 10	Regional	Middle
Regional - 11	Regional	Big
Albino - 1	Forastero	Middle
Albino - 2	Forastero	Middle
CCN-51	Forastero	Middle
FMT-1	Forastero	Middle
FTU-6	Forastero	Middle
CAU-37	Forastero	Small
EET-8	Trinitario	Big
EET-96	Trinitario	Middle
FMAC-11	Trinitario	Middle
FMAC-12	Trinitario	Middle
ICS-1	Trinitario	Big
ICS-39	Trinitario	Big
ICS-95	Trinitario	Small
TSH-565	Trinitario	Small

Bis-Tris (bis(2-hydroxyethyl) amino-tris(hydroxymethyl)methane) were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Methylene chloride was purchased from Labscan Ltd (Dublin, Ireland). All LC–MS grade solvents, methanol, and acetonitrile were purchased from J.T. Baker (Phillipsburg, New Jersey, USA). The SPE cartridges (Strata X-AW 33 μ , 100 mg/3 mL) were acquired from Phenomenex (Torrance, CA, USA), and the Sep-Pak® classic C18 cartridges from Waters (Milford, MA).

2.2. Geographical origin of the samples

Cocoa (*Theobroma cacao*) trees were cultivated, and their cobs fermented and extracted, at the “Cannes” (Maceo) experimental farm, located 132 km from Medellín, Department of Antioquia, Colombia (Supplementary Material). Cannes is situated 1000 m above sea level (6°31'16.62"N and -74°49'25.57"W), the annual average temperature is 23 °C, the annual precipitation is 2700 mm, and the mean relative humidity is 80%. The study included 31 different clones of cocoa beans grown on the experimental farm, which were classified according to their denomination on the farm (three criollos, three criollos modernos, four forasteros, two forasteros albinos, ten regionals, and nine trinitarios) (Table 1). For the harvest of samples, the period of cocoa grown known as “traviesa” (crossbred) was selected (from May to June of 2016); this was after the first flowering of the year, but before the main harvest. The fruit ripeness was determined by an expert farmer; healthy and representative cobs were selected from each clone batch grown on the experimental farm. The extraction of the cocoa beans was done the same day for all the samples. The beans were separated inside plastic meshes and labelled for subsequent identification. The meshes with the beans were arranged in fermentation drawers. The fermentation process was developed in a place covered in the same area of cocoa crop and was performed at room temperature spontaneously and in wooden drawers designed in three columns by three rows with a capacity of

150 kg per drawer. The three central horizontal drawers were used, and labelled as drawer 1, drawer 2 and drawer 3. The monitoring and evolution was developed under the guidance of a farmer and a FEDE-CACAO (National Federation of Cocoa-Colombia) technician with expertise in the cocoa fermentation process. The fermentation was performed with beans that were healthy and free of impurities such as leaves or insects, for six days, guaranteeing the death of the embryo in each bean.

A natural drying process was performed on wooden platforms in a marquee dryer. The meshes were opened and the beans were carefully extended, ensuring that the clones were not mixed. The drying was completed on the seventh day, as indicated by the cracking of the beans and the development of a kidney shape and brown colour. Samples (18 cocoa beans in triplicate) were processed and analysed up to one month after collection on the farm. The samples were stored at -80 °C before being extracted for further analyses.

2.3. Processing of cocoa beans

The PhytoPs and PhytoFs from the cocoa beans samples were isolated using a dispersive liquid–liquid extraction followed by an SPE, according to a procedure described by Collado-González, Medina et al. (2015) with modifications. Each sample was ground in a coffee mill to obtain a semi-fine cocoa powder (< 1000 μ m), homogenized, and weighed (about 2 g), before being added to a mortar together with 10 mL of BHA dissolved in methanol (1 g L⁻¹). Then, the homogenized sample was transferred to a centrifuge tube and centrifuged for 10 min at 4 °C and 2000g. The supernatant was filtered in a C18 Sep-Pak (previously activated with 10 mL of methanol and 10 mL of water). Exactly 1 mL of the extract was diluted with 10 mL of hexane, 2 mL of methanol, and 2 mL of BIS–TRIS buffer (0.02 M, HCl, pH = 7); between each addition the mixture was stirred vigorously. The emulsion obtained was subjected to SPE using a Strata X-AW cartridge (100 mg/3 mL); prior to this, the cartridge was conditioned and equilibrated with 2 mL of hexane, 2 mL of methanol, and 2 mL of milliQ water. After removal of the non-subject compounds, the cartridge was washed with 2 mL of hexane, 2 mL of milliQ water, 2 mL of a solution of methanol/milliQ water (1/3, v/v), and 2 mL of acetonitrile. The target compounds were eluted with 1 mL of methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry extracts were reconstituted with 200 μ L of mobile phase (solvent A, a mixture of milliQ water/0.01% (v/v) acetic acid, and solvent B, a solution of methanol/0.01% (v/v) acetic acid, in a proportion of 90:10 v/v, respectively). The samples were filtered through 0.45- μ m PVDF filters (Millipore, MA, USA) and 20 μ L of each sample were analysed. The samples were analysed in triplicate.

Stock solutions of PhytoFs and PhytoPs were prepared in methanol/water (50:50, v/v), to facilitate the ionization process in the mass spectrometer, at a concentration of 1000 nM for each compound and stored in Eppendorf tubes at -80 °C. Twelve successive dilutions were prepared for the calibration curve (with R² value higher than 0.99).

2.4. Measurements

2.4.1. Phytofurans and phytoprostanes analyses

The identification and quantification of PhytoFs and PhytoPs were performed using a UHPLC coupled to a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany), with a BEH C₁₈ analytical column (2.1 \times 50 mm, 1.7 μ m) (Waters, Milford, MA). The column temperature was 6 °C. The mobile phases consisted of water/acetic acid (99.99:0.01, v/v) (A) and methanol/acetic acid (99.99:0.01, v/v) (B) and 20 μ L of the diluted extract was injected at a flow of 0.2 mL min⁻¹. The following gradient program was used: 60% B at 0 min, 62% B at 2 min, 62.5% B at 4 min, 65% B at 8 min, and 60% B at 8.01 min. The acquisition time was 8.01 min for each sample, with a post-run of 1.5 min for column equilibration. Analysis was performed in the negative mode by

Table 2

UHPLC-QqQ-MS/MS parameters for the quantification and confirmation of phytoprostanes and phytofuranos. ^Z Quantification transition. ^Y Confirmation transition. ^X Coeluting diastereoisomers quantified together.

Compound	Retention time (min)	ESI mode	MRM transition (<i>m/z</i>)	Fragmentor (V)	Collision energy (V)
<i>Phytoprostanes</i>					
<i>Ent</i> -16- <i>epi</i> -16- <i>F</i> _{1r} -PhytoP ^X	1.583	Negative	327.1 > 283.2 ^Z	80	15
			327.1 > 225.1 ^Y	80	15
9- <i>F</i> _{1r} -PhytoP	1.631	Negative	327.2 > 273.1	110	15
			327.2 > 171.0	110	15
<i>Ent</i> -16- <i>F</i> _{1r} -PhytoP ^X	1.712	Negative	327.2 > 283.2	80	10
			327.2 > 225.1	80	10
9- <i>epi</i> -9- <i>F</i> _{1r} -PhytoP	1.785	Negative	327.2 > 272.8	110	10
			327.2 > 171.0	110	10
9- <i>D</i> _{1r} -PhytoP	1.791	Negative	325.2 > 307.3	100	4
			325.2 > 134.7	100	4
9- <i>epi</i> -9- <i>D</i> _{1r} -PhytoP	2.022	Negative	325.2 > 307.2	100	7
			325.2 > 134.9	100	7
16- <i>B</i> ₁ -PhytoP	2.620	Negative	307.2 > 223.2	100	10
			307.2 > 235.1	100	100
9- <i>L</i> ₁ -PhytoP	3.079	Negative	307.2 > 185.1	110	7
			307.2 > 196.7	110	7
<i>Phytofurans</i>					
<i>Ent</i> -9-(<i>RS</i>)-12- <i>epi</i> - <i>ST</i> - Δ^{10} -13-PhytoF	0.906	Negative	344.0 > 300.0	110	10
			344.0 > 255.9	110	10
<i>Ent</i> -16-(<i>RS</i>)-9- <i>epi</i> - <i>ST</i> - Δ^{14} -10-PhytoF	1.501	Negative	343.9 > 209.0	90	12
			343.9 > 201.1	90	12
<i>Ent</i> -16-(<i>RS</i>)-13- <i>epi</i> - <i>ST</i> - Δ^{14} -9-PhytoF	1.523	Negative	343.0 > 171.1	90	22
			343.0 > 97.2	90	22

multiple reaction monitoring (MRM). The MS parameters fragmentor (ion optics: capillary exit voltage), collision energy, and preferential MRM transition of the corresponding analytes were optimized for each analyte (Table 2). The source parameters used were gas flow: 8 L min⁻¹; gas temperature: 325 °C; sheath gas temperature: 350 °C; jetstream gas flow: 12 L min⁻¹; nebulizer: 30 psi; capillary voltage: 3000 V; and nozzle voltage: 1750 V. Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies). The elution gradient and mass spectrometric parameters were as described previously (Collado-González, Medina, et al., 2015). The quantification of PhytoPs and PhytoFs detected in cocoa clones was performed using authentic standard according to standard curve freshly prepared as mentioned in the previous Section 2.3.

2.4.2. Fatty acid profile

The analysis of fatty acids was performed by methylating these compounds *in situ*, following the procedure described by Taber, Morrow, and Jackson Roberts (1997) and later improved by Trigueros and Sendra (2015). The cocoa beans were ground, homogenized, and weighed. A sample of approximately 40 mg was transferred into a test tube. 60 μ L of C17:0 in n-hexane solution as internal standard (-20 mg mL⁻¹ solution in HPLC grade n-hexane) was added. Then, 100 mL of methylene chloride and 1 mL of 0.5 M NaOH in methanol were added, and the tubes were heated in a water bath at 90 °C for 10 min. One milliliter of BF₃ in methanol was added and the mixture was left at room temperature (25 °C) for 30 min. One milliliter of distilled water and 600 mL of hexane were added, and then the fatty acids methyl esters (FAMES) were extracted by vigorous shaking for about 1 min. Following centrifugation, aliquots were dried with anhydrous sodium sulfate and the top layer was transferred into a vial flushed with nitrogen, which was stored at -20 °C until analysis. The FAMES were analysed using GC-MS with a SupraWax-280 column, 100% polyethylene glycol (Teknokroma S. Co. Ltd., 165 Barcelona, Spain; 30 m \times 0.25 mm \times 0.25 μ m film thickness). The analyses were carried out using helium as carrier gas at a flow rate of 1.1 mL min⁻¹ and a program as follows: initial temperature 80 °C, hold for 2 min; rate of increase of 8.0 °C min⁻¹ to 160 °C; rate of 4 °C min⁻¹ from 160 to 220 °C, hold for 13 min; and rate of 10 °C min⁻¹ from 220 to 260 °C, hold for 6 min. The injector and detector temperatures were held at 230

and 260 °C, respectively; 0.5 μ L of the extract was injected. The analyses were run in duplicate.

2.4.3. Statistical analysis

To determine the effect of each factor on each individual PhytoP, PhytoF, and PUFA, an analysis of variance (ANOVA, $p \leq 0.05$), Pearson's correlation analysis of the different variables studied and multiple range test (Tukey's test) were carried out using the SPSS program, version 23.0 (SPSS Inc., Chicago, IL, USA). Mean values were compared by the LSD (Least Significant Difference) test when significant interactions between factors were found.

3. Results and discussion

Oxidative stress is a complex chemical and physiological phenomenon that accompanies practically all the biotic and abiotic stresses in plants (Hasanuzzaman, Hossain, da Silva, & Fujita, 2012). It develops as a result of the overproduction and accumulation of reactive oxygen species (ROS) (Demidchik, 2015).

In this study, we selected the Cannes experimental farm because it is located in an area of cocoa production in the region of Antioquia, Colombia. This farm has a rich clone plantation; the cocoa trees are the same age and there are many different clones. In addition, the abiotic stresses (such as drought, salinity, cold, high light/UV-B, heat, air pollution, heavy metals, mechanical wounding, and nutritional deficiency (Nakabayashi & Saito, 2015)) influencing the beans were considered homogeneous for all samples, as was the biotic stress experienced during fermentation. These variables, although influencing the formation of the target metabolites, were assumed to be constant and, although they may have exerted some influence on the results obtained, the impact of the genetic resource (clones) was assumed to be far superior. However, as far as we know, this is the first report where different classes of PhytoPs have been detected and quantified in fermented and dried cocoa beans of different clones; and as a result it was determined statistically different PhytoP and PhytoF levels were found among the different cocoa beans clones (Tables 3 and 4). They were independent of a classification like criollo (and criollo moderno), regional, forastero, or trinitario cocoa. The results of statistical tests are shown in Supplementary Table S2.

Table 3

Individual series of PhytoFs levels (ng g^{-1}) in cacao beans. The values are represented as means \pm SD (standard deviation). ND: not detected or not quantified.

Samples	<i>ent</i> -16-(RS)-9- <i>epi</i> -ST- Δ^{14} -10-PhytoF	<i>ent</i> -9-(RS)-12- <i>epi</i> -ST- Δ^{10} -13-PhytoF + <i>ent</i> -9-(S)-12- <i>epi</i> -ST- Δ^{10} -13-PhytoF + <i>ent</i> -9-(R)-12- <i>epi</i> -ST- Δ^{10} -13-PhytoF	<i>ent</i> -16-(RS)-13- <i>epi</i> -ST- Δ^{14} -9-PhytoF
Criollo moderno - 1	1.13 \pm 0.08	0.99 \pm 0.05	1.51 \pm 0.15
Criollo moderno - 2	ND	ND	1.26 \pm 0.34
Criollo moderno - 3	1.09 \pm 0.06	ND	1.55 \pm 0.62
Criollo - 1	ND	ND	ND
Criollo - 2	1.18 \pm 0.07	0.97 \pm 0.22	3.31 \pm 0.12
Criollo - 3	ND	ND	ND
Regional - 1	ND	ND	ND
Regional - 2	1.48 \pm 0.13	1.12 \pm 0.10	2.96 \pm 0.36
Regional - 3	0.99 \pm 0.05	ND	2.19 \pm 0.07
Regional - 4	1.49 \pm 0.07	1.55 \pm 0.04	1.11 \pm 0.05
Regional - 5	0.95 \pm 0.08	ND	1.00 \pm 0.09
Regional - 6	1.88 \pm 0.18	2.21 \pm 0.18	ND
Regional - 7	ND	ND	ND
Regional - 8	2.70 \pm 0.39	2.94 \pm 0.21	7.49 \pm 0.30
Regional - 9	1.08 \pm 0.15	ND	1.74 \pm 0.30
Regional - 10	1.33 \pm 0.28	1.37 \pm 0.10	2.38 \pm 0.37
Regional - 11	1.09 \pm 0.00	ND	2.24 \pm 0.24
Albino - 1	1.20 \pm 0.03	ND	2.13 \pm 0.35
Albino - 2	1.56 \pm 0.21	1.20 \pm 0.08	2.79 \pm 0.35
CCN-51	1.80 \pm 0.11	ND	3.29 \pm 0.07
FMT-1	1.39 \pm 0.03	1.10 \pm 0.13	3.06 \pm 0.26
FTU-6	1.00 \pm 0.17	ND	1.01 \pm 0.13
CAU-37	1.38 \pm 0.13	1.58 \pm 0.31	2.06 \pm 0.06
EET-8	ND	ND	1.38 \pm 0.17
EET-96	1.05 \pm 0.03	ND	2.14 \pm 0.80
FMAC-11	1.38 \pm 0.20	1.08 \pm 0.11	1.82 \pm 0.18
FMAC-12	1.01 \pm 0.02	1.14 \pm 0.06	1.60 \pm 0.47
ICS-1	ND	ND	ND
ICS-39	ND	ND	ND
ICS-95	1.18 \pm 0.19	ND	ND
TSH-565	1.58 \pm 0.09	1.30 \pm 0.12	2.23 \pm 0.32

PhytoPs and PhytoFs were detected in all the cocoa beans samples analysed, except for some specific samples in which they were below the LOD (Tables 3 and 4) and with respect to recovery percentages were in the range of 85 and 123% for all the analytes (PhytoPs and PhytoFs) according to our recent published validated method (Domínguez-Perles et al., 2018). The identification of these free PhytoPs was confirmed according to their pseudomolecular ions, the characteristic MS/MS fragmentation product ions (quantification and confirmation transitions), and their corresponding retention times (Collado-González, Medina, et al., 2015). PhytoPs and PhytoFs are formed non-enzymatically as regio- and stereoisomeric mixtures, so the analytical conditions employed in our study did not allow enantiomeric separation; therefore, they are quantified in the cocoa beans samples as racemic mixtures of PhytoPs and PhytoFs (Fig. 1). It is important to note that *ent*-16-*epi*-16- F_{11} -PhytoP + *ent*-16- F_{11} -PhytoP are epimers at position C16; *ent*-9-(RS)-12-*epi*-ST- Δ^{10} -13-PhytoF + *ent*-9-(S)-12-*epi*-ST- Δ^{10} -13-PhytoF + *ent*-9-(R)-12-*epi*-ST- Δ^{10} -13-PhytoF are a racemic mixture at position C9 (50% 9R and 50% 9S); and 16- B_{11} -PhytoP + *ent*-16- B_{11} -PhytoP and 9- L_1 -PhytoP + *ent*-9- L_1 -PhytoP represent racemic mixtures that are not separable in our chromatographic conditions (Collado-González, Medina, et al., 2015).

The total PhytoPs and PhytoFs amounts in the cocoa beans ranged from 221.46 to 1589.83 and 1.18 to 13.13 ng g^{-1} , respectively, being Criollo-2 and Regional-8 the clones with the highest concentration of PhytoPs and PhytoFs, respectively (Tables 3 and 4). Moreover, the greatest proportion of the PhytoPs profile of the samples was represented by the series F_{11} , while the series B_{11} , L_1 , and D_{11} were detected at lower levels, corresponding to ranges of 209.46 to 1558.44, 3.04 to 46.35, 2.11 to 26.34, and 1.82 to 21.79 ng g^{-1} , respectively. These results are consistent with those reported previously for *Passiflora edulis* Sims shell, macroalgae, and red wine (Barbosa et al., 2015; Marhuenda et al., 2015; Medina et al., 2017). In relation to PhytoFs, there are few studies of these compounds. Cuyamendous et al. (2015) described for the first time the synthesis of PhytoFs and quantified the

compound *ent*-16(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF in nuts (pine nuts and walnuts) and seeds (flaxseeds and chia seeds). Yonny et al. (2016) studied PhytoFs in melon leaves, but they did not find *ent*-16(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF; conversely, in cocoa beans, this PhytoF was the most abundant (Table 3). Accordingly, the profile of the individual series differed significantly among the samples of the cocoa clones analysed. Furthermore, differences in genotype could also affect the apparent PhytoPs content and series in plant samples (Carrasco-Del Amor et al., 2016; Collado-González, Medina et al., 2015; Collado-González, Moriana et al., 2015). On this matter, a study of different types of nuts (walnut, macadamia, and pecan) was reported by Carrasco-Del Amor et al. (2017). This work showed that the PhytoP profile varied according to the nut type, and possibly according to the given genotype within specie. This could explain the results in our study - where the qualitative and quantitative PhytoP profiles varied greatly among the cocoa clones, but they were also affected by factors such as the growing conditions and chemical composition with respect to ALA, OA, LA and other lipids, and tocopherols. These compounds varied tremendously within the species, according to environmental variability, geographical origin, temperature, and irrigation (Zhu, Taylor, Sommer, Wilkinson, & Wirthensohn, 2015). Further studies are needed to investigate if the cultivation of the same cocoa clones in different areas has a real effect on the production of specific PhytoPs and PhytoFs.

The importance of PhytoPs and PhytoFs is related not only to plant physiology but also to their biological effects in humans, since they are part of our diet and their presence has already been reported in human biofluids (Barden et al., 2009). They can reach the gastrointestinal tract and interact with the gut microflora. In fact, there is evidence that these bioactive lipid derivatives can modify the functions of the immune systems (Durand et al., 2011) and neuronal systems (Minghetti et al., 2014). Furthermore, there is similarity between the plant and mammalian stress responses. In this sense, Medina et al. (2017) tentatively proposed that the effect of PhytoPs on human physiological

mechanisms could be related to different metabolic processes. For instance, PhytoPs can activate the stress-sensing mammalian transcription factor Nrf2 (nuclear factor erythroid-2) (Heiss et al., 2014). This transcription factor is an emerging regulator of cellular resistance to oxidants and it controls and induces expression of the antioxidant response. Accordingly, Nrf2 activation contributes to several beneficial bioactivities of natural products. In this sense, the benefits of fruit consumption may be due to the interactions of different bioactive compounds (flavonoids, other polyphenols, and PhytoPs, among others), since they could activate the Nrf2 pathway and increase expression of the antioxidant response (Waltenberger, Mocan, Šmejkal, Heiss, & Atanasov, 2016).

In relation to cocoa or its derivatives, the capacity of this food has been hypothesized to activate the Nrf2 factor, but it is associated with flavan-3-ol(-) - epicatechin (Shah et al., 2010). Accordingly, the results of the present study show the presence and diverse concentrations of the different series of PhytoPs in cocoa beans, and can formulate the contribution of PhytoPs in favor of the reduction of the risk of ischemic heart disease. As previously stated by Carrasco-Del Amor et al. (2015) and Marhuenda et al. (2015), this study also supports the importance of PhytoPs and PhytoFs as relevant OS biomarkers in plants.

Furthermore, the FAME profile was determined with the objective of comparing it with the concentrations and profiles of PhytoPs and PhytoFs; as mentioned above, ALA is the precursor of both PhytoPs and PhytoFs, so some relationship between this fatty acid and the oxylipins under study here was expected. Other fatty acids from cocoa beans were measured in order to find possible correlations with PhytoPs and PhytoFs synthesis. The result of this analysis was expressed as g Kg^{-1} and it is presented in Table 5. The most important fatty acids, for types of cocoa beans, were C16:0, and C18:0 and C18:1. Pearson correlations were made between the totals and each of the series of PhytoPs, PhytoFs, and FAMES. The results showed medium linear correlations, with a level of significance of $p < 0.05$ between the total PhytoPs and C18:1

($r = 0.357$); total PhytoFs and C16:1 and C20:0 ($r = 0.406$ and $r = -0.477$, respectively); 9-F₁₁-PhytoP and C18:1 ($r = 0.433$); 9-*epi*-9-D₁₁-PhytoP and C14:0 ($r = -0.439$); *ent*-16-B₁-PhytoP + 16-B₁-PhytoP and C16:1 ($r = -0.395$); *ent*-9-L₁-PhytoP + 9-L₁-PhytoP and C16:1 ($r = -0.391$); *ent*-9-(RS)-12-*epi*-ST- Δ^{10} -13-PhytoF + *ent*-9-(S)-12-*epi*-ST- Δ^{10} -13-PhytoF + *ent*-9-(R)-12-*epi*-ST- Δ^{10} -13-PhytoF and C14:0 ($r = 0.648$); *ent*-16-(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF and C14:0, C16:0; C18:0, and C18:3 ($r = 0.438$, $r = 0.424$, $r = -0.416$, and $r = 0.485$, respectively). Although the correlation of the results is positive in this preliminary analysis, it should not be attributed solely to this correlation among the series of the PhytoPs, PhytoFs and FAMES. In future assays, it should be considered other physicochemical phenomena linked to the biotic and abiotic factors that can occur both in the harvest and postharvest periods of the cocoa fruits. According to previous studies, the formation of these compounds develops from the attack of ROS on ALA. But, as we indicated previously in the results, only *ent*-16-(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF showed a positive correlation, in accordance with Cuyamendous et al. (2015). Notably, flaxseeds showed the highest C18:1 level but had a relatively low *ent*-16-(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF level compared to walnuts and chia seeds, that contained less C18:1. However, pine nuts had the lowest ALA level and the lowest *ent*-16-(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF level; but, it was also established in the same study that the relative concentrations of *ent*-16-(RS)-13-*epi*-ST- Δ^{14} -9-PhytoF do not necessarily express the relative C18:1 concentrations in walnuts and chia seeds.

4. Conclusions

The study of PhytoPs and PhytoFs can be considered recent, and it has been carried out in several matrices; such as rice, walnuts, almonds, melon plants (leaves), macroalgae, wines and musts, vegetables oils, among others. However, this is the first identification and quantification of these compounds in dry fermented cocoa beans.

Table 5
FAMES levels in cacao beans. The values are represented as g Kg^{-1} .

Sample	Myristic acid	Palmitic acid	Palmitic acid	Stearic acid	Oleic acid	Isomer	Linoleic acid	α -Linolenic acid	Gadoleic acid
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:1	C18:2	C18:3	C20:0
Criollo moderno - 1	0.13	48.06	0.51	51.04	51.07	0.75	7.98	0.40	1.80
Criollo moderno - 2	0.24	105.06	1.24	114.15	114.80	1.93	13.30	0.67	4.27
Criollo moderno - 3	0.24	92.34	1.21	110.41	121.60	2.11	11.94	0.86	3.60
Criollo - 1	0.54	90.18	0.83	135.60	127.94	2.04	12.56	0.88	4.51
Criollo - 2	0.11	92.45	0.89	104.09	113.62	2.07	9.75	0.73	3.28
Criollo - 3	0.49	106.09	0.97	128.48	130.84	1.56	13.62	0.92	4.61
Regional - 1	0.21	99.18	0.74	106.02	114.11	1.55	13.53	0.84	3.64
Regional - 2	0.13	77.79	0.93	83.44	89.37	1.47	10.92	0.72	2.44
Regional - 3	0.13	102.95	0.96	146.62	133.79	2.24	13.17	0.76	5.05
Regional - 4	0.13	78.45	1.01	94.56	94.82	1.45	10.21	0.58	3.36
Regional - 5	0.11	73.67	0.79	77.58	92.88	1.96	10.14	0.66	2.73
Regional - 6	0.18	86.97	0.72	111.82	108.42	1.44	12.22	0.75	3.49
Regional - 7	0.18	77.35	0.67	101.38	96.95	1.25	8.70	0.46	3.43
Regional - 8	0.33	79.24	1.31	72.74	84.95	1.20	9.02	0.71	2.45
Regional - 9	0.15	78.32	1.11	104.83	98.65	1.90	11.37	0.61	3.60
Regional - 10	0.17	66.16	0.74	79.17	81.47	1.34	8.20	0.52	2.57
Regional - 11	0.15	78.73	0.93	119.17	107.22	1.98	14.12	0.79	4.03
Albino - 1	0.15	78.73	0.93	119.17	107.22	1.98	14.12	0.79	4.03
Albino - 2	0.12	89.27	0.87	107.04	116.61	1.66	10.89	0.69	3.67
CCN-51	0.09	93.43	0.91	106.23	114.75	1.69	9.91	0.87	3.66
FMT-1	0.15	57.34	0.41	69.10	72.32	0.85	5.73	0.42	2.04
FTU-6	0.17	70.59	1.09	88.17	85.30	1.68	8.88	0.57	2.92
CAU-37	0.07	30.29	0.21	36.27	36.64	0.47	4.35	0.24	1.09
EET-8	0.17	103.32	0.67	151.59	138.63	1.58	12.33	0.72	4.74
EET-96	0.14	73.89	0.72	102.46	99.76	1.62	10.00	0.62	3.10
FMAC-11	0.11	99.56	1.01	117.90	124.88	1.94	14.75	0.84	3.64
FMAC-12	0.16	88.01	0.91	92.59	99.55	1.40	8.30	0.55	3.13
ICS-1	0.15	72.71	0.70	94.49	91.99	1.35	12.10	0.62	3.03
ICS-39	0.14	58.91	0.52	75.29	72.40	1.01	9.06	0.49	2.36
ICS-95	0.40	103.33	0.67	97.73	116.13	1.42	11.90	0.86	3.98
TSH-565	0.26	84.24	0.82	102.16	103.55	1.28	9.77	0.62	3.86

The results obtained show dry fermented cocoa beans to be a rich source of PhytoPs and PhytoFs. In fact, the individual concentrations of the PhytoPs and PhytoFs reported in the present study are higher than in other matrices studied. Moreover, the concentrations of the PhytoPs and PhytoFs determined in the different cocoa clones showed significant differences among them, but unrelated to the classification: criollo, criollo moderno, regional, forastero, and trinitario. One well known characteristic of cocoa is the quantity and quality of its fatty acids and fats, which implies their theoretical correlation with the profiles of the studied compounds. But, taking into account the lack of correlation with ALA and other fatty acids, the variation in the concentrations of PhytoPs and PhytoFs may be attributed to the biotic and abiotic stressors to which the plants are exposed. Also, this variation may be due to other characteristics of the matrix, such as the presence of antioxidants and equivalent molecules, followed by variations in the genetic resource, the origin of the cocoa crop, and the fermentation and drying processes. This information may be very useful for cocoa industries and for the development of the cocoa growing regions.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.12.072>.

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