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Origin-based polyphenolic fingerprinting of *Theobroma cacao* in unfermented and fermented beans

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ABSTRACT

A comprehensive analysis of cocoa polyphenols from unfermented and fermented cocoa beans from a wide range of geographic origins was carried out to catalogue systematic differences based on their origin as well as fermentation status. This study identifies previously unknown compounds with the goal to ascertain, which of these are responsible for the largest differences between bean types. UHPLC coupled with ultra-high resolution time-of-flight mass spectrometry was employed to identify and relatively quantify various oligomeric proanthocyanidins and their glycosides amongst several other unreported compounds. A series of biomarkers allowing a clear distinction between unfermented and fermented cocoa beans and for beans of different origins were identified. The large sample set employed allowed comparison of statistically significant variations of key cocoa constituents.

1. Introduction

The health and nutritional benefits of cocoa products have been the subject of intense research, including several recent monographs (Paoletti, Poli, Conti, & Visioli, 2012; Watson et al., 2013). Major contributors to these benefits, in the form of anti-oxidative agents, have been attributed to the abundance of polyphenolic compounds in cocoa (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999; Natsume et al., 2000; Sanbongi et al., 1998; Wollgast & Anklam, 2000; Wollgast, Pallaroni, Agazzi, & Anklam, 2001), which can account for up to 18% of the dried bean mass (Bravo, 2009). Consequently, a health claim on cocoa flavanols referring to their properties beneficial to cardiovascular health was granted by EFSA in 2012 (Commission Regulation (EU) No 432/2012). Interestingly, the antioxidant capacity of cocoa has been shown to surpass that of tea as well as red wine (Lee, Kim, Lee, & Lee, 2003). Furthermore, polyphenols not only impart some of the bitter and astringent tastes in cocoa (Stark, Bareuther, & Hofmann, 2005, 2006), but also contribute to the signature reddish-brown color of cocoa-derived products (Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012). The importance of investigating the diversity of cocoa polyphenols has, therefore, already resulted in several key studies, where, aside from well-known polyphenols including catechins, procyanidins (and their oligomers), anthocyanidins as well as flavanols and their glycosides (Sánchez-Rabaneda et al., 2003; Wollgast & Anklam, 2000), several other phenolic compounds, such as clovamide (Arlorio et al., 2008; Sanbongi et al., 1998), *N*-phenylpropenoyl-L-amino acids (Stark & Hofmann, 2005; Stark, Justus, & Hofmann, 2006), and C-glycosylated flavan-3-ols (Stark & Hofmann, 2006; Stark, Keller, Wenker, Hillmann, & Hofmann, 2007), have been identified and, at times, quantified.

The quantity and variety of cocoa polyphenols have previously been shown to be highly dependent not only on intrinsic factors, such as genotype (Hansen, Mañez, Burri, & Bousbaine, 2000; Jonfia-Essien, West, Alderson, & Tucker, 2008), but also extrinsic factors such as origin (Elwers, Zambrano, Rohsius, & Lieberei, 2009), harvest season (Oracz, Zyzelewicz, & Nebesny, 2015), fermentation methods (Afoakwa, Quao, Takrama, Budu, & Saalia, 2013; Nazaruddin, Seng, Hassan, & Said, 2006), and subsequent processing steps (Bordiga et al., 2015; Counet, Ouwerx, Rosoux, & Collin, 2004; Kothe, Zimmermann, & Galensa, 2013; Redovnikovic et al., 2009). Several methods have been used to profile cocoa polyphenols in order to investigate these differences, including near infrared spectroscopy (Huang et al., 2014; Hue et al., 2014; Krähmer et al., 2015; Teye, Huang, Lei, & Dai, 2014; Teye et al., 2015), nuclear magnetic resonance spectroscopy (Caligiani, Palla, Acquotti, Marseglia, & Palla, 2014; Diomande et al., 2015), countercurrent chromatography (Esatbeyoglu, Wray, & Winterhalter, 2015), high resolution mass spectrometry (Milev, Patras, Dittmar, Vrancken, & Kuhnert, 2014), and most importantly,

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normal- and reverse-phase high performance liquid chromatography coupled to mass spectrometry (Adamson et al., 1999; Counet & Collin, 2003; Hammerstone et al., 1999; Lee et al., 2003; Patras, Milev, Vrancken, & Kuhnert, 2014; Sánchez-Rabaneda et al., 2003). Additionally, multivariate statistical techniques, such as principal component analysis (PCA), have been instrumental in processing the large set of chemometric data acquired from these experimental methods (Aculey et al., 2010; Caligiani et al., 2014; Marhuenda-Egea, Gonsálvez-Álvarez, Lledó-Bosch, Ten, & Bernabeu, 2013; Teye et al., 2015; Vázquez-Ovando, Molina-Freaner, Nuñez-Farfán, Betancur-Ancona, & Salvador-Figueroa, 2015).

Herein, we present a statistical analysis of the most diverse set of cocoa beans yet reported, paired in their respective unfermented and fermented form, from six different origins including the Ivory Coast, Tanzania, Malaysia, Indonesia, Ecuador, and Brazil. These countries were chosen based on geographical diversity, their global cocoa production, as well as sourcing opportunities. Previously unidentified compounds in unfermented beans have been tentatively assigned. We have compared the polyphenolic profiles of selected beans and have employed PCA to visualize the difference between the various bean types. The scoring plots reveal a selection of markers responsible major differences between the beans. Additionally, we have supplemented these results with total phenolic contents measured using the Folin-Ciocalteu assay (Singleton & Rossi, 1965) using gallic acid and TROLOX as standards.

2. Material and methods

2.1. Chemicals and reagents

Sodium acetate, dichloromethane, HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Carl Roth (Germany). Milli-Q water (18.2 MΩ·cm at 25 °C) was used throughout all experiments. Acetic acid, sodium carbonate, Folin & Ciocalteu's phenol reagent, gallic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), and hesperetin were obtained from Sigma-Aldrich. Hydrochloric acid was obtained from Merck (Germany).

2.2. Sample collection

Cocoa bean samples were collected from 6 countries (Ecuador, Indonesia, Malaysia, Tanzania, Ivory Coast, and Brazil) over 3 continents by Barry Callebaut AG, in unfermented and fermented form. Information on fermentation and drying times has been present in Table 1. Unfermented samples were stored at − 80 °C and fermented dry samples were stored at 4 °C prior to defatting and both stored at 4 °C after defatting.

2.3. Sample preparation

At least 30 g of cocoa beans from a sample were first de-shelled and then ground to a fine powder using a Retsch Grindomix GM200 knife mill (Haan, Germany) at 10,000 rpm. 5 g of powder was defatted using dichloromethane in an automated Büchi B-811 Soxhlet extraction apparatus (Essen, Germany) for 18 h. The defatted powder was dried under vacuum and stored at 4 °C until further use.

Polyphenol extraction was carried out using an acidified methanolic solution ($MeOH:H_2O:CH_3COOH$::70:28:2). 50 mg of defatted power was mixed with 5 mL of the extraction solution and this mixture was sonicated in an ultrasonication bath for 10 mins, stirred for a further 30 mins, and finally syringe-filtered through a PTFE membrane filter (0.45 μm). The methanolic extract was spiked with hesperetin as an internal standard (final concentration of 2 mg/L) and used directly after for HPLC-MS experiments. The unspiked extract was diluted 5 times for total phenolics assays, which were also performed directly after extraction.

2.4. Total phenolics assay

Total phenolic content was determined in a 96-well plate using an optimized Folin-Ciocalteu assay that has been previously reported (Cicco, Lanorte, Paraggio, Viggiano, & Lattanzio, 2009). Gallic acid and TROLOX were used as standards with concentrations ranging from 0 to 0.5 mg/mL in up to 8 steps. All standard solutions were prepared using the same methanolic extraction solution used for polyphenol extraction. Briefly, 30 μL of diluted polyphenol extract (or standard) was made up to 100 μL using Milli-Q water. 100 μL of Folin-Ciocalteu reagent was added the resulting mixture was vortexed for 2–5 s. To this solution was added 800 μL of a 5% solution of sodium carbonate. The resulting mixture was vortexed again and placed in an oven for 20 mins at 40 °C. These solutions were cooled to room temperature, vortexed once more, and transferred onto a 96-well plate (200 μL per well). Absorbance was measured at 725 nm on a 96-well Biochrom EZ Read 2000 microplate reader (Cambridge, UK).

2.5. HPLC-TOF-MS

HPLC experiments were performed on an Agilent 1260 HPLC system using a ZORBAX Eclipse Plus C18 column (RRHD, 2.1 × 100 mm, 1.8 μm particle size) along with the recommended guard column. The binary solvent system used consisted of Milli-Q water (Solvent A) and acetonitrile (Solvent B), both containing 0.05% formic acid. The sample injection volume was 2 μL. The gradient employed was adapted from a previously reported method (Sánchez-Rabaneda et al., 2003) at a constant flow rate of 0.5 mL/min and column temperature of 40 °C. The gradient was (t (min), %B): (0, 5); (1, 5); (8, 16.5); (9, 17); (10, 17.5); (11, 17.5); (12, 18.5); (13, 18.5); (23, 95); (25, 95).

The effluent HPLC system was connected to an Impact HD ultra-high resolution ESI-Q-q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an electrospray ionization source (nebulizer pressure of 1.8 bars, dry gas flow rate of 9 L/min, and dry gas temperature of 200 °C). All data were acquired in the negative ion mode. The TOF analyzer was calibrated with a 0.1 M sodium formate solution before each chromatographic run. Calibrations were carried out in the enhanced quadratic mode. MS data were recorded in negative ion mode, where full scan spectra and selected MS/MS datasets were recorded. Monoisotopic molecular masses were calculated from molecular formulae using reported NIST monoisotopic atomic masses of the elements (Coursey, Schwab, Tsai, & Dragoset, 2015).

2.6. Statistical analysis

Analysis of HPLC-TOF-MS unfermented data was performed using the R package XCMS (Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006; Tautenhahn, Bottcher, & Neumann, 2008) and MAIT (Fernandez-Albert, Llorach, Andres-Lacueva, & Perera, 2014). Peak areas were normalized to the internal standard (hesperetin) and used for relative quantification. The intensity threshold was set to 10^4. Only peaks detected in more than 75% of all samples were taken into account.

The resulting 57 peaks were used to perform principal component analysis (PCA). The data was centered and Pareto scaling was applied (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006).

3. Results and discussion

Within this contribution, we present a detailed and comprehensive analysis and comparison of the polyphenol profiles of unfermented (also frequently referred to as raw or wet cocoa beans) and fermented beans from different origins comparing a total of 86 samples. Several previous papers in the literature introduced a related exercise, however, in our view, a significant number of them suffered from shortcomings which include small and restricted sample sets, lack of details on sample origins, low levels of compound assignments, particularly after

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