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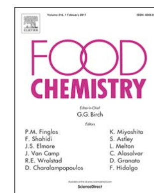

Bioactive compounds in Mexican genotypes of cocoa cotyledon and husk

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ABSTRACT

A characterization of the phenolic profile of 25 cocoa genotypes established in a Mexican gene bank was carried out. From five different extraction methods commonly used for phenols, extraction with acidified methanol–-water was chosen as the best to quantify the concentrations of theobromine and individual phenols in cocoa beans. High concentrations of individual and total phenols were found for genotypes native to Mexico (like RIM105, M031, and M033) or from Peru and Ecuador (INI10), but not the commercial mix (CAF), and were directly associated with their antioxidant activities. Despite the loss of some theobromine and phenols during fermentation, epicatechin remained in the fermented cotyledon in high concentrations. This study could help promote the commercialization of Mexican genotypes of cocoa and reports the possibility of upcycling fermented cocoa husks, which are rich in bioactive compounds and fiber, as novel functional extracts for use in food formulation or for nutraceutical purposes.

1. Introduction

Cocoa (*Theobroma cacao* L.) is a perennial tree widely cultivated in America, Asia, and Africa, whose beans are extracted in order to produce the main ingredient of cocoa powder and chocolate (Cuatrecasas, 1964). Phenolic compounds constitute about 10% of cocoa beans and are responsible for their antioxidant, cardiovascular protective, antitumor, anti-inflammatory (Aprotosoaie, Luca, & Miron, 2016; Corti, Flammer, Hollenberg, & Luscher, 2009; Jolic, Redovnikovic, Markovic, Sipusic, & Delonga, 2011; Keen, Holt, Oteiza, Fraga, & Schimitz, 2005; Rusconi & Conti, 2010; Selmi, Cocchi, Lanfredini, Keen, & Gershwin, 2008; Steinberg, Bearden, & Keen, 2003), antineurodegeneratives (Nehlig, 2013), antibacterial, and anticariogenic properties (Ferrazzano, Amato, Ingenito, De Natale, & Pollio, 2009). Phenolic compounds are secondary metabolites that are stored in the pigmented cells of the cotyledon and which perform various functions at the plant, such as the regulation of its development, among others. Flavonoids are the largest and most diverse group of phenolic compounds found in the cocoa bean, which, depending on the structural composition of its central heterocyclic ring, oxidation state and glycosylation pattern, may be divided into six different subclasses, including flavones, isoflavones, flavanones, flavon-3-ols, flavanols, and anthocyanins (Ignat, Volf, & Popa, 2011; Oracz, Nebesny, & Żyżelewicz, 2015; Rice-Evans, 2004).

The most common compounds in cocoa beans are the flavanols epicatechin, catechin, and procyanidins. According to various studies, epicatechin is the compound found in the greatest amounts in cocoa beans, representing about 35% of the total phenolic content, but other polyphenolic compounds have also been identified, including gallocatechin, epigallocatechin, epicatechin-3-*O*-gallate, quercetin, quercetin 3-glucoside, quercetin-3-arabinoside, clovamide, and deoxyclovamide (Oracz, Nebesny et al., 2015; Sanbongi et al., 1998).

Theobromine and caffeine are two compounds from the group of methylxanthines that are also present in cocoa (Belscak, Komes, Horzic, Kovacevic, & Karlovic, 2009; Timbie, Schrist, & Kenney, 1978), whose physiological effects on the central nervous, cardiovascular, gastrointestinal, respiratory, and renal systems are well known (Nehlig, 2013; Nehlig, Daval, & Debry, 1992; Spiller, 1998).

The phenolic profile in foods, including cocoa, may be affected by various factors: environmental (origin of the sample, variety, maturity, and climate) and processing (roasting, tempering, alkalinization, and storage) (Jolic et al., 2011; Oracz, Żyżelewicz, & Nebesny, 2015; Rusconi & Conti, 2010). Flavonoids are water soluble and highly susceptible to enzymatic and non-enzymatic oxidation (Oracz, Nebesny et al., 2015). It is estimated that the phenolic content can be reduced from 100 to 10% during the manufacturing process of cocoa (fermentation, drying, and roasting) (Afoakwa, Ofosu-Ansah, Budo, Mensah-Brown, & Takrama 2015; Oracz, Nebesny et al., 2015; Rusconi & Conti,

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2010). This can be caused by the presence of the enzyme polyphenol oxidase, epicatechin epimerization caused by pH changes during the fermentation process, and the polymerization of quinones during drying, among other reasons (Afoakwa et al., 2015; Oracz, Żyżelewicz et al., 2015).

According to Nazaruddin, Seng, Hassan, and Said (2006) phenolic compounds are stored in the cotyledons of the cocoa seeds; however, during the fermentation loss of these compounds occurs due to diffusion out of the cotyledon. As a result, the husk becomes a material rich in these bioactive compounds. Phenolic compounds that have been identified in methanol extracts have been epicatechin and *p*-hydroxybenzoic acid. Similarly, theobromine is an important compound in the husk, whose content is around 12.9 mg/g (Arlorio et al., 2005).

Each genotype of cocoa has its own distinguishing characteristics, including bean size, chemical composition, organoleptic properties, and degree of fermentation. In Mexico one of the agricultural products with the greatest potential for cultivation in tropical regions is cocoa. In fact, *Theobroma cacao* is now grown in an area of about 60,000 hectares, mainly distributed in the states of Tabasco and Chiapas, and yields a production volume of 27,000 tons of cocoa beans (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, 2015). Although several studies have focused on the study of the phenolic compounds of the cocoa beans commercially produced in Mexico (Quiroz, Aguilar, Ramírez, & Ronquillo, 2013; Ramírez, Cely Niño, & Ramírez, 2013; Romero-Cortes et al., 2013), no study has yet exists showing characterization by genotype.

Several studies on different genotypes of cocoa in other countries report varied results for the content of phenols and methylxanthines after processing, which give rise to big differences in terms of sensory characteristics and the final quality of the beans (Cruz, Paula Bacelar, Sergio Eduardo, & Eliete da Silva, 2015). Therefore, the characterization of the phenolic profile of Mexican cocoa genotypes established in a national gene bank could have utility in clonal selection, and the phenolic profile and antioxidant capacity of the genotypes could be used as a marker of nutraceutical quality. To this end, this study contributes to the characterization of the phenolic profile of 25 genotypes and one commercial mix of fermented cocoa harvested in the Gene Bank of the Mexican National Institute of Forestry, Agriculture and Livestock. A comparison of different methods of extraction of phenolic compounds in a homogeneous sample of cocoa beans was first performed; then the most effective extraction methods were chosen for the subsequent characterization of phenols and the phenolic profile of the 25 different Mexican genotypes of cocoa selected and a commercial mix used in Mexico.

2. Materials and methods

2.1. Samples

Twenty-five different genotypes of dry and fermented cocoa were used (Table 1) in comparison with a commercial mix sample fermented (CAF) or non fermented (CAL). All of them were harvested in the 2012–2013 season and belong to the Germplasm Bank of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP), Huimanguillo, Mexico. The samples were fermented and dried following the industrial method (Arana-Sánchez et al., 2015; Hernandez-Hernandez, Lopez-Andrade, Ramirez-Guillermo, Guerra Ramirez, & Caballero Perez, 2016), namely the fermentation of the cocoa beans for six days in wooden boxes and subsequent natural drying for five days in wooden platforms with up to 7% humidity. During the natural drying, the temperature was maintained at 55 °C and the air humidity at 70% (Arana-Sánchez et al., 2015; Hernandez-Hernandez, Lopez-Andrade, Ramirez-Guillermo, Guerra Ramirez, & Caballero Perez, 2016). Cocoa beans husk was obtained from the same fermented and some non-fermented samples.

2.2. Chemicals

The standards of gallic acid (GA), caffeic acid, (−)-epicatechin, (+)-catechin hydrate and theobromine, trifluoroacetic acid (TFA), anthrone and Folin-Ciocalteu's phenol, 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich (Madrid, Spain). 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).

2.3. Extraction methods

The samples of cocoa seed and cocoa husk were manually separated, milled, and homogenized to a particle size of 0.5 μm for the extraction. Seeds and husk were milled separately by a mill (Culatti, Italia), then were winnowed by a 0.5-μm sieve. The cocoa seed samples were defatted by extraction with *n*-hexane for six hours using a Soxhlet apparatus. Five different extraction methods were compared.

2.3.1. Method A

Methanol-water extraction. 1 gram of cocoa seed or cocoa husk sample was extracted twice with 3 mL of methanol-water 80:20 (*v/v*) at 70 °C for one hour with stirring. The two filtered liquids were pooled to form the final extract.

2.3.2. Method B

Ethanol-acidified water extraction. 1 gram of cocoa seed or cocoa husk sample was extracted with 5 mL of ethanol:water 30:70 (*v/v*) acidified to pH 3 with concentrated HCl. After stirring for two hours at room temperature, the mix was filtered and the solid was extracted again with acetone:water 70:30 (*v/v*). The liquids obtained from both extractions were pooled.

2.3.3. Method C

Water extraction. 1 gram of cocoa seed or cocoa husk sample was extracted twice with 6 mL of distilled water with stirring at 70 °C for one hour. The liquids obtained from each extraction were pooled.

2.3.4. Method D

Methanol-acidified water extraction. 1 gram of cocoa seed or cocoa husk sample was extracted using method A but acidified to pH 3 with HCl.

2.3.5. Method E

Acidified water extraction. 1 gram of cocoa seed or cocoa husk sample was extracted using method C but acidified to pH 3 with HCl.

2.4. Determination of total phenols

Total phenolic content was determined by the Folin-Ciocalteu spectrophotometric method and was expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965). A 20-μL aliquot of the Method D extract (described above), then 80 μL of Na2CO3 0.7 M and 100 μL of Folin-Ciocalteu reactive 0.2 M were added to a microplate. A microplate reader (Model 550; Bio-Rad, Hercules, CA) was used for the absorbance measurements at 490 nm. Results were calculated with an equation obtained from a calibration curve whose concentrations ranged from 0 to 100 mg of gallic acid.

2.5. Analysis of phenols

2.5.1. HPLC-DAD

Phenols were quantified using a Varian ProStar liquid chromatography system with a C-18 column (Kinetex® Biphenyl 100 Å, 250 mm × 4.6 mm, i.d. 5 μm) and diode array detector (DAD; the

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