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Short communication

Characterization of new flavan-3-ol derivatives in fermented cocoa beans

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ABSTRACT

Two series of compounds showing mass signals at *m/z* 605 and 893 (negative ionization mode) have been detected in fermented cocoa beans. This study objective is to identify these mass signals and characterize their structure in fermented cocoa samples. Our hypothesis is that these signals may correspond to ethyl-bridged flavan-3-ols resulting from flavan-3-ol condensation with acetaldehyde which is a microbial metabolite. Mass spectrometry was used to compare the retention times and mass fragmentation patterns between a model solution using epicatechin and procyanidin dimer B2, the major flavan-3-ols of cocoa, as precursors and extracts of fermented cocoa. Their identification was confirmed: four isomers of ethyl-linked epicatechin as well as several isomers of epicatechin-ethyl-procyanidin B2, in which B2 was mostly linked through its upper unit, were characterized in cocoa. This study demonstrates the presence of flavan-3-ol acetaldehyde condensation products in fermented cocoa beans and provides the first report of epicatechin-ethyl-procyanidin B2.

1. Introduction

Polyphenols are abundant plant secondary metabolites, including a large variety of compounds with over ten thousand structures identified to date (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011; Cheynier, Comte, Davies, Lattanzio, & Martens, 2013). They are important bioactive compounds and contribute to the health benefits associated with dietary consumption of plant foods and to organoleptic properties, such as color, astringency and bitterness. Among polyphenols, proanthocyanidins, which are oligomers and polymers of flavan-3-ols, are particularly abundant in most plant-derived foods.

Cocoa beans, the seeds of the cocoa tree (*Theobroma cacao),* are the essential ingredient to make chocolate. They contain large amounts of polyphenols (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999) and are among the richest dietary sources of proanthocyanidins (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). The major polyphenols in unfermented cocoa beans are the flavan-3-ol monomer (−)-epicatechin and procyanidins (i.e. oligomers of catechin and epicatechin), up to decamers (Hammerstone & Lazarus, 2000; Hammerstone et al., 1999). They are also major contributors to the bitterness and astringency of raw cocoas bean (Stark, Bareuther, & Hofmann, 2005). Before they can be sold to high quality chocolate makers, raw cocoa beans go through different processes, all of which are important for cocoa quality (Albertini et al., 2015; de Brito et al., 2001; De Taeye, Caullet, Eyamo Evina, & Collin, 2017; Stark et al., 2005; Zahouli, Tagro, Fae, Ban-Koffi, & Nemlin, 2010). In particular, the fermentation process, lasting from 4 to almost 7 days and involving wide temperature (up to 50 °C) and pH variations (down to pH 4) (Schwan, Rose, & Board, 1995), as well as generation of microbial metabolites such as acetic acid, lactic acid, ethanol, and acetaldehyde (Albertini et al., 2015; Eyamo Evina et al., 2016), is a key step, as many flavor precursors are formed during this phase. The combination of acidic conditions and high temperatures leads to major changes in cocoa beans. Polyphenols are particularly affected by these transformations as they are highly reactive molecules, potentially yielding hundreds of products through a diversity of reactions. Indeed, during this step, the polyphenol concentration decreases (Kim & Keeney, 1984). Epimerization, cleavage of the interflavanic bonds and oxidation have been suggested to affect procyanidin oligomers during cocoa processing (De Taeye et al., 2017). Dehydrodiepicatechin A, arising from oxidation of epicatechin, and two of its isomers were detected in fermented cocoa beans (Eyamo Evina et al., 2016). Degradation of procyanidin trimer C1, under conditions mimicking cocoa processing, yielded epimers and oxidation products containing additional A-type linkages, suggesting that nonpolar compounds detected in chocolate result from similar reactions (De Taeye et al., 2017). Nevertheless, most flavan-3-ol derivatives formed in cocoa

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and chocolate are still unknown.

After comparison of the composition of unfermented and fermented cocoa beans, several polyphenols have been proposed as biomarkers for fermentation (D'Souza et al., 2017). These include known compounds, such as epicatechin or caffeoyl and coumaroyl aspartates, but also a series of unknown compounds, tentatively described as dimethyl *O*-flavanol oligomers.

For some of these compounds, detected at *m/z* 605 in the negative ion mode, several structural hypotheses have been postulated. Thus, a molecular formula ($C_{31}H_{25}O_{13}$) and structures similar to procyanidin dimers in which the lower flavan-3-ol unit is replaced with a methylated flavanone with the formula $C_{16}H_{14}O_7$ has been proposed from mass fragmentation spectra (Patras, Milev, Vrancken, & Kuhnert, 2014). More recently, the signals, detected at *m/z* 605.1661 by high-resolution mass spectrometry, have been reattributed to the ionized molecular formula $C_{32}H_{29}O_{12}$ and postulated to correspond to dimethyl-*O*-procyanidin B dimers (D'Souza et al., 2017). However, this molecular formula may also correspond to (*epi*)catechin dimers in which the two flavan-3-ol units are linked through a methylmethine bridge (Cádiz-Gurrea et al., 2014). These compounds, commonly called ethyl-bridged (*epi*)catechin dimers, result from (*epi*)catechin condensation with acetaldehyde (Fulcrand, Doco, Es-Safi, Cheynier, & Moutounet, 1996). They are well known to occur in fermented beverages such as wine (Cheynier, Fulcrand, Sarni-Manchado, & Moutounet, 1997; Saucier, Little, & Glories, 1997), which contains flavan-3-ols and acetaldehyde, and have been reported in plants (Pinasseau et al., 2017; Tanaka, Takahashi, Kouno, & Nonaka, 1994). They may also form during fermentation of cocoa beans, which exhibits many favourable conditions, namely the presence of acetaldehyde, catechin/epicatechin, as well as low pH, high temperature, and long exposure time. However, they have not yet been identified in cocoa.

Considering that flavonoids react randomly in this condensation process, as recently shown (Vallverdú-Queralt et al., 2017a, 2017b), the compounds detected at *m/z* 893.2290 in the negative ion mode, assigned to dimethyl-*O*-procyanidin B trimer ($C_{47}H_{41}O_{18}$) (D'Souza et al., 2017), may correspond to similar compounds formed by acetaldehyde condensation of (*epi*)catechin with a procyanidin dimer, such as epicatechin dimers B2 and B5, which are abundant in cocoa. To the best of our knowledge, these molecules have never been characterized.

The purpose of the present work was to confirm this structural hypothesis, demonstrate that these molecules detected in fermented cocoa beans arise from flavan-3-ol condensation with acetaldehyde and identify them.

2. Materials and methods

2.1. Reagents and materials

Methanol (CH_3OH), formic acid (HCO_2H) and acetic acid (CH_3COOH) were obtained from Prolabo (Fontenay S/Bois, France). (−)-Epicatechin and procyanidin B2 were purchased from Sigma (St. Louis, MO). Acetaldehyde (CH_3CHO) was obtained from Merck (Darmstadt, Germany). Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA) prior to use.

2.2. Polyphenol extraction from fermented and dried cocoa beans

The sample of fermented and dried cocoa beans was provided by Valrhona SA (Tain l'Hermitage, France), ground under liquid nitrogen and stored at –20 °C until extraction. The cocoa bean powder (0.24 g) was first defatted with hexane (6 mL, extraction in an ultrasonic bath at 40 °C for 90 min). After centrifugation, the pellet was dried under vacuum and extracted using an adapted protocol (Mané et al., 2007): 0.015*g* of defatted cocoa powder were added with 75 µL of methanol-acetic acid (98:2, v/v) vortexed and put in an ultrasonic bath for 5 min at 28 °C. Then 900 µL of acetone-H_2O-acetic acid (70:28:2, *v/v/v*) were added and samples were vortexed and put in the ultrasonic bath for 30 min. Samples were then centrifuged at 17,640 RCF for 15 min at 4 °C. The supernatant was removed and dried under vacuum (GENEVAC EZ-2; 35 °C for 2 h). The polyphenol extract was stored at –20 °C until analysed.

2.3. Hemisynthesis of ethyl-bridged flavan-3-ol dimers and trimers

The following protocol was adapted from Es-Saffi et al. (Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999). An acidic solution was prepared with 12 µL of acetic acid in 225 µL of water and 100 µL of ethanol, giving a pH value of 2.2. Then 3.6 mg of (−)-epicatechin (34.5 mmol/L) and 1.8 mg of proanthocyanidin B2 (8.67 mmol/L) were mixed and 22 µL of acetaldehyde (1093 mmol/L) were then added. The solution was left at 22 °C for 20 min before the reactions were monitored by UHPLC-ESI-IT-MS (ultra-high-performance liquid chromatography with ion trap mass spectrometry).

2.4. UHPLC-MS analysis

The analyses were carried out on two different UHPLC-MS machines: a UHPLC-ESI-IT-MS for determination of the fragmentation schemes and a UHPLC-ESI-HRMS (ultra-high-performance liquid chromatography with high-resolution mass spectrometry) for determination of the exact mass and molecular formula.

UHPLC-ESI-IT-MS analyses were carried out with an ultra-high-performance liquid chromatography system (UHPLC) UPLC Acquity system from Waters (Milford, MA) hyphenated to a Bruker Daltonics AmaZonX (Bremen, Germany) ion trap mass spectrometer (IT-MS) equipped with an electrospray ion source (ESI). Chromatographic separation was achieved with a BEH C_{18} column (150 mm length, 1 mm internal diameter, 1.7 µm particle size) equipped with a 0.2-µm prefilter (Waters, Milford, MA). The mobile phase consisted of two solvents: solvent **A** being water/formic acid (99/1, *v/v*) and solvent **B** being methanol/formic acid (99/1, *v/v*). The elution program was a linear multi-step gradient (0 min: 2% **B**; 1 min: 2% **B**; 6.5 min: 15% **B**; 9 min: 15% **B**; 12 min: 30% **B**; 14 min: 30% **B**; 27 min: 75% **B**; 32 min: 95% **B**; 37 min: 95% **B**; 40 min: 2% **B**; 45 min: 2% **B**). The flow rate was 0.08 mL/min with an injection volume set at 0.5 µL. Mass spectrometry analyses were conducted in negative ionization mode and the specific conditions were a scan between *m/z* 100 and 2000; target mass was set at *m/z* 500.

UHPLC-ESI-HRMS analyses were carried out using an Accela UHPLC system from Thermo Fisher Scientific (San Jose, CA) consisting of an autosampler, a quaternary pump, a vacuum degasser, and a thermostated column compartment, coupled with a high-resolution (HRMS) linear ion trap-Orbitrap (LTQ-Orbitrap Velos) equipped with an electrospray ionization (ESI) source. Chromatographic separation was achieved under the same conditions as described above. The specific HRMS conditions were adapted from a previous method (Vallverdú-Queralt et al., 2015). Instrument control and data acquisition were performed with Xcalibur 3.0. software (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out the same day as the analysis according to the manufacturer's guidelines.

Tentative annotation of the chromatographic peaks was made according to the measured accurate masses and the isotopic patterns. Under these conditions, all proposed molecular formulas were estimated with mass errors below 2 ppm (2 mmu). Metabolite identification was performed by comparing retention times and mass fragmentation spectra to those of the available standards, including ethyl-bridged flavanol oligomers obtained by hemisynthesis.

3. Results and discussion

UPLC-ESI-IT-MS analysis of fermented cocoa bean extracts showed the presence of numerous compounds, including series of molecules