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# Identification and quantification of phytoprostanes and phytofurans of coffee and cocoa by- and co-products†

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Phytoprostanes (PhytoPs) and phytofurans (PhytoFs) are isoprostanoids that result from the peroxidation of  $\alpha$ -linolenic acid and are biomarkers of oxidative stress in plants and humans. These compounds exhibit several interesting biological activities (e.g. neuroprotection and anti-inflammatory activities). The aim of this research was to add value to coffee pulp (CP), cocoa husk (CH) and cocoa pod husk (CPH) by identifying and quantifying PhytoPs and PhytoFs by liquid chromatography–tandem mass spectrometry. The contents of PhytoPs and PhytoFs in CP, CH, and CPH were, respectively, 654.6, 474.3 and 179.9, and 543.2, 278.0 and 393.8 ng per g dry weight (dw). The main PhytoP found in CP (171.37 ng per g dw) and CPH (37.12 ng per g dw) was 9-*epi*-9- $F_{1t}$ -PhytoP, while *ent*-9- $L_{1t}$ -PhytoP was the most abundant in CH (109.78 ng per g dw). The main PhytoF found in all sources was *ent*-16(*RS*)-13-*epi*-ST- $\Delta^{14}$ -9-PhytoF, at 196.56, 126.22, and 207.57 ng per g dw in CP, CH, and CPH, respectively. We provide the first complete profile of PhytoPs and PhytoFs for these agro-residues, which could be used in the functional food industry for enriching food or as nutritional supplements.

## Introduction

Coffee is one of the most widely traded agricultural commodities and the most frequently consumed beverages in the world.<sup>1</sup> Chocolate and other derived products are widely distributed and consumed around the world (e.g. milk chocolate drinks, solid bars of chocolate, cookies, cakes, and candies).<sup>2</sup> Both products are the most important agro-tropical crops; in 2016, the world production of green coffee and cocoa reached 9.22 and 4.47 million tons, respectively.<sup>3</sup>

Most Arabica green coffee beans (*Coffea arabica* L.) in the world are obtained through the coffee wet process. In this process, the damaged and unripe berries are first eliminated by flotation, and then the outer skin and most of the pulp of

the sunken fruits are mechanically removed by pressing the fruit in water using a pulper. The obtained beans are fermented to remove the layer of pulp remnants and the mucilage layer. After 12–48 h of fermentation, the beans are washed and dried to about 12% moisture content and de-hulled to remove the parchment (Fig. 1). The resulting solid wastes (parchment and silverskin) are collectively termed parchment husks. On average, for every one ton of clean coffee produced, 0.28 tons of parchment husk and 0.5 tons of pulp would be generated.<sup>4</sup> The main coffee waste is thus the pulp (CP) which represents 40% of the fresh weight of coffee cherries.<sup>5</sup>

In the case of cocoa processing significant quantities of side products, mostly cocoa pod husk (CPH) and cocoa husk (CH), are produced.<sup>6</sup> CPH is immediately discarded after the cocoa's harvest and represents 74% of the cocoa fruit weight.<sup>7</sup> After fermentation, drying, and roasting, CH is removed from the cocoa beans, and then the nibs are used for the production of chocolate (Fig. 1). CH represents between 12% and 20% of the total weight of the dry cocoa bean.<sup>8</sup>

Due to the great demand for green coffee and cocoa beans, large amounts of residues are generated after processing in both producing and processing countries, and if badly managed they present a serious disposal problem. For instance, when they are left on the fields, they can lead to

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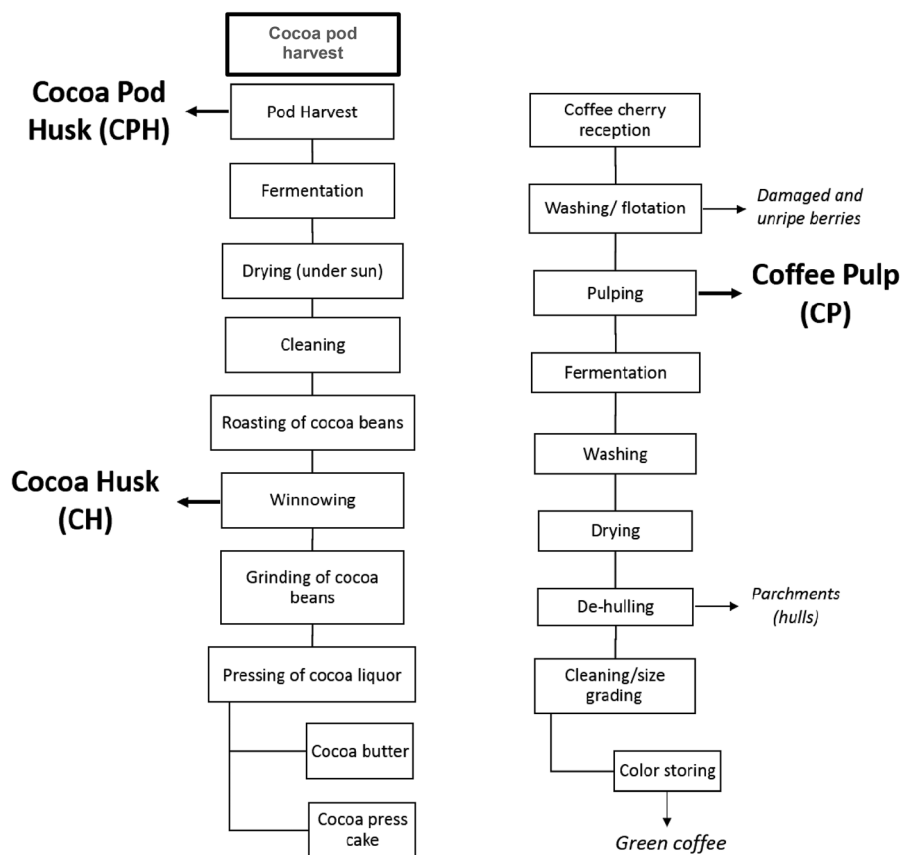


Fig. 1 Diagram of green coffee wet processing (adapted from ref. 40) and cocoa processing (adapted from ref. 8).

environmental problems, creating bad odors, and causing mold diseases and landscape deterioration.<sup>9</sup>

Mexico ranked eleventh and thirteenth in the worldwide production of green coffee (151 714 ton) and cocoa beans (26 836 ton), respectively, in 2016.<sup>10</sup> Considering this, in 2016, the coffee industry generated around 159.9 thousand tons of waste (including hulls, skin mucilage, and pulp).<sup>11</sup> In the case of cocoa, the generation of agricultural waste is estimated to be 241.3 thousand tons including cocoa husk and cocoa pod husk.<sup>8,12</sup>

To face this situation, several studies have been proposed with the aim of adding value to these derived co- and by-products, while decreasing their negative environmental impact. These residues are an interesting and cheap renewable source of biomolecules (carbohydrates such as cellulose, hemicellulose, and pectin, proteins, lipids, and bioactive phytonutrients like polyphenols, pigments, *etc.*), which can be used in the formulation of food and non-food products as they exhibit interesting physicochemical and/or biological activities (*e.g.* surface active agents, gelifying agents, antioxidants,<sup>13,14</sup> and antimicrobials).<sup>15</sup> However, as far as we know, to date, no report has considered coffee and cocoa by- and co-products as a potential source of phytoprostanes (PhytoPs) and phytofurans (PhytoFs).

Isoprostanes (IsoPs) and isofurans (IsoFs) are the products of free radical-induced peroxidation of polyunsaturated fatty

acids (PUFAs), such as arachidonic acid, in animal cells. Similarly, in plants, isoprostanoids such as PhytoPs and PhytoFs are formed by non-enzymatic oxidation of  $\alpha$ -linolenic acid (C18:3 n-3, ALA).<sup>16</sup> The absorption of such molecules by the human body can be through food intake or pollen aspiration.<sup>17</sup> These lipid mediators are likely to play a role in the development of the brain and central nervous system, could also be involved in the prevention of metabolic disorders,<sup>18</sup> seem to present cytoprotective responses in immature brain cells such as neuroblasts and oligodendrocytes,<sup>19</sup> or could act as mediators of oxidative stress.<sup>20</sup> In addition, they could be involved in anti-inflammatory and apoptosis-inducing activities.<sup>21</sup> Consequently, there is growing interest in studying the molecular profile and amount of these kinds of compounds in plant foods and their response to cultural and postharvest transformation practices and/or environmental conditions. These compounds have been found in an array of medicinal plants, and natural or unprocessed and processed plant foods, such as cereals, nuts and oilseeds, macroalgae, some fruits and legumes (for a review of the occurrence of these compounds see ref. 22 and 23). Recently, León-Perez *et al.*<sup>22</sup> reported the presence of PhytoPs and PhytoFs in dry fermented cocoa beans, but as far as we know, no reports exist on the content of these molecules either in coffee or in cocoa by- and co-products. Hence, the interest of this work that aims at the identification and quantification of PhytoPs and PhytoFs from

coffee pulp (CP), cocoa husk (CH) and cocoa pod husk (CPH) is to add value to these agro-residues from the coffee and cocoa industry. Indeed, PhytoP and PhytoF rich extracts could be obtained from these agro-residues to enrich food or to be used as nutritional supplements due to their interesting biological activities.

## Materials and methods

### Biological material

CP (from *Coffea arabica* var. sachimor) was collected in March in Huatusco (Veracruz, Mexico), while CH and CPH (from *Theobroma cacao* L. Criollo) were collected in June in Comalcalco (Tabasco, Mexico), both in 2016. CH was obtained as a by-product of the conventional industrial roasting process from the collection center of AMCO Company located in Tabasco, Veracruz, Mexico. CP and CPH were chopped and dried in a fluidized bed dryer (GRACO, TGA101) in lots of 3 kg, for 90 min at 60 °C. The dried raw material was milled in a kitchen-type grinder (Moulinex Deluxe Stainless Steel Mill 843, France), sieved to particle sizes between 315 and 630  $\mu\text{m}$ , and then stored at  $-20$  °C before experiments.

### Materials

The internal standards (IS) d4-15-F<sub>2t</sub>-IsoPs were purchased from Cayman Chemical (Ann Arbor, MI, USA). The internal standards C19-16-F<sub>1t</sub>-PhytoPs, and the external standards *ent*-16-F<sub>1t</sub>-PhytoP, *ent*-16-*epi*-16-F<sub>1t</sub>-PhytoP, 9-F<sub>1t</sub>-PhytoP, 9-*epi*-9-F<sub>1t</sub>-PhytoP, *ent*-16-B<sub>1t</sub>-PhytoP, and *ent*-9-L<sub>1t</sub>-PhytoP were synthesized according to the previously described procedure. The PhytoP stock solutions were prepared in methanol at a concentration of 100  $\mu\text{g mL}^{-1}$  and stored at  $-20$  °C.

LC-MS methanol, acetonitrile, and HPLC chloroform were purchased from Fisher Scientific (Loughborough, UK). Hexane, formic acid, acetic acid, absolute ethanol, ammonia, and potassium hydroxide (KOH) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ethyl acetate (HPLC grade) and butylated hydroxytoluene (BHT) were supplied by VWR (EC) and Acros Organics (Geel, Belgium), respectively. The water used in this study was purified on a MilliQ system (Millipore) or provided by Fisher Scientific (Loughborough, UK). SPE Oasis MAX 3cc cartridges (60 mg) were obtained from Waters (Milford, MA, USA).

### Extraction of Isoprostanoids

PhytoP and PhytoF extraction was performed in triplicate with approximately 0.1 g of each sample. The plant material was mixed with 2 mL of MeOH containing 1% (v/v) of butylated hydroxytoluene (BHT). Then, the mixture was vortexed and 1.5 mL of phosphate buffer (pH 2) saturated in NaCl, and 6 ng of each IS were added. This solution was stirred for 1 h at 100 rpm at room temperature. Then, the extract was centrifuged at 4000 rpm for 5 min at 25 °C, the supernatant was separated, and 4 mL of cold chloroform were added. This mixture was vortexed for 30 s and then centrifuged at 1500 rpm for

5 min at room temperature. Subsequently, the chloroform phase was separated and concentrated under a nitrogen flow at 40 °C for 2 h. Afterward, 950  $\mu\text{L}$  of KOH were added, the mixture was incubated for 30 min at 40 °C, and 1 mL of formic acid (40 mM, pH 4.6) was added before running the SPE separation. SPE Oasis MAX cartridges were conditioned with 2 mL of MeOH and equilibrated with 2 mL of formic acid (20 mM, pH 4.5). After loading the sample, the cartridges were successively washed with: 2 mL of NH<sub>3</sub> 2% (v/v), 2 mL of a mixture of formic acid (20 mM):MeOH (70:30, v/v), 2 mL of hexane, and finally 2 mL of hexane:ethyl acetate (70:30, v/v). The targeted metabolites were eluted with 2 mL of a hexane:ethanol:acetic acid mixture (70:29.4:0.6, v/v). Lastly, all samples were evaporated to dryness under a nitrogen flow at 40 °C for 30 min, and 100  $\mu\text{L}$  of H<sub>2</sub>O:ACN (0.1% (v/v) formic acid) (83:17, v/v) were added prior to injection into the micro-HPLC-QTRAP system.

### Identification and quantification of isoprostanoids

PhytoPs and PhytoFs, extracted from CP, CH, and CPH, were chromatographed using a micro-LC equipped with CTC Analytics AG (Zwingen, Switzerland), as described by Yonny *et al.*<sup>24</sup> The autosampler vial tray was kept at 10 °C and the column (HALO C18 0.5  $\times$  100 mm, 2.7  $\mu\text{m}$ , Eksigent Technologies, CA, USA) was set at 40 °C. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile:methanol (80:20, v/v; with 0.1% (v/v) of formic acid) (B). The gradient elution program was performed as follows (min/%B): 0/17; 1.6/17; 2.85/21; 7.3/25; 8.8/28.5; 11/33.3; 15/40; 16.5/95; 18.9/95; 19/17; 21/17.

5  $\mu\text{L}$  of the sample was injected and eluted at a flow rate of 0.03  $\text{mL min}^{-1}$ . An AB SCIEX QTRAP 5500 mass spectrometer (Sciex Applied Biosystems) was used to analyze isoprostanoids. The ionization source was an electrospray (ESI), operated in the negative mode. The source voltage was maintained at  $-4.5$  kV and nitrogen was used as the curtain gas. Multiple reaction monitoring (MRM) of each compound was performed, allowing the determination of two transitions, one for quantification (T1), the other for specification (T2) (Table 1). The analysis was conducted by monitoring the precursor ion to product ion (T1) transition. Peak detection, integration and quantitative analysis were performed using MultiQuant 3.0 software (Sciex Applied Biosystems).

Isoprostanoid quantification was based on the calibration curves obtained from the ratio of analyte to IS area under the curve. Linear regression of 6 concentration levels of the PhytoP and PhytoF mixture (16, 32, 64, 128, 256 and 512  $\text{pg } \mu\text{L}^{-1}$ ) of each analyte is presented in Table 2. The sensitivity of the method is evaluated through LOD and LOQ parameters which were defined as the lowest concentration with a signal to noise ratio above 3 and 10 respectively. According to the type of isoprostanoids, LOD and LOQ range from 0.16 to 0.63  $\text{pg injected}$  and 0.16 to 1.25  $\text{pg injected}$ , respectively.

In order to validate the methodology, the extraction yield (EY) and matrix effect (ME) were evaluated in order to assess the efficiency of sample processing. These parameters, specific to each compound (external standards and IS), allow the evalu-

**Table 1** PhytoP and PhytoF quantification by micro-LC-MS/MS<sup>a</sup>

Compounds	Calibration curve	R <sup>2</sup>	Weighting
<i>ent</i> -16-F <sub>1c</sub> -PhytoP	$y = 1.06857x$	0.997	1/x
<i>ent</i> -16- <i>epi</i> -16-F <sub>1c</sub> -PhytoP	$y = 0.87312x$	0.998	1/x
9-F <sub>1c</sub> -PhytoP	$y = 0.79830x$	0.998	1/x
9- <i>epi</i> -9-F <sub>1c</sub> -PhytoP	$y = 0.71005x$	0.998	1/x
<i>ent</i> -16-B <sub>1c</sub> -PhytoP	$y = 1.92920x$	0.991	1/x
<i>ent</i> -9-L <sub>1c</sub> -PhytoP	$y = 2.87653x$	0.983	1/x
<i>ent</i> -16(RS)-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF	$y = 1.71852x$	0.998	1/x
<i>ent</i> -9(RS)-12- <i>epi</i> -ST-Δ <sup>10</sup> -13-PhytoF	$y = 0.72222x$	0.998	1/x
<i>ent</i> -16(RS)-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF	$y = 1.95197x$	0.997	1/x

<sup>a</sup> Six data points ( $n = 3$ );  $y$ : the peak area of the analyte;  $x$ : the concentration of the analyte (pg μL<sup>-1</sup>). The deuterated form of IsoPs (D<sub>4</sub>-15F<sub>2c</sub>-IsoP) and C19-16-F<sub>1c</sub>-PhytoP were used as internal standards for quantification of samples in this study.

**Table 2** Multiple reaction monitoring (MRM) of PhytoPs and PhytoFs

Compounds	R <sub>t</sub> (min)	Precursor ions ( $m/z$ )	Product ions ( $m/z$ )
<i>Ent</i> -16-B <sub>1c</sub> -PhytoP_T1	10.50	307.2	235
<i>Ent</i> -16-B <sub>1c</sub> -PhytoP_T2	10.50	307.2	223
9/16-F <sub>1c</sub> -PhytoP_T1	5.58–6.27	327.2	283.2
9-F <sub>1c</sub> -PhytoP_T2	5.75	327.2	171.2
9- <i>epi</i> -9-F <sub>1c</sub> -PhytoP_T2	6.27	327.2	171.2
16-F <sub>1c</sub> -PhytoP_T2	6.04	327.2	251.2
16- <i>epi</i> -16-F <sub>1c</sub> -PhytoP_T2	5.58	327.2	251.2
<i>Ent</i> -9-L <sub>1c</sub> -PhytoP_T1	10.67	307.2	185.1
<i>Ent</i> -9-L <sub>1c</sub> -PhytoP_T2	10.67	307.2	197
<i>Ent</i> -16(RS)-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF_T1	4.68	343.2	201
<i>Ent</i> -16(RS)-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF_T2	4.68	343.2	127
<i>Ent</i> -16(RS)-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF_T1	4.68	343.2	209.1
<i>Ent</i> -16(RS)-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF_T2	4.68	343.2	199
<i>Ent</i> -9(RS)-12- <i>epi</i> -ST-Δ <sup>10</sup> -13-PhytoF_T1	4.56	343.2	237.1
<i>Ent</i> -9(RS)-12- <i>epi</i> -ST-Δ <sup>10</sup> -13-PhytoF_T2	4.56	343.2	86.9

ation of product losses due to the extractive process or a skew in the mass quantification due to ion suppression or an increase caused by the sample matrix and interference from metabolites. The experiments described below were performed in triplicate for each plant material (CP, CH, and CPH). The EY is calculated by comparing the area under the peak observed for a sample spiked by a known concentration of metabolites at the end of the extractive process ( $A_{\text{After}}$ ) and the area for an equivalent sample spiked at the beginning of the extractive process ( $A_{\text{Before}}$ ). The results, expressed in percentage ( $EY = A_{\text{Before}}/A_{\text{After}} \times 100$ ), are given for two concentrations of standards (32 and 256 pg μL<sup>-1</sup> of compounds with IS). The ME is calculated by comparing the area under the peak observed for a sample spiked by a known concentration of metabolites at the end of the extractive process ( $A_{\text{After}}$ ) and the area under the peak observed for pure standards in the mobile phase at the same concentration ( $A_{\text{Pure}}$ ). The calculation is:  $ME = A_{\text{After}}/A_{\text{Pure}} \times 100$ .

### Fatty acid identification and quantification

The CP, CH, and CPH lipid determination was performed by Soxhlet extraction using chloroform as a solvent according to the AOAC method.<sup>41</sup> Afterward, the extracts were converted into

methyl esters (FAMES) according to the NF T60-233 method.<sup>42</sup> The fatty acid profile was determined on a GC Agilent 6890 (Bios Analytique, France) series, equipped with an INNOWax capillary column (SGE, Courtaboeuf, France), with the following characteristics: length, 30 m; internal diameter, 0.32 mm, and film thickness, 0.25 mm. Helium was used as the carrier gas with a flow of 1 mL min<sup>-1</sup> and a splitting ratio of 1/80 was used. The temperature of the injector was 250 °C and the flame ionization detector was set at 270 °C. The oven was heated from 185 °C to 225 °C at 5 °C min<sup>-1</sup> and maintained at 225 °C for 20 min. FAMES were identified by comparison with commercially available standards (Sigma-Aldrich, France). The ALA content was expressed as mg per g dw of each plant material, according to the relative amount (% w/w) of this fatty acid in the lipid fraction.

### Statistical analysis

Statistical analysis (one-way analysis of variance ANOVA) was performed using Minitab software version 18 (Minitab Inc., USA). The level of significance was set at  $p < 0.05$ . The results are expressed as the mean of triplicates (mean values ± standard deviations). Tukey's test was used to determine which mean values were different.

## Results and discussion

PhytoPs and PhytoFs are produced by non-enzymatic oxidation of ALA which are primary metabolites commonly found in the plant membranes. Thus, it would not be surprising to identify such molecules in coffee and cocoa by- or co-products. The identification and quantification assays were performed on Mexican coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH).

The recovery of PhytoPs and PhytoFs can be influenced by several factors, such as the efficiency of separation during the liquid/liquid extraction step, the affinity of compounds for the selected phases or the selectivity of the used SPE cartridges.<sup>25</sup> In addition, the possible PhytoP and PhytoF co-elution with the compound(s) present in the raw CP, CH, and CPH materials may affect the ionization during LC-MS/MS analysis, therefore leading to an inaccuracy in measurements.<sup>26</sup> Thus, taking into account these crucial factors, EY and ME parameters are determined. These data allow us to better define the method and deduce by calculation a value closer to reality of metabolite rates present in the raw material. In this study, we observed an excellent recovery rate for each matrix, notably for PhytoFs of CP with the EY ranging from 95 to 97% (Table 3). In this matrix, the PhytoPs' EY varies between 71 and 96%. In the case of CH, the EYs were observed to range from 72 to 97% and 76 to 86% for PhytoPs and PhytoFs, respectively. Finally, for CPH, the values are between 95 and 111% for PhytoPs and 88 and 96% for PhytoFs. The EY of a few metabolites reaching more than 100% may correspond to the co-elution of a compound that presents the same MRM transition.



**Table 3** Percentage of matrix effect and extraction yield of isoprostanoid derivatives

Compounds	Concentration (pg $\mu\text{L}^{-1}$ )	ME <sup>a</sup> (%)			EY <sup>b</sup> (%)		
		CP	CH	CPH	CP	CH	CPH
16-F <sub>1t</sub> -PhytoP	32	63.0	50.3	65.4	84.9	78.4	110.7
	256	55.7	49.8	42.8	95.7	79.6	102.2
16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP	32	36.7	52.1	55.8	70.8	71.7	111.5
	256	30.4	50.2	41.4	92.0	74.1	102.4
9-F <sub>1t</sub> -PhytoP	32	52.9	64.5	65.5	78.2	73.6	103.2
	256	44.6	54.0	44.1	92.9	76.3	94.6
9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP	32	57.8	64.7	66.5	77.8	77.9	105.9
	256	51.6	62.0	48.8	92.9	74.1	98.8
<i>Ent</i> -16-B <sub>1t</sub> -PhytoP	32	31.3	50.0	50.0	75.5	94.2	103.6
	256	38.6	52.8	42.4	92.2	80.6	98.6
<i>Ent</i> -9-L <sub>1t</sub> -PhytoP	32	39.9	52.1	49.8	74.9	96.6	101.4
	256	48.4	54.2	42.3	94.1	83.1	100.6
<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST- $\Delta^{14}$ -10-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	52.9	46.7	33.4	97.2	84.3	87.6
<i>Ent</i> -9( <i>RS</i> )-12- <i>epi</i> -ST- $\Delta^{10}$ -13-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	50.1	46.2	52.3	95.2	75.9	95.6
<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST- $\Delta^{14}$ -9-PhytoF	32	ND	ND	ND	ND	ND	ND
	256	49.7	63.1	103.2	97.3	86.1	93.6

<sup>a</sup> Matrix effect (%) measured by micro-LC-MS/MS for coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH). <sup>b</sup> Extraction yield (%) measured by micro-LC-MS/MS for coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH). ND: not determined.

In this study, an important matrix effect (CP, CH, and CPH) on the isoprostanoid estimation was observed (Table 3), either by decreasing (for PhytoPs) or by increasing (for PhytoFs) the content of the analyzed compounds. For instance, the matrix effect for different PhytoPs ranged from 30.4% to 63%, 49.8% to 64.7%, and 41.4% to 66.5%, in CP, CH, and CPH, respectively, whereas it ranged from 49.7% to 52.9%, 46.2% to 63.1%, and 33.4% to 103.2%, in CP, CH, and CPH, respectively, for PhytoFs (Table 3). Thus, one may say that the quantity of PhytoPs is actually underestimated with respect to the actual quantity in the starting materials, whereas it is likely overestimated for PhytoFs.

#### Analysis of phytoprostanes in CP, CH, and CPH

CP, which is obtained after depulping during coffee processing (Fig. 1), has the highest PhytoP concentration. In traditional

primary green coffee wet processing, CP undergoes natural fermentation, as it is left overnight at ambient temperature before being treated or discarded. To avoid uncontrolled fermentation, which could influence the PhytoP concentration, the CP used in this study was immediately dried after the depulping step. The total PhytoP content in CP was 654.6 ng per g dw, 9-*epi*-9-F<sub>1t</sub>-PhytoP (171.4 ng per g dw) and 9-F<sub>1t</sub>-PhytoP (145.6 ng per g dw) being the major-quantified PhytoPs (Table 4, ESI†). These two compounds have already been reported in cocoa beans<sup>22</sup> and as olive oil biomarkers of water stress in olive trees, as their content in extra virgin olive oil correlated with water deficiency in olive tree plantations.<sup>27</sup> Similarly, an important variation of 9-F<sub>1t</sub>-PhytoP was identified in melon leaves when the plant was exposed to thermal stress.<sup>24</sup> In our case, the presence of these PhytoPs was not dependent on the hydric stress in coffee trees, as coffee was harvested in a region located 1300 m above the

**Table 4** Qualitative and quantitative profile of isoprostanoids in coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH)

Compounds	Isoprostanoid concentration <sup>a</sup> (ng per g dw)		
	CP	CH	CPH
<i>Ent</i> -16-F <sub>1t</sub> -PhytoP	123.4 ± 3.1 <sup>a</sup>	41.2 ± 2.7 <sup>a</sup>	19.0 ± 1.6 <sup>a</sup>
16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP	86.8 ± 2.5 <sup>b</sup>	39.6 ± 2.6 <sup>a</sup>	20.2 ± 1.6 <sup>a</sup>
9-F <sub>1t</sub> -PhytoP	145.6 ± 6.2 <sup>c</sup>	76.5 ± 3.0 <sup>b,c</sup>	37.0 ± 0.8 <sup>b</sup>
9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP	171.4 ± 4.8 <sup>d</sup>	67.7 ± 1.4 <sup>c</sup>	37.1 ± 1.1 <sup>b</sup>
<i>Ent</i> -16-B <sub>1t</sub> -PhytoP	60.7 ± 2.2 <sup>c</sup>	110.5 ± 9.5 <sup>b</sup>	34.0 ± 4.0 <sup>b</sup>
<i>Ent</i> -9-L <sub>1t</sub> -PhytoP	66.7 ± 3.0 <sup>c</sup>	138.8 ± 12.5 <sup>d</sup>	32.6 ± 3.3 <sup>b</sup>
<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> -ST- $\Delta^{14}$ -10-PhytoF	163.2 ± 5.8 <sup>f</sup>	62.8 ± 5.6 <sup>f</sup>	43.5 ± 1.2 <sup>f</sup>
<i>Ent</i> -9( <i>RS</i> )-12- <i>epi</i> -ST- $\Delta^{10}$ -13-PhytoF	183.4 ± 7.1 <sup>g</sup>	89 ± 0.7 <sup>g</sup>	142.7 ± 8.9 <sup>g</sup>
<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> -ST- $\Delta^{14}$ -9-PhytoF	196.6 ± 0.6 <sup>g</sup>	126.2 ± 6.6 <sup>h</sup>	207.6 ± 11.7 <sup>h</sup>

<sup>a</sup> Expressed as dry weight and quantified by micro-LC-MS/MS. The results are the mean of triplicates (mean values ± standard deviations measured according to Pearson (divided by *n*), values followed by the same letter are not significantly different in Tukey's test using 95% confidence level). Coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH).

sea level, with an average annual temperature and rainfall of 19.1 °C and 1825 mm, respectively,<sup>28</sup> corresponding to good Arabica growing conditions. Indeed, according to Chapagain and Hoekstra, the water requirement of a coffee plant is about 1277 mm per year.<sup>29</sup> Thus, the PhytoP amounts extracted from CP could be considered as base values under the optimal climate conditions for growing Arabica coffee trees. Monitoring the PhytoP concentration in plants grown under hydric and/or thermal stress could allow the determination of the relationship between the PhytoP content and these factors. Hence, this information could also be used as biomarkers of hydric and/or thermal stress in coffee trees, as previously demonstrated for olive trees and melon leaves.

On the other hand, it would also be interesting to investigate how different edaphoclimatic conditions influence the qualitative and quantitative profile of PhytoPs and PhytoFs in the coffee plant and each by- and co-product.

Regarding cocoa products, the total content of PhytoPs in CH and CPH was, respectively, 474.3 and 179.9 ng per g dw (Table 4, ESI<sup>†</sup>). Post-harvest processing could partially explain this value, since CH was obtained after the fermentation and roasting steps (Fig. 1). The main compound found in CH was *ent*-9-*L*<sub>1t</sub>-PhytoP (138.8 ng per g dw), followed by *ent*-16-*B*<sub>1t</sub>-PhytoP (110.5 ng per g dw) and 9-*F*<sub>1t</sub>-PhytoP (76.5 ng per g dw). In comparison, in cocoa beans, the most abundant PhytoP is 9-*F*<sub>1t</sub>-PhytoP (114.52–1134.08 ng g<sup>-1</sup>).<sup>22</sup> These com-

Chemical structure	Name
<b>Phytosteranes</b>	
	16- <i>F</i> <sub>1t</sub> -PhytoP
	16- <i>epi</i> -16- <i>F</i> <sub>1t</sub> -PhytoP
	9- <i>F</i> <sub>1t</sub> -PhytoP
	9- <i>epi</i> -9- <i>F</i> <sub>1t</sub> -PhytoP
	<i>Ent</i> -16- <i>B</i> <sub>1t</sub> -PhytoP
	<i>Ent</i> -9- <i>L</i> <sub>1t</sub> -PhytoP
<b>Phytofurans</b>	
	<i>Ent</i> -16( <i>RS</i> )-9- <i>epi</i> - <i>ST</i> -Δ <sup>14</sup> -10-PhytoF
	<i>Ent</i> -9( <i>RS</i> )-12- <i>epi</i> - <i>ST</i> -Δ <sup>10</sup> -13-PhytoF
	<i>Ent</i> -16( <i>RS</i> )-13- <i>epi</i> - <i>ST</i> -Δ <sup>14</sup> -9-PhytoF

Fig. 2 Chemical structures of phytosteranes and phytosterols present in coffee pulp (CP), cocoa husk (CH), and cocoa pod husk (CPH).

pounds have already been identified in different rice flours.<sup>30</sup> The 9-*L*<sub>1t</sub>-PhytoP series has been reported in melon leaves,<sup>24</sup> extra virgin olive oil,<sup>27</sup> walnut, macadamia nuts, and pecan nuts as minor components.<sup>17</sup> In addition, it was observed that frying at 130 °C increased the concentration of these compounds in macadamia nuts by 2.3-fold.<sup>17</sup>

9-*F*<sub>1t</sub>-PhytoP and 9-*epi*-9-*F*<sub>1t</sub>-PhytoP (~37 ng per g dw), *ent*-16-*B*<sub>1t</sub>-PhytoP (34.0 ng per g dw), and *ent*-9-*L*<sub>1t</sub>-PhytoP (32.6 ng per g dw) were the main compounds extracted from CPH. The stereoisomer 9-*F*<sub>1t</sub>-PhytoP was found in CP (145.6 ng per g dw) and CPH (37.0 ng per g dw). The 9-*F*<sub>1t</sub>-PhytoP series was predominant in kernel nuts, up to 7.8 ng g<sup>-1</sup>.<sup>17</sup> In olive trees, hydric stress has been shown to increase the content of certain *F*<sub>1t</sub>- and *B*<sub>1t</sub>-PhytoPs. Accordingly, the authors proposed that 9-*F*<sub>1t</sub>-PhytoP, 9-*epi*-9-*F*<sub>1t</sub>-PhytoP, and 16-*B*<sub>1t</sub>-PhytoP + *ent*-16-*B*<sub>1t</sub>-PhytoP could be considered as early biomarkers of water stress.<sup>27</sup> On the other hand, the *B*<sub>1t</sub>-PhytoP series has been found in high levels in linseed oil,<sup>31</sup> and is likely involved in the regulation of gene expression in *Arabidopsis thaliana* and tobacco.<sup>32</sup>

Several vegetable matrices have been characterized by their total PhytoP content. The retrieved concentrations of PhytoPs in CP, CH and CPH suggest that they are valuable sources of these compounds. Our results are consistent with those found in cocoa beans (221.46–1589.83 ng g<sup>-1</sup>),<sup>22</sup> brown macroalgae (*Ectocarpus siliculosus*) (310 ng g<sup>-1</sup>),<sup>39</sup> and leaves, flowers and roots of different plants (43–1380 ng g<sup>-1</sup>),<sup>16</sup> and they are similar to or higher than those found in almonds (40.25–238.09 ng g<sup>-1</sup>),<sup>33</sup> musts (21.4–447.1 ng g<sup>-1</sup>),<sup>34</sup> rice bran (22.20–118.00 ng g<sup>-1</sup>) and white and brown rice flours (2.55–32.89 ng g<sup>-1</sup>),<sup>33</sup> *Passiflora edulis* Sims shell (1.3–67.6 ng g<sup>-1</sup>),<sup>35</sup> and raw green table olive fruits (5.81–9.99 ng g<sup>-1</sup>).<sup>36</sup> Dry leaves of melon plants subjected or not subjected to thermal stress (2620–3210 and 4620–6280 ng g<sup>-1</sup>, respectively),<sup>24</sup> fertigated French beans (*Phaseolus vulgaris*) (22 641 ng g<sup>-1</sup>), pea (132 755 ng g<sup>-1</sup>), and “manget-out” (*Pisum sativum* spp. *arvense*) (263 134 ng g<sup>-1</sup>)<sup>37</sup> are the ones with the highest total PhytoP concentration found in the literature. More recently, León-Perez *et al.*<sup>22</sup> reported a total PhytoP and PhytoF content ranging from 221.46 to 1589.83 ng g<sup>-1</sup> and from 1.18 to 13.13 ng g<sup>-1</sup>, respectively, in dry fermented cocoa beans.

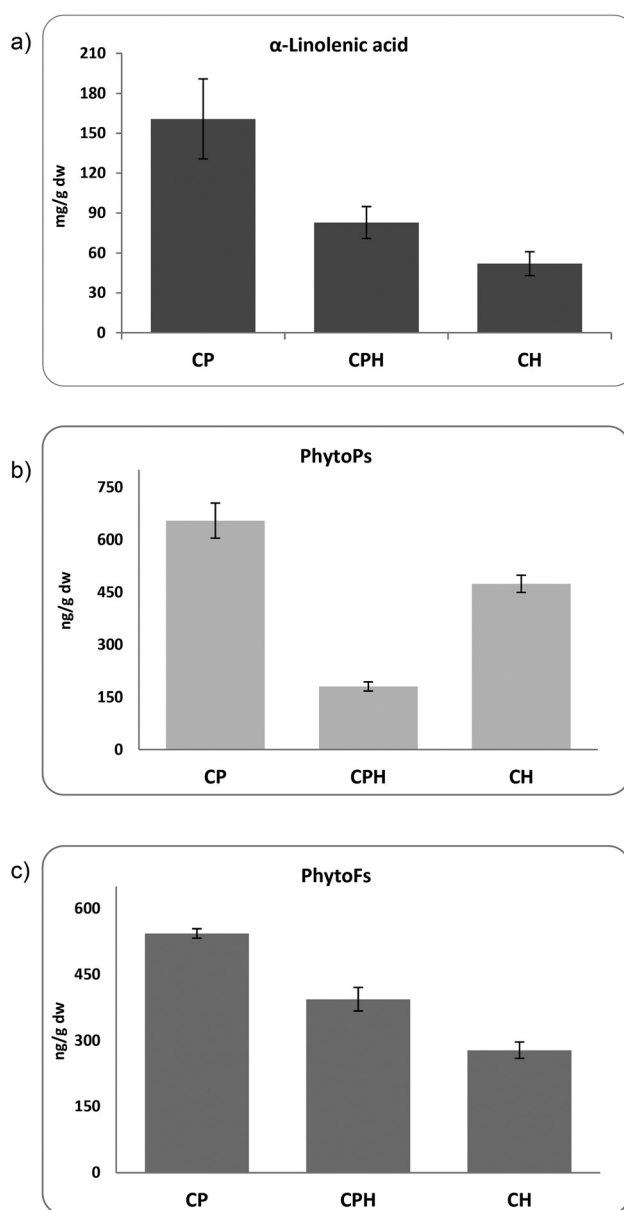
In our study, CP was the material with the highest content of PhytoPs (654.6 ng per g dw). This result could be ascribed to the ALA concentration since CP has the highest content of ALA (160.7 mg per g dw), in comparison with CPH and CH (82.8 and 52.1 mg per g dw, respectively) (Fig. 3). Moreover, CPH showed the lowest PhytoP concentration (181.2 ng per g dw), likely because this by-product did not undergo further processing steps, except a moderate drying before being stored (Fig. 3).

In conclusion, considering the isoprostanoids' interest (biological activities among others), the total amount of PhytoPs in CP, CH, and CPH (ranging from 179.9 to 654.6 ng g<sup>-1</sup>) is very interesting and could confer new applications for these unexploited agro-residues.

### Phytofurane analysis in CP, CH, and CPH

Three different PhytoFs were characterized in our samples (Table 4, Fig. 2). Similar to PhytoPs, these molecules are produced by non-enzymatic oxidation of PUFAs *via* a parallel pathway after the initial endoperoxide formation and further oxygenation is required.<sup>38</sup>

*Ent*-16(*RS*)-9-*epi*-ST-Δ<sup>14</sup>-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ<sup>10</sup>-13-PhytoF, and *ent*-16(*RS*)-13-*epi*-ST-Δ<sup>14</sup>-9-PhytoF were identified in CP, CH, and CPH. As shown in Table 4, the total PhytoF content in ng per g dw was: 543.2 (CP), 278.0 (CH), and 393.8 (CPH), showing that these by- and co-products are



**Fig. 3** Comparison between the concentrations of α-linolenic acid, phytoprostanes (PhytoPs) and phytofurans (PhytoFs) in coffee pulp (CP), cocoa husk (CH) and cocoa pod husk (CPH). The values of each column are the mean of triplicates (mean values ± standard deviations).



an interesting source of PhytoFs. These values are higher than the ones found in, e.g. dry fermented cocoa beans (1.18 to 13.13 ng g<sup>-1</sup>)<sup>22</sup> and white and brown rice flours and rice bran (0.69, 4.2, and 10.3 ng per g dw, on an average, respectively),<sup>30</sup> and similar to those reported in brown macroalgae (*Ectocarpus siliculosus*) (486 ng g<sup>-1</sup>),<sup>39</sup> but lower than those found in pea (*Pisum sativum* var. BGE-033620) (4258 ng g<sup>-1</sup>)<sup>37</sup> and dry leaves of melon plants subjected or not subjected to thermal stress (1960–2750 and 3250–4810 ng g<sup>-1</sup>, respectively).<sup>24</sup> The epimeric mixture of *ent*-16(*RS*)-13-*epi*-ST- $\Delta$ <sup>14</sup>-9-PhytoF predominates in the three samples. These compounds were identified and quantified for the first time in different nuts and seeds in concentrations up to 10 and 16.06 ng g<sup>-1</sup> in walnut<sup>18</sup> and brown rice flour,<sup>30</sup> respectively. The second main compound extracted from CP (183.4 ng per g dw), CH (89.0 ng per g dw), and CPH (142.7 ng per g dw) was *ent*-9(*RS*)-12-*epi*-ST- $\Delta$ <sup>10</sup>-13-PhytoF, which was already detected in melon leaves<sup>24</sup> and in brown rice bran (4.6 ng per g dw).<sup>30</sup> The less predominant PhytoF in CP, CH, and CPH was *ent*-16(*RS*)-9-*epi*-ST- $\Delta$ <sup>14</sup>-10-PhytoF, present at 163.2, 62.8, and 43.5 ng per g dw, respectively. This compound was identified in different rice cultivars and the highest concentrations detected were 1.25, 4.21, and 12.88 ng per g dw for white flour, brown flour, and bran, respectively.<sup>30</sup>

The highest concentration of PhytoFs was detected in CP, followed by CPH and CH. Interestingly, the same order in the concentration of ALA was observed in our investigated samples (160.7, 82.8, and 52.1 mg per g dw) in CP, CPH, and CH, respectively (Fig. 3). It would be interesting to quantify these compounds from freshly harvested samples of CP, CH, and CPH, to determine the influence of primary and/or secondary processing on the content of ALA, PhytoFs, and PhytoFs, in order to demonstrate whether a correlation exists. Some authors working on nuts, seeds, and animal tissues show that the PhytoF concentration was not ALA-dependent, but probably oxidative stress condition-dependent.<sup>33</sup> Anyhow, the quantification of PhytoPs and PhytoFs could provide a unique sensitive indication of oxidant injury (crop conditions and postharvest factors).

## Conclusions

The coffee and cocoa co- and by-products present a major problem for waste disposal, leading to disease propagation or environmental issues, both in the producing countries and in the processing countries. To the best of our knowledge, this is the first time that a qualitative and quantitative profile of PhytoPs and PhytoFs in CP, CH, and CPH has been determined. The concentrations of these compounds retrieved from coffee and cocoa by- and co-products suggest that these residues are a valuable source of PhytoPs and PhytoFs. Thus, this research contributed to complete the actual portfolio of interesting biomolecules derived from coffee and cocoa by- and co-products. Further research is needed to investigate how different edaphoclimatic and processing conditions, as well as

the species, influence the qualitative and quantitative profile of PhytoPs and PhytoFs in coffee and cocoa co- and by-products. The use of such residues as a source of bioactive compounds could provide an economical alternative to the farmers and smallholders in the producing countries. Indeed, taking into account the wide range of biological activities shown here (anti-inflammatory activities, apoptosis, immune function regulation, and neuroprotection) and those which still remain to be elucidated, there is scope for the implementation of such compounds. In this context, PhytoP and PhytoF rich extracts could be obtained from these residues to be used in the functional food industry for enriching food or as nutritional supplements.

Although additional studies are necessary, this work opens new insights and prospects, not only to add value to these agro-wastes but also to pave the way towards the interest of enlarging this study to green coffee bean and cocoa nibs and their derived products.

## Abbreviations

ACN	Acetonitrile
ALA	$\alpha$ -Linolenic acid (C18:3 n-3)
BHT	Butylated hydroxytoluene
CH	Cocoa husk
CP	Coffee pulp
CPH	Cocoa pod husk
dw	Dry weight
ESI	Electrospray
EY	Extraction yield
FAMES	Fatty acid methyl esters
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IS	Internal standards
IsoFs	Isosofurans
IsoPs	Isoprostanes
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix effect
MeOH	Methanol
MRM	Multiple reaction monitoring
PhytoPs	Phytosterols
PhytoFs	Phytosterols
PUFAs	Polyunsaturated fatty acids
QTRAP	Triple-quadrupole mass spectrometer combined with a linear ion trap
SPE	Solid phase extraction

## Conflicts of interest

There are no conflicts of interest to declare.

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