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Title: Cocoa bean husk: industrial source of antioxidant phenolic extract

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ABSTRACT

BACKGROUND: Cocoa bean husk (CBH) is the principal by-product of the cocoa industry and a significant agro-industrial residue. Using different hydrothermal treatments of CBH, we show that CBH is an important source of bioactive compounds including theobromine, epicatechin, and catechin.

RESULTS: Treatment over 150 °C significantly increased the yield of total and individual phenols and theobromine, as well as the antioxidant capacity of the liquid fraction. A total of 52 different genotypes of cocoa bean husk harvested in two seasons of production were analyzed. Overall, higher amounts of total phenols, theobromine, and epigallocatechin were detected in samples from the 2015 season while samples from 2014 had higher quantities of catechin and similar quantities of epicatechin.

CONCLUSION: CBH treatment at 170 °C for 30 minutes produces an antioxidant-rich extract, high in phenols (5.5 %), sugars (22 %), and theobromine (5.6 %) that is suitable for applications in the food, cosmetic or nutraceutical industries.

Keywords: cocoa bean husk, epicatechin, theobromine, hydrothermal treatment, cocoa genotypes.

INTRODUCTION

Cocoa bean husk (CBH) is the principal by-product of the cocoa industry and is considered an agro-industrial residue. CBH is part of the cocoa bean, separated from the cotyledons together with the germ during the pre-roasting process or after the roasting process of *Theobroma cacao*.¹ About 27,000 tons of dried cocoa beans are produced annually in Mexico (<http://infosiap.siap.gob.mx>), and if the CBH constitutes approximately 12 % of the beans' weight,² a total of 3240 tons of CBH residue are generated. In the cocoa producing regions of Mexico, there are some examples of artisanal CBH use, such as the making of cookies, and occasionally the production of organic fertilizer (bokashi) for local use. However, the vast majority of husks are disposed of as waste.

Our preliminary study on cocoa beans suggested that after its fermentation, CBH may be an important source of bioactive compounds, such as theobromine and phenols, which, together with the high fiber content and antibacterial activity of CBH make it an interesting novel ingredient for the food industry.³ Other studies also highlighted the potential application of some of CBH's properties within the field of nutraceutical foods, ranging from its high content of phenolic compounds, to around 18.2 mg g⁻¹ dietary fiber¹, such as cellulose, pectin, and lignin;⁴ high nutritional quality of fat (6.7 %), with a particularly high linoleic acid composition;⁵ and protein (17 %).

The phenolic compounds that have been identified in methanol extracts of CBH include epicatechin and p-hydroxybenzoic acid, and the theobromine content of CBH is around 12.9 mg g⁻¹.¹ It has been proved that phenolic compounds are stored in the cotyledons of the cocoa seeds.⁶ However, during fermentation, a significant amount of these compounds is lost by diffusion out of the cotyledon. In fact, part of the phenols present

in the cotyledon migrate to the husk, converting CBH into a bioactive compound-rich material after fermentation.⁴

Recent studies on the potential uses of CBH indicate that it could be effectively employed in the development of activated carbon monoliths due to its lignocellulosic structure.⁷ In feed formulations for pigs in which CBH has been added, positive nutritional benefits have also been found, including an improvement of intestinal microbial balance.⁸

Other studies have focused on demonstrating the anticaries activity of CBH extracts, revealing that it reduces the development of almost all oral streptococci studied in humans by decreasing acid production. In addition, the synthesis of insoluble glucans by the glucosyltransferases of *Streptococcus mutans* and *S. sobrinus* is significantly inhibited by CBH extract.⁹⁻¹³ In the study carried out by Osawa et al.,¹⁰ in which the cariostatic substances present in the CBH were isolated and characterized, it was found that the fraction with the highest antiglucosyl-transferase activity was the one with the highest polyphenol content and the presence of epicatechin polymers with intermolecular bonds C-4 and C-8 (C-6). In other studies, the presence of proanthocyanidins like procyanidin B2 and cinnamtannin A2 were detected in CBH.¹⁴ Furthermore, Kopp and Bradbury¹⁵ patented a method for the extraction of a polyphenol-enriched or a theobromine-enriched fraction from CBH by extraction with an acidified ethanol solution.

Several studies have presented CBH as a potential source of bioactive compounds like theobromine, phenols, and the cell wall lignin, cellulose, and hemicellulose for use in the food, cosmetic or pharmaceutical industries, but no industrial treatment exists for the utilization of these valuable by-products. The aim of this work is to test industrial technologies like thermal treatment from 50 °C to 200 °C – currently applied for the

utilization and valorization of other lignocellulosic by-products from the olive oil, asparagus or date industries^{16,17} – for the extraction of bioactive compounds from the cocoa bean husks of several Mexican genotypes.

MATERIALS AND METHODS

Samples.

CBH was removed from cotyledon by hand, crushed with a mill (Culatti, Italy), and separated using a 0.5 micrometer screen.

A total of 52 CHB samples (at 7 % humidity) were extracted from different varieties of cacao, which were harvested in the 2013-2014 and 2014-2015 seasons, belonging to the Germplasm Bank of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP), Huimangillo, Mexico.

Chemicals.

The standards of gallic acid (GA), caffeic acid, (–)-epicatechin, (+)-catechin hydrate, theobromine, trifluoroacetic acid (TFA), anthrone, Folin-Ciocalteu's phenol reagent, and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich (Madrid, Spain). Sodium bicarbonate (Na_2CO_3), ethanol, and methanol were from Panreac Quimica S.A. (Barcelona, Spain). Concentrated HCl was purchased from Fluka (Switzerland). Acetonitrile was of HPLC-grade purity (Romyl, Teknokroma, Barcelona, Spain) and acetone was obtained from Scharlau (Barcelona, Spain).

Phenolic extraction methods.

For the characterization, extracts were produced from a homogeneous sample of CHB by four different methods previously used with cacao cotyledon³ with the following solvents: methanol [Method A], ethanol-acetone at pH3 [Method B], water [Method C], methanol at pH 3 [Method D], and water at pH3 [Method E]. Methods A, B, C, D and E were carried out as described.³

Total phenolic contents.

The total phenol content was determined by the Folin-Ciocalteu spectrophotometric method¹⁸ and expressed as grams of gallic acid equivalents for the extracts obtained by Method D.

Total sugars.

Total sugars were analyzed by the Anthrone colorimetric method¹⁹ using a spectrophotometer (BIO-RAD iMark Microplate Reader, USA) for the extracts obtained by Method D.

Antiradical activity.

The antioxidant activity of each phenolic extract was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described in a previous study.¹⁸ This method is based on the measurement of the free-radical scavenging capacity of the antioxidant against the stable radical DPPH. A microplate reader (Bio-Rad 550 model, Hercules, CA, US) was used for the absorbance measurements. The result of the activity of each extract was expressed as an EC₅₀ (effective concentration, mg/mL). The amount of antioxidant necessary to decrease the initial absorbance by 50 % (EC₅₀) was calculated for each antioxidant solution from a calibration curve by linear regression.

Analysis of individual phenols

HPLC-DAD. Phenols were quantified using a Varian ProStar liquid chromatography system with a C-18 column (Kinetex[®]Biphenyl100 Å, 250 mm x 4.6 mm, i.d. 5 µm) and diode array detector (DAD; the wavelengths used for quantification were 254, 280, and 340 nm) with automatic Rheodyne injection valves (20 µL loop). The mobile phases used were 0.01% trichloroacetic acid in water (A) and acetonitrile (B), using the following gradient over a total run time of 55 min: 95 % A and 5 % of B initially, 75 % A and 25 % of B in 30 min, 50 % A and 50 % of B in 45 min, 0 % A and 100 % of B in 47 min, 75 % A and 25 % of B in 95 min, and 95 % A and 5 % of B in 52 min until completion of the run. Quantification was carried out by integration of the peaks at different wavelengths in function of the compounds, with reference to calibrations made using external standards. Calibration curves were made for theobromine at concentrations ranging from 0 to 2 mg/mL ($r=0.991$), and for (-)-epicatechin and (+)-catechin from 0 to 1 mg/mL ($r\geq 0.990$). All determinations were carried out in triplicate and the samples were filtered (0.45 µm) before analysis. For the theobromine and epicatechin quantifications, the initial extract was diluted 1:4; for the other quantifications, no dilution was needed.

Hydrothermal treatments.

For the extraction of valuable compounds, CBH was diluted 1:6 in water. The mixture was heated in a water bath to 50 °C, 70 °C or 90 °C, or heated to 120 °C in a Stuart sample concentrator (United Kingdom). The characteristics of the equipment used for hydrothermal treatments at 150 °C, 170 °C and 200 °C were described in a previous study.¹⁹ In brief, the reactor has a 2 L capacity

stainless steel reservoir that can operate at temperatures up to 240 °C and a maximum pressure of 4.2 MPa. Heating of the CBH was performed by direct injection of high-pressure and high-temperature steam. Samples (50 g) of CBH were loaded into the reactor and treated for 5 or 30 minutes for the treatment at 150 or 200 °C, respectively.

Statistical analysis.

Results were expressed as the mean $\pm$ standard deviation of three replicates. SAS/STAT v9.2 (Cary, NC, USA) software for Windows was used for statistical analyses. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the Tukey test. A p-value of 0.05 was considered to be statistically significant. Correlation coefficients between parameters were assessed by means of the Pearson Correlation test. Canonical discriminant analyses were carried out in 52 genotypes between eleven variables.

RESULTS AND DISCUSSIONS

Comparison of methods for quantifications.

Of the four phenolic extraction methods, Method B and Method D were the most effective for extracting total phenols from CBH (Figure 1A), yielding 13 and 11 mg g⁻¹ of total phenols, respectively (without a statistically significant difference). This experiment showed that ethanol-acetone and methanol were the most effective solvents for phenolic extraction from CBH. Our result demonstrating the effectiveness of methanol for phenol extraction from CBH corroborates the previous finding.²⁰ Using these extraction methods, a greater amount of phenolic acids were released by acid hydrolysis, which separates sugars and esters during hydrochloric acid treatment.²¹

Methods B and D yielded a greater amount of total phenols than reported by other studies, like the values reported by Manzano et al.²² (6.04 mg g⁻¹) for CHB extracted with water. For example, Martínez and colleagues²⁴ compared a mix of methanol:acetone with ethanol as extraction solvents, recovering a greater quantity of total phenols for methanol:acetone than ethanol, with yields of 1.50 mg g⁻¹ and 0.8 mg g⁻¹, respectively.²³

The antioxidant activity (Figure 1B), calculated as the 50 % effective concentration (EC₅₀), was higher in the extracts of Method B and Method D, with no significant differences between them, and corresponded to the higher amounts of total phenols in these extracts. These results correspond to those indicated by Azizah et al.,²⁰ who found that extraction with methanol produced the CBH extract with the highest antioxidant activity.

A significantly higher amount of total sugars (67.89 mg g⁻¹) was extracted using Method E (Figure 1C) with water acidified to pH 3 as solvent, following by Method B, then methods A, C, and D.

Characterization of genotypes.

The total phenols and sugars in the CBH of the different cacao genotypes extracted using Method D, in which 80 % methanol at pH 3 was used as the solvent, are shown in Table 1. Method D was selected because it provided the optimum quantification of total phenols and sugars as well as the bioactive profile obtained by HPLC-DAD. For the 2014 genotypes, the highest amount of total phenols was extracted from INI 9 (17.34 mg g⁻¹), followed by M033 (14.02 mg g⁻¹) and M026 (13.61 mg g⁻¹) (p<0.05). The amount of total phenols in INI 9 was four times greater than in M031, the genotype with the lowest amount of total phenols (p<0.05). Arlorio et al.¹ reported similar quantities of

total phenols (18.2 mg g^{-1}) in the CBH of commercial samples supplied by the Factory Ferrero SpA (Alba, Italy). The CAF and M033 husks had the highest antioxidant activity (19.87 and 19.88 mg mL^{-1} , respectively; $p < 0.05$), although this value did not correspond with the phenolic content of the CAF genotypes, which have a relatively low phenolic content (6.33 mg g^{-1}). However, the lowest antioxidant activity was recorded in M031 whose phenolic content was also one of the lowest. The highest total sugars were extracted from the CRP (110.42 mg g^{-1}), RIM 44 (93.26 mg g^{-1}), and CRC (73.84 mg g^{-1}) genotypes. Lower amounts were recorded in the INI 4 (16.83 mg g^{-1}), INI 8 (17.00 mg g^{-1}), and INI 10 (18.04 mg g^{-1}) genotypes. The correlation between total phenols and DPPH, as determined by the Pearson Correlation test, was -0.56 , which would indicate a moderate correlation between the total phenol content and antioxidant activity of CBH extracts.

Overall, theobromine, catechin, and epicatechin were the major compounds found in the genotypes of CBH analyzed (Table 2). Smaller quantities of four epicatechin derivatives and epigallocatechin were also detected. These results are similar to those reported in cotyledon,³ although they were found in smaller amounts in husk. Arlorio et al.¹ pointed out that epicatechin is one of the major components in husk, but the contents reported (2.75 mg g^{-1}) were lower than those found in this work. The INI 9 genotype had the highest content of epicatechin and catechin, and one of the highest contents of theobromine (31.56 mg g^{-1} , 4.66 mg g^{-1} , and 11.18 mg g^{-1} , respectively; $p < 0.05$) (Table 2). A similar quantity of theobromine was found in CBH (12.9 mg g^{-1}).¹ According to Timbie et al.,²⁴ changes in theobromine occur during sweat box fermentation, thus the decrease in the concentration of theobromine in the husk following fermentation is accountable. Theobromine is not soluble in water like the soluble polyphenols;

nevertheless, cytological studies indicate that diffusion of this compound is promoted during fermentation by a morphological rearrangement of lipid and aqueous phases.²⁵

Concerning the 2015 genotypes, the highest total phenol content in husks was recorded in INI 8 03/15 (34.88 mg g⁻¹), followed by SPA 9 (30.76 mg g⁻¹), and CRC (27.14 mg g⁻¹) (p<0.05). The 2015 genotypes with the lowest amounts of total phenols were ICS 95 06/15 (9.47 mg g⁻¹), INI 2 03/15 (9.58 mg g⁻¹), SIAL 22 (11.27 mg g⁻¹), and F7 07/15 (11.96 mg g⁻¹). Total sugars ranged from 12.35 mg g⁻¹ (F14 03/15) to 743.78 mg g⁻¹ (INI 1 03/15). The maximum antioxidant activity corresponded to the genotypes with the highest phenolic content, INI 8 03/15 (p<0.05); similarly, the lowest antioxidant activity corresponded to the genotype with the lowest phenolic content, INI 2 03/15 (p<0.05).

The correlation coefficient between the antioxidant activity (Ec₅₀) and the total phenol content as determined by the Pearson Correlation test was -0.68, which indicates the high antioxidant power of CBH extracts with higher phenolic contents. These results are in agreement with those carried out by other groups, who also found a linear correlation between the content of *T. cacao* phenolic compounds and their antioxidant capacity.²⁴⁻²⁶

For the cocoa bean husks of the 2015 harvest, epicatechin, theobromine, and catechin were again the major compounds found (Table 2). Four epicatechin derivatives and epigallocatechin were also identified. Theobromine content ranged from 14.16 to 18.27 mg g⁻¹ for CRC 04/15 and F7 12/14, respectively, and no significant differences between the genotypes' theobromine contents were found. The highest content of epicatechin was found in INI 1 03/15 (34.97 mg g⁻¹) and the lowest in PA169 06/15 (4.40 mg g⁻¹). Catechin content ranged from 0.55 mg g⁻¹ to 3.33 mg g⁻¹ to, in INI 1 03/15 and INI 8 03/15, respectively.

Theobromine was present in higher concentration in the genotypes analyzed in 2014-2015 than the 2013-2014 season. This difference may be due to the varieties analyzed, the season, agronomic factors or the fermentation process; hence, further studies will be necessary to determine how the concentration of these bioactive compounds depend on these factors.

Canonical discriminant analyses

Discriminant canonical analysis is a multivariate statistical technique whose purpose is to analyze if there are significant differences between groups of objects with respect to a measured set of variables. The analysis permits the systematic classification of new observations of unknown origin in one of the analyzed groups, if they exist. A discriminant analysis was applied in order to compare the husks of the 52 harvested cocoa genotypes. The variables analyzed were antioxidant activity (DPPH) and the content of total phenols, total sugars, theobromine, catechin, epicatechin, epicatechin derivative I, epicatechin derivative II, epicatechin derivative III, epicatechin derivative IV, and epigallocatechin. In this analysis, a maximum of 11 canonical variables (CV) were generated. The first three canonical variables explained the greatest amount of total variability among treatments. CV1 explains for 47.7 %, CV2 explains for 16.6 %, and CV3 explains for 13.6 % of variability. Together, the three canonical variables account for a total of 78% of the variability among the genotypes.

As show in Figure 2, canonical variable 1 (CV1) is mainly influenced by total phenols, theobromine, and total sugars because they have the highest standardized canonical coefficients; whereas CV2 is mainly influenced by total sugars and CV3 by theobromine and DPPH.

The genotypes in the upper right quadrant are those with the highest CBH total sugar content (SPA 9 06/15, F20 11/15, INI 1 03/15, F9 12/14, CRP, and M033). The genotypes located in the upper and lower left quadrants correspond to genotypes for which CBH has the lowest antioxidant activity (CRC, CONA 1, INI 4, INI 8, INI 10, M008, M023, M031, F14 03/15, F33 12/15 RIM 44, and RIM 88). The genotypes in the lower right quadrant correspond to those CBHs with the highest total phenol content (CRC 04/15, F0 12/14, F21 12/14, F21 03/15, F28 12/14, F33 12/15, INI 8 03/15, ICS 95 12/14, and PA 169 06/15).

Extraction of bioactive compounds by hydrothermal treatments.

In order to recover a greater amount of bioactive compounds in a cheaper and more effective way, CBH was submitted to different hydrothermal pretreatment conditions with a range of temperatures from 50 °C to 200 °C. Hydrothermal treatments at 150 °C, 170 °C, and 200 °C were carried out in an industrial steam reactor used for the extraction of phenolic compounds from olive oil waste.¹⁹ Results from the hydrothermal treatments were compared with those of solvent extraction (Method D) and a control extraction using water. Higher amounts of total phenols were recovered from CBH by hydrothermal treatments of 150 °C, 170 °C, and 200 °C as compared to Method D and control (Figure 3). Overall, the amount of total phenols increased significantly with higher temperatures, probably because hydrothermal treatment solubilizes a greater amount of phenols.²⁹ The largest amount of total phenols (82.11 mg g⁻¹ sample) was obtained in the treatment performed at 170 °C for a reaction time of 30 minutes, followed very closely by the treatment at 200 °C for a reaction time of five minutes, in which 79.37 mg g⁻¹ was recovered without significant difference between the two treatments. A previous experiment with dates that compared two hydrothermal

treatments at 140 °C and 160 °C showed that treatment at 140 °C extracted the largest amount of total phenols for other agricultural waste. This is probably because the more severe treatment solubilized a greater portion of the cell wall material, mainly hemicelluloses, which may be linked to simple phenols.¹⁸ The treatment at 170 °C and 30 minutes produced an extract with an EC₅₀ of 8.18 mg mL⁻¹, yielding the extract with the second highest antioxidant power, only surpassed by treatment at 200 °C during 5 minutes, which produced an extract with an EC₅₀ of 5.58 mg mL⁻¹ (Figure 3B), without a significant difference between the two treatments. The highest total sugars were recovered in the treatment at 170 °C with water for 30 minutes (Figure 3C).

A high negative correlation, as determined by the Pearson Correlation test (-0.81), was again observed between total phenol content and the EC₅₀ as an inverse measure of the antioxidant activity, and no clear correlation was found neither between the phenolic content and total sugar content nor between the antioxidant activity and total sugar content. These results agreed with those of other studies carried out with cocoa, in which the EC₅₀ was strongly correlated with total phenol and flavonoid contents.^{26,30,31}

The effect of hydrothermal treatments at different temperatures on the HPLC profile of cocoa bean husk is shown in Figure 3D. The highest content of theobromine was found in the extract obtained from treatment at 200 °C for 5 min and 170 °C+water for 30 min treatment, yielding 43.93 mg g⁻¹ and 40.94 mg g⁻¹, six-fold higher than the theobromine content obtained with the control treatment. The 200 °C for 5 min and 170 °C for 30 min treatments also yielded extracts with the highest amount of catechin (5.67 mg g⁻¹ and 3.38 mg g⁻¹, respectively). Epicatechin content ranged from 0.99 to 9.75 mg g⁻¹, yet only thermal treatment at 50 °C yielded significantly higher epicatechin content than the control.

Mass balance of the extraction of bioactive compounds at 170 °C for 30 minutes.

The treatment at 170 °C for 30 minutes was selected because it led to high solubilization of the desired compounds from CBH and because the treatment is easy to scale up for industrial use. In fact, these are the conditions currently used by industry to revalorize the olive oil by-product pomace.³²

The amounts of phenols, sugars, and theobromine recovered from 100 kg of cocoa bean husk are shown in Figure 4. From 100 kg of CBH, 29.35 kg of crude extract was obtained that was rich in phenols (5.5%), sugars (22%), and theobromine (5.6%). From this extract, it is easy to purify the bioactive components (or use the extract directly) for application in the food, cosmetic or pharmaceutical fields. This extraction method is completely natural, without the addition of any solvent or chemical, obtaining in this way a final inexpensive product that can be used in both organic and conventional products in the case of the food industry.

CONCLUSION

The characterization of CBH from different Mexican genotypes confirmed the presence of bioactive compounds that could be obtained to revalorize this cacao by-product. Phenolic extraction using 80 % methanol or ethanol-acetone mixture at pH 3 were the most effective methods for the analytical characterization of the phenolic content of CBH. The significant differences between genotypes in terms of total and individual phenols, sugars, and antioxidant activity should help to promote the industrial use of the best genotypes, also taking into account the genotypes' agronomical issues.

Hydrothermal treatments over 150 °C increased the bioactive compounds' solubilization and the antioxidant activity of the liquid phase. Treatment of 170 °C with water for 30 minutes was the most effective condition for extracting both phenolic compounds and sugars from the CBH. However, 200 °C for 5 minutes was the best treatment in order to extract theobromine and catechin. Thus, the application of this industrial treatment can help to revalorize the CBH with the extraction of bioactive compounds, as well as providing a rich source of sugar and fiber for human or animal food. Epicatechin, theobromine, and catechin were the main bioactive compounds found in CBH. The application of thermal treatment permitted the natural solvent-free extraction of bioactive compounds with antioxidant properties for the valorization of CBH and their use in the food, cosmetic or nutraceutical industries. Further studies are necessary to establish the biological potential of the phenolic and sugar content of CBH and determine other interesting components in order to maximize the use of this by-product. It is also important to characterize and identify the biologically active oligosaccharides formed by the thermal treatment of CBH or initially present in the raw material, as well as the activity of the fiber concentrated in the final solid.

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Figure Captions

Figure 1. Total phenols (A), total sugars (B), and antioxidant activity (DPPH) (C) of samples extracted from CBH by different methods.

Figure 2. Canonical discriminant classification of two canonical variables for the 52 genotypes studied.

Figure 3. Effect of several hydrothermal treatments on total sugars (A), total and individual phenols (B and D, respectively) and antioxidant activity (DPPH) (C) of CBH extracts.

Figure 4. Mass balance of extraction of bioactive compounds from thermally treated CBH (170 °C for 5 minutes).

489 **Table 1.** Total sugars, total phenols, and radical scavenging of DPPH (EC₅₀ expressed as
490 mg/mL) in CBH extracts of different cocoa genotypes supplied by INIFAP, Mexico, in 2014
491 and 2015.

GENOTYPES 2014				GENOTYPES 2015			
Sample	Total phenols (mg g ⁻¹ of sample)	Antioxidant activity (mg mL ⁻¹ EC ₅₀)	Total sugars (mg g ⁻¹ of sample)	Sample	Total phenols (mg g ⁻¹ of sample)	Antioxidant activity (mg mL ⁻¹ EC ₅₀)	Total sugars (mg g ⁻¹ of sample)
CAF	6.33±0.98 ^a fghi	19.87±1.04 m	24.07±1.95 ijk	CRC 04/15	27.14±0.93 abcd	16.87±0.55 fgh	36.13±2.49 fghi
CONA 1	5.91±0.59 ghi	33.32±1.60 ghij	25.95±2.51 ghijk	F0 12/14	24.20±1.32 bcde	15.34±0.96 gh	23.67±9.12 hi
CONA 2	8.92±0.62 defgh	23.07±0.07 lm	31.64±0.75 fghijk	F7 12/14	15.23±1.77 efghij	38.35±3.84 bcd	35.53±2.36 fghi
CRC	8.50±0.81 defgh	37.73±2.24 efgh	73.84 ±3.50 bc	F7 07/15	11.96±1.93 hij	27.83±3.91 cdefg	44.82±2.08 fgh
CRP	13.26±1.19 bc	39.32±0.93 defg	110.42±3.15 a	F9 12/14	24.75±2.30 bcd	25.12±1.28 defg	100.0±17.11 cd
INI 1	7.27±0.14 efghi	38.86±0.73 defg	20.44±1.03 jk	F14 12/14	22.70±1.10 bcde	26.06±0.86 defg	35.70±2.33 fghi
INI 4	8.42±0.70 defghi	27.74±1.69 jkl	16.83±1.37 k	F14 03/15	12.67±0.70 ghij	40.93±1.28 bc	12.35±3.81 i
INI 8	10.58±1.96 bcde	29.75±1.99 ijk	17.00±0.33 k	F14 11/15	19.11±0.93 defgh	24.61±0.60 efg	61.99±8.68 ef
INI 9	17.34±3.49 a	32.40±1.58 hij	33.21±1.12 efghijk	F20 11/15	12.78±0.76 fghij	71.38±11.08 a	121.57±5.57 c
INI 10	10.22±0.87 bcde	25.93±0.06 klm	18.04±2.55 k	F21 12/14	21.39±4.67 cdefg	23.53±0.98 efg	41.70±1.90 fghi
M001	6.20±0.55 fghi	44.12±2.28 bcd	23.38±0.37 ijk	F21 11/15	24.76±1.50 bcd	25.04±0.62 defg	97.23±15.48 cd
M002	9.90±1.35 cdef	38.48±0.84 defgh	56.06±0.93 cde	F21 03/15	19.60±2.00 defgh	26.50±1.18 defg	29.20±2.85 ghi
M003	7.52±1.25 efghi	43.02±1.34 bcde	46.74±2.13 defghi	F28 12/14	17.92±0.95 defghij	27.82±1.38 cdefg	33.59±1.75 fghi
M008	6.15±0.38 fghi	42.04±1.58 bcde	44.76±3.49 defghi	F33 03/15	18.99±2.00 defghi	17.32±0.55 fgh	24.61±0.85 hi
M022	9.14±1.10 defg	29.38±1.50 ijkl	48.45±3.27 defgh	F33 12/15	18.53±0.95 defghij	19.52±0.12 fg	36.33±2.03 fghi
M024	8.98±0.90 defg	29.18±1.73 ijkl	40.18±7.73 defghijk	F42 03/15	24.06±2.95 bcde	26.54±0.60 defg	75.74±5.35 de
M025	4.48±1.45 i	41.61±0.52 cdef	25.20±5.12 hijk	F44 03/15	22.27±2.34 bcde	21.28±0.59efg	49.32±4.24 efgh
M026	13.61±1.46 abc	27.57±0.98 jkl	42.11±5.40 defghij	ICS95 12/14	24.42±2.70 bcde	20.73±1.87 fg	55.68±0.47 efg
M028	4.30±0.54 i	47.92±2.95 bc	52.33±5.13 cdef	ICS 95 06/15	9.47±0.71 j	44.56±1.73 b	20.84±1.60 hi
M029	5.12±0.97 hi	48.36±3.56 b	25.97±3.11 ghijk	INI 1 03/15	24.18±0.45 bcde	34.56±1.91 bcde	743.78±36.06 a
M031	3.86±0.54 i	72.23±5.27 a	32.53±4.91 efghijk	INI 2 03/15	9.58±0.87 ij	76.88±12.74 a	28.90±1.25 ghi
M032	6.29±0.22 fghi	35.41±2.59 fghi	61.64±8.30 cd	INI 8 03/15	34.88±3.34 a	5.59±0.31 h	28.42±1.03 ghi
M033	14.02±1.18 ab	19.88±2.45 m	51.51±6.68 cdef	PA 169 06/15	18.03±2.48 defghij	21.00±0.92 fg	25.60±2.01 ghi
RIM 44	5.83±0.16 ghi	38.56±0.43	93.26±0.00 ab	SIAL 22	11.27±0.60 hij	29.42±1.24 cdef	35.17±3.23 fghi

		defgh					
RIM 88	10.85±0.53 bcde	28.42±1.91 jkl	49.59±1.65 defg	SPA 9 06/15	30.76±2.02 abc	22.81±0.72 efg	200.82±14.06 b
RIM 105	11.91±2.04 bcd	25.93±1.49 klm	45.78±17.18 defghi	UF273 05/15	22.17±1.86 bcdef	19.81±0.68 fg	24.63±2.91 hi

*Standard deviations (n=3). n.d. not determined. Means with the same letter between lines are not significantly different. $\alpha = 0.05$

494 **Table 2.** Bioactive compounds profile (mg gallic acid equivalents g⁻¹ of sample) of CBH from
495 different cocoa genotypes supplied by INIFAP, Mexico, in 2014 and 2015.

Year of harvest	Genotype	Theobromine	Catechin	Epicatechin	Epicatechin derivative I	Epicatechin derivative II	Epicatechin derivative III	Epicatechin derivative IV	Epigallocatechin
2014	CAF	9.25 ± 0.00 ^a abcde	1.79 ± 0.00 defghi	9.53 ± 0.00 cd	0.03 ± 0.00 ab	0.13 ± 0.00 bcd	0.02 ± 0.00 b	0.02 ± 0.00 ab	0.05 ± 0.00 bc
	CONA 1	9.88±0.77 abcde	2.99 ± 0.82 bcd	15.63 ± 4.26 bcd	0.07 ± 0.01 ab	0.07 ± 0.02 cd	n.d. c	0.03 ± 0.00 ab	0.09 ± 0.10 bc
	CONA 2	10.26± 0.11 abcde	2.86 ± 0.32 bedef	18.46 ± 0.33 abcd	0.12 ± 0.05 ab	0.09 ± 0.00 cd	Traces b	0.03 ± 0.00 ab	0.06 ± 0.01 bc
	CRC	11.74 ± 0.84 a	3.09 ± 0.14 bcd	25.23 ± 0.95 ab	0.12 ± 0.05 ab	0.10 ± 0.00 cd	Traces	Traces	traces
	CRP	10.59 ± 0.38 abcde	3.44 ± 0.11 abc	19.03 ± 8.04 abcd	0.27 ± 0.26 ab a	0.11 ± 0.10 cd	0.16 ± 0.11 b	n.d.	0.12 ± 0.12 bc
	INI1	7.62 ± 0.92 de	1.27 ± 0.18 ghi	13.70 ± 4.37 bcd	0.05 ± 0.07 ab	0.05 ± 0.07 cd	0.08 ± 0.06 b	n.d.	0.08 ± 0.09 bc
	INI4	10.13 ± 0.84 abcde	1.72 ± 0.21 defghi	17.48 ± 1.86 abcd	0.04 ± 0.05 ab	0.04 ± 0.05 cd	n.d.	traces	traces
	INI8	11.40 ± 1.03ab	3.59 ± 0.66 abc	19.67 ± 2.69 abcd	0.14 ± 0.04 ab	0.14 ± 0.04 bcd	0.11 ± 0.02 b	0.11 ± 0.05 ab	0.10 ± 0.03 bc
	INI9	11.18 ± 1.37 abc	4.66 ± 0.49 a	31.56 ± 5.09 a	0.16 ± 0.02 ab	0.16 ± 0.02 abcd	0.13 ± 0.01 b	0.19 ± 0.04 ab	0.13 ± 0.03 bc
	INI10	9.97 ± 2.00 abcde	1.71 ± 0.10 defghi	12.52 ± 0.29 bcd	0.11 ± 0.01 ab	0.11 ± 0.00 cd	0.04 ± 0.05 b	0.07 ± 0.02 ab	0.19 ± 0.00 abc
	M001	7.86 ± 0.66 cde	1.33 ± 0.02 ghi	12.16 ± 3.28 bcd	0.04 ± 0.00 ab	0.05 ± 0.06 cd	n.d.	n.d.	0.03 ± 0.05 bc
	M002	7.64 ± 0.20 de	0.83 ± 0.02 i	5.44 ± 0.04 d	0.25 ± 0.03 ab	0.02 ± 0.01 cd	0.02 ± 0.01 b	0.10 ± 0.06 ab	0.06 ± 0.02 bc
	M003	10.19 ± 0.51 abcde	1.08 ± 0.04 hi	13.80 ± 2.33 bcd	0.09 ± 0.11 ab	n.d.	n.d.	0.02 ± 0.00 ab	0.02 ± 0.00 bc
	M008	7.39 ± 0.51 e	1.52 ± 0.06 efghi	10.33 ± 0.18 cd	0.16 ± 0.06 ab	0.13 ± 0.01 bcd	0.13 ± 0.05 b	0.07 ± 0.04 ab	0.05 ± 0.04 bc
	M022	9.75 ± 0.48 abcde	2.90 ± 0.38 bcde	15.40 ± 6.19 bcd	0.23 ± 0.01 ab	0.19 ± 0.10 abc	0.11 ± 0.01 b	0.10 ± 0.02 ab	0.24 ± 0.19 ab
	M024	9.53 ± 0.64 abcde	2.41 ± 0.34 bcdefgh	14.22 ± 1.24 bcd	0.06 ± 0.04 ab	0.09 ± 0.07 cd	0.10 ± 0.00 b	0.08 ± 0.11 ab	0.08 ± 0.10 bc
	M025	8.20 ± 1.00 bcde	1.24 ± 0.06 ghi	9.76 ± 1.49 cd	Traces	n.d.	0.02 ± 0.02 b	0.05 ± 0.07 ab	0.02 ± 0.00 bc
	M026	11.05 ± 0.82 abc	3.69 ± 0.61 ab	18.87 ± 9.72 abcd	0.03 ± 0.04 ab	0.14 ± 0.05 bcd	0.13 ± 0.05 b	0.07 ± 0.10 ab	0.15 ± 0.02 bc
	M028	8.77 ± 0.09 abcde	3.47 ± 0.02 abc	16.04 ± 1.27 bcd	0.19 ± 0.02 ab	n.d.	n.d.	n.d.	n.d.
	M029	7.92 ± 1.30 cde	1.51 ± 0.10 fghi	11.72 ± 2.51 bcd	0.03 ± 0.00 ab	n.d.	Traces	0.08 ± 0.04 ab	0.05 ± 0.01 bc
	M031	8.77 ± 0.57 abcde	2.23 ± 0.08 cdefgh	12.21 ± 1.89 bcd	0.08 ± 0.00 ab	n.d.	Traces	n.d.	n.d.
	M032	9.92 ± 0.00 abcde	1.17 ± 0.00 ghi	15.81 ± 0.00 bcd	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.00 bc
	M033	10.50 ± 0.77 abcde	3.59 ± 0.46 abc	12.34 ± 1.56 bcd	0.15 ± 0.07 ab	0.34 ± 0.01 a	0.41 ± 0.13 a	0.21 ± 0.16 a	0.41 ± 0.04 a
	RIM 44	7.67 ± 0.55 de	1.19 ± 0.04 ghi	12.18 ± 4.33 bcd	Traces	Traces	0.02 ± 0.03 b	traces	0.04 ± 0.02 bc
	RIM 88	10.59 ± 1.23 abcde	2.48 ± 0.71 bcdefg	21.32 ± 1.24 abc	0.04 ± 0.00 ab	0.31 ± 0.11 ab	0.03 ± 0.01 b	0.03 ± 0.02 ab	0.06 ± 0.03 bc
	RIM 105	10.89 ± 0.04 abcd	1.75 ± 0.03 defghi	17.71 ± 0.22 abcd	0.07 ± 0.01 ab	0.09 ± 0.01 cd	0.09 ± 0.02 b	0.06 ± 0.00 ab	0.06 ± 0.00 bc
2015	CRC 04/15	18.27 ± 2.01 a	2.85 ± 0.00 ab	21.17 ± 5.46 abc	0.27 ± 0.02 b	0.22 ± 0.09 a	0.12 ± 0.05 a	0.02 ± 0.03 d	0.28 ± 0.01 ab
	F0 12/14	15.35 ± 0.22 a	1.45 ± 0.07 ab	10.70 ± 0.91 bc	0.17 ± 0.01 b	n.d.	n.d.	n.d.	n.d.
	F7 12/14	14.16 ± 0.59 a	1.21 ± 0.16 ab	10.68 ± 0.07 bc	n.d.	n.d.	n.d.	n.d.	n.d.
	F7 07/15	15.27 ± 0.04 a	1.33 ± 1.89 ab	9.19 ± 0.44 bc	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.17 ab
	F9 12/14	16.62 ± 2.75 a	1.46 ± 0.15 ab	19.22 ± 5.90 abc	n.d.	n.d.	0.18 ± 0.03 a	n.d.	0.16 ± 0.04 ab
	F14 12/14	17.19 ± 0.99 a	1.31 ± 0.07 ab	16.49 ± 5.09 abc	n.d.	n.d.	n.d.	n.d.	0.26 ± 0.10 ab
	F14 03/15	16.51 ± 0.62 a	2.23 ± 0.18 ab	19.35± 14.16 abc	0.15 ± 0.22 b	n.d.	n.d.	n.d.	0.34 ± 0.38 ab
	F14 11/15	16.59 ± 1.46 a	1.41 ± 0.03 ab	13.87 ± 3.57 bc	0.12 ± 0.17 b	n.d.	n.d.	n.d.	0.03 ± 0.04 b
	F20 11/15	15.01 ± 0.70 a	1.04 ± 0.06 ab	13.97 ± 0.22 bc	n.d.	n.d.	n.d.	n.d.	n.d.
	F21 12/14	17.47 ± 1.61 a	1.85 ± 0.00 ab	20.25 ± 4.59 abc	n.d.	n.d.	n.d.	0.23 ± 0.14 abcd	0.22 ± 0.05 ab
	F21 11/15	17.50 ± 1.94 a	1.20 ± 0.08 ab	21.17 ± 7.35 abc	0.11 ± 0.15 b	n.d.	n.d.	0.16 ± 0.04 bcd	0.11 ± 0.02 ab
	F21 03/15	17.86 ± 1.06 a	2.06 ± 0.00 ab	16.59 ± 3.78 abc	0.06 ± 0.03 b	n.d.	n.d.	0.22 ± 0.15 bcd	0.14 ± 0.03 ab

	F28 12/14	14.60 ± 0.70 a	1.69 ± 0.00 ab	13.97 ± 1.53 bc	n.d.	n.d.	n.d.	0.03 ± 0.02 cd	0.19 ± 0.00 ab
	F33 03/15	16.62 ± 0.62 a	1.57 ± 0.03 ab	7.62 ± 0.07 bc	0.06 ± 0.09 b	n.d.	n.d.	0.04 ± 0.06 cd	0.02 ± 0.03 b
	F33 12/15	15.04 ± 0.66 a	1.18 ± 1.68 ab	16.70 ± 0.87 abc	n.d.	n.d.	n.d.	n.d.	n.d.
	F42 03/15	15.74 ± 1.65 a	1.25 ± 0.21 ab	16.39 ± 4.80 abc	0.08 ± 0.08 b	n.d.	n.d.	0.10 ± 0.10 bcd	0.06 ± 0.00 ab
	F44 03/15	16.49 ± 0.37 a	1.19 ± 0.06 ab	14.54 ± 2.18 bc	0.03 ± 0.04 b	n.d.	n.d.	0.29 ± 0.03 abc	n.d.
	ICS95 12/14	18.20 ± 2.05 a	1.89 ± 0.07 ab	21.38 ± 3.57 abc	0.24 ± 0.14 b	0.07 ± 0.03 a	0.13 ± 0.00 a	0.22 ± 0.01 bcd	0.12 ± 0.09 ab
	ICS 95 06/15	14.29 ± 0.18 a	2.19 ± 0.00 ab	10.84 ± 0.44 bc	n.d.	n.d.	n.d.	0.05 ± 0.08 bcd	n.d.
	INI 1 03/15	15.76 ± 3.74 a	0.55 ± 0.77 b	34.97 ± 8.81 a	n.d.	0.07 ± 0.10 a	n.d.	0.30 ± 0.07 ab	0.08 ± 0.12 ab
	INI 2 03/15	14.99 ± 0.81 a	1.97 ± 0.07 ab	12.37 ± 0.44 bc	0.02 ± 0.03 b	n.d.	n.d.	n.d.	0.08 ± 0.11 ab
	INI 8 03/15	16.93 ± 0.04 a	3.33 ± 0.14 a	18.03 ± 3.49 abc	0.88 ± 0.38 a	0.21 ± 0.30 a	0.25 ± 0.35 a	0.47 ± 0.13 a	0.46 ± 0.07 a
	PA 169 06/15	14.93 ± 0.73 a	0.99 ± 1.40 ab	4.40 ± 6.22 c	n.d.	n.d.	n.d.	n.d.	0.09 ± 0.13 ab
	SIAL 22	14.86 ± 0.40a a	2.42 ± 0.03 ab	26.68 ± 0.73 ab	0.11 ± 0.03 b	0.03 ± 0.04 a	n.d. b	0.04 ± 0.01 cd	n.d.
	SPA 9 06/15	15.19 ± 1.68 a	0.99 ± 0.05 ab	20.30 ± 5.24 abc	0.16 ± 0.05 b	n.d.	n.d.	0.19 ± 0.08 bcd	n.d.
	UF273 05/15	16.90 ± 1.98 a	2.42 ± 0.03 ab	15.41 ± 0.22 abc	0.24 ± 0.02 b	0.02 ± 0.03 a	n.d.	0.21 ± 0.08 bcd	0.15 ± 0.09 ab

496 * Standard deviations (n=3). n.d. not determined. Means with the same letter between lines are not significantly different. α =0.05

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Means with the same letter are not significantly different. $\alpha = 0.05$

Total phenols are expressed as gallic acid equivalents.

Figure 1.

Pooled Within-Class Standardized Canonical Coefficients

Variable	Total phenols	Total sugars	DPPH (EC50)	Theobromine	Catechin	Epicatechin	Derivative I	Derivative II	Derivative III	Derivative IV	Epigallocatechin
CV1	0.7856	0.5279	-0.4107	0.8003	-0.2105	-0.4352	0.1718	-0.0838	0.3375	-0.2601	-0.0430
CV2	0.7856	0.5279	-0.4107	0.8003	-0.2105	-0.4352	0.1718	-0.0838	0.3375	-0.2601	-0.0430
CV3	-0.0204	0.0270	0.6274	0.6664	-0.0406	-0.6436	-0.4108	-0.4917	0.0233	-0.2903	0.2319

Figure 2.

*Standard deviations (n=3); **traces: values detected $\leq 0.01 \text{mg g}^{-1}$ of sample.

Means with the same letter are not significantly different. $\alpha = 0.05$

Total phenols are expressed as gallic acid equivalents.

Figure 3.

Figure 4.