Two molecules newly identified by Mass Spectrometry in fermented cocoa beans impact chocolate sensory quality

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Abstract

Polyphenols are a wide and diverse group of plant secondary metabolites found in large amount in cocoa beans. They can be further modified under specific conditions such as fermentation or oxidation. The aim of this study was to characterize this group of molecules and evaluate their impact on chocolate taste. Sixteen cocoas bean samples and the sixteen chocolate samples associated were analyzed. The chocolates were made by a standard process. They were divided into four sensory groups by sensory analysis.

The polyphenols have been extracted from the ground and defatted cocoa beans and analyzed by UHPLC-HRMS. Two new series of polyphenolic compounds have been detected in fermented cocoa beans. Two mass signals at *m/z* 605 and 893 (in the negative ionization mode) have been assigned to compounds known as ethyl bridged flavanols that had never been reported in cocoa. These molecules resulting from condensation of flavanols with acetaldehyde can be formed in planta or more likely during fermentation as acetaldehyde is a microbial metabolite.

Chemometrics applied to cocoa polyphenol composition showed that ethyl-bridged flavanols are key molecules to discriminate cocoas according to the sensorial groups of the associated chocolate. Future studies will aim at determining the contribution of these molecules to cocoa and chocolate taste, especially bitterness and astringency.

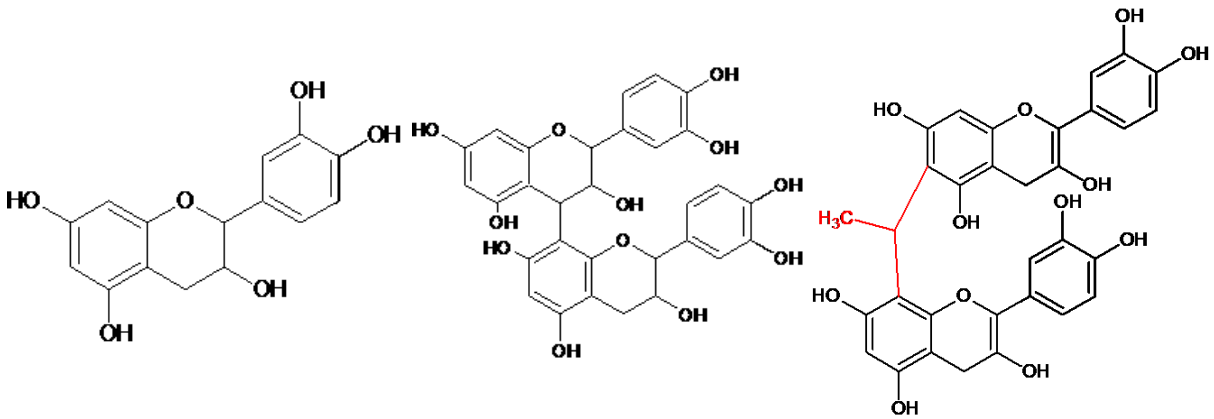
Introduction

Cocoa beans, the seeds of the cocoa tree (*Theobroma cacao),* are the essential ingredient to make chocolate. They contain large amounts of polyphenols, particularly flavonoids such as flavan-3-ols (Hammerstone *et al*, 1999). Polyphenols are a wide family of molecules with different classes and sub-classes, having multiple roles for the plant survival: they can protect the plant from predators or from the sun and are responsible of some tissue colour (Andersen and Markham, 2006). Polyphenols are also responsible for the bitterness and astringency in raw cocoas bean (Stark *et al*, 2005): they can be found in such high quantities that the raw bean is practically inedible.

Before they can be sold to manufacturers, raw cocoas beans go through different processes, all of which are important for cocoa quality (de Brito *et al*, 2001, Stark *et al*, 2005, Zahouli *et al*, 2010, Albertini *et al*, 2015, de Taeye *et al*, 2017). In particular, the fermentation process, lasting from 2 to almost 10 days and involving wide temperature and pH variations, is a key step as many flavour precursors are formed during this phase. Polyphenols are particularly concerned by the transformation as they are highly reactive molecules, potentially yielding hundreds of products through a diversity of reactions. Polyphenol composition is strongly modified during fermentation, so that several polyphenols have been recently proposed as relevant biomarkers for fermentation (Dsouza *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.

In particular, molecules detected at *m/z* 605. 1661 in the negative ion mode have been attributed the molecular formula $C_{32}H_{30}O_{13}$ on the basis of high resolution mass spectrometry analysis and postulated to be dimethyl-*O*-procyanidin B dimers. 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, commonly designated as ethyl-bridged (epi)catechin dimers (Figure 1) as postulated by Cadiz-Gurrea *et al*, 2014. These compounds, resulting from (epi)catechin condensation with acetaldehyde (Fulcrand *et al*, 1996), are well known to occur in fermented beverages such as wine and are likely to form during fermentation of cocoa beans. Indeed, the cocoa bean fermentation process regroups many favourable

conditions allowing the formation of ethyl bridged flavanol oligomers, namely the presence of acetaldehyde, catechin/epicatechin as well as low pH, high temperature, and long exposure time.



(epi)catechin
M=290.271 g/mol

Procyanidin dimer
M=578.526 g/mol

Ethyl Bridged Dimer
M=605.166 g/mol

Figure 1: Structures of some flavan-3-ols ((epi)catechin monomer and dimer) and new ethyl-bridged epicatechin dimer present in cocoa beans.

Similarly, the compounds detected at *m/z* = 893.2290 in the negative ion mode, assigned to dimethyl-*O*-procyanidin B trimer ($C_{47}H_{42}O_{18}$) (Dsouza *et al*, 2017), likely correspond to ethyl-bridged flavan-3-ol trimers in which one (epi)catechin is linked though an ethyl bridge to a B-type procyanidin dimer. Indeed, procyanidin dimers, and especially epicatechin dimers B2 and B5, are abundant in cocoa and could react with acetaldehyde in the same way as the monomers, yielding several isomers of ethyl bridged trimers.

The aim of this study was to characterize these compounds, analyse them in cocoa, and relate them to chocolate sensory characteristics.

Materials and methods

Samples

16 cocoas beans from different origins and harvest years have been used in this work. The beans have been fermented and dried and are therefore commercial beans. Part of each of the 16 cocoa bean samples has been transformed into chocolate using a standard process, leading to the corresponding 16 chocolates. These chocolates have been divided into four sensory poles after sensory analysis by a trained panel. The four sensory poles have then been associated to the corresponding cocoa beans.

Reagents

Hexane and acetic acid were purchased from VWR Prolabo® (Radnor, PA, USA). Methanol, acetone and formic acid were purchased from Sigma Aldrich (Saint Louis, MI, USA). Deionized water has been purified with a Milli-Q water system (Millipore, Bedford, MA, USA). (-)-epicatechin, and procyanidin dimer B2 were purchased from Sigma (St. Louis, MO). Acetaldehyde was obtained from Merck (Darmstadt, Germany).

Extraction

Polyphenol extraction from powders of the fermented dried cocoa beans has been performed in two successive steps: delipidation with hexane and polyphenol extraction with methanol and acetone, following a protocol adapted from Mane *et al* (2007). Each polyphenol extraction has been performed in triplicate. The polyphenols extracts were stored at -20°C until analysed.

UPLC-DAD-MS analysis

UPLC-DAD-ESI-IT-MS: The analyses were carried out with a UHPLC Acquity system from Waters (Milford, MA, USA). Chromatographic separation was achieved with a BEH C_{18} 1.7 µm x 1 mm x 150 mm (35°C) column equipped with a 0.2µm pre-filter (Waters). The mobile phase consisted of two solvents: solvent A water/formic acid (99/1, v/v) and solvent B = methanol/formic acid (99/1, v/v). The elution program was (min; %B) (0;2) (1;2) (6.5;15) (9;15) (12;30) (14;30) (27;75) (32;95) (37;95) (40;2) (45;2). The flow rate was 0.08mL/min with an injection volume set at 0.5µL. UV detection was set from 200nm to 650nm.

Mass analysis was performed with a Bruker Daltonics AmaZonX (Bremen, Germany) mass spectrometer equipped with an electrospray source and an ion trap mass analyser. Analyses were conducted in negative ionisation mode and the specific conditions were a scan between 100 *m/z* and 2000 *m/z*, target mass set at 500 *m/z*.

UPLC- High resolution MS - The analyses were carried out using an Accela UHPLC system from Thermo Fisher Scientific (San Jose, CA, USA). Chromatographic separation was performed on an Acquity BEH C_{18} column (150 mm length, 1 mm internal diameter, 1.7 µm particle size; Waters) at 35 °C. The mobile

phase consisted of H_2O/HCO_2H (99/1, V/V) (solvent A) and CH_3OH/HCO_2H (99/1, V/V) (solvent B). The flow rate was 0.08 mL/min and the injection volume 0.5 µL. The elution program was as follows: (min; %B) (0;2) (1;2) (6.5;15) (9;15) (12;30) (14;30) (27;75) (32;95) (37;95) (40;2) (45;2).

The mass spectrometer was a linear ion trap-Orbitrap (LTQ-Orbitrap Velos) from Thermo Fisher Scientific (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source.

Hemisynthesis of ethyl-bridged flavanol dimers and trimers

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. 3.6 milligrams of (-)-epicatechin and 1.8 mg of proanthocyanidin B2 were mixed and 22 µL of acetaldehyde (1157 mol/L) were then added. The reactions were monitored by UHPLC coupled with the low resolution mass spectrometer (ion trap).

Chemometrics analysis

Chemometrics analysis was applied to the average low resolution mass spectra recorded for each sample along the first 24minutes of the elution profile. After recalibrating the m/z values using the Scilab (Version 6.0.0) software (Scilab Enterprises, Orsay, France), a list of *m/z* signals (around 900 values) was established and the corresponding intensities were recorded for each sample. Analysis of this data using PLS-DA (Partial Least Square Discriminant Analysis) was performed using Unscrambler® X (Version 10.4.1) (CAMO Software AS, Oslo, Norway).

Results

Characterisation of ethyl-bridged flavanol oligomers in cocoa

Analysis of cocoa polyphenols was performed by both UPLC-HRMS and UPLC-ESI-IT-MS, giving access respectively to exact mass and thus molecular formula and to fragmentation patterns. In addition, reactions of acetaldehyde with epicatechin and procyanidin B2, selected as they are the main flavan-3-ols present in cocoa, were investigated in model solution and the products thus formed analysed by UPLC-ESI-IT-MS and compared to those present in the cocoa beans to confirm identification.

High resolution mass spectrometry analysis of cocoa samples enabled detection of the target molecules, at *m/z* 605.1641 and *m/z* 893.2282, in the negative ion mode, with several isomers eluted at different retention times. These experimental masses are very close to the theoretical masses (respectively 605.1659 and 893.2292) as the mass defect is only of 1.8 and 1 mDa.

The mass signal at *m/z* 605 was detected four times (at 17.6min, 21.2min, 21.9min, and 24.6min) along the chromatogram obtained by UPLC-ESI-IT-MS analysis of the cocoa sample, indicating that at least 4 isomers are present (Figure 2).

UPLC-ESI-IT-MS analysis of the model solution containing epicatechin, procyanidin B2, and acetaldehyde after 12 hours of incubation (Figure 2), also showed four compounds at *m/z* 605, corresponding to the four expected isomers of ethyl-linked epicatechin dimers. On the basis of their relative retention times on the C_{18} column reported earlier (Es-Safi *et al*, 1999), the compounds eluted at 17.6 min and 24.6 min, respectively, likely correspond to the 8-8 and 6-6 linked dimers and the other two, eluted at 21.2 min and 21.9 min, to the R and S enantiomers of the 8-6 linked dimer. These retention times are the same as those observed in the cocoa extract, suggesting that the four isomers detected in cocoa are the four ethyl-bridged epicatechin dimers.

The different proportions observed (*i.e*. lower proportions of the first eluting one and higher proportion of the last one in cocoa, likely reflects the higher instability of the 8-linkages as reported earlier (Tanaka *et al*, 1994).

Analysis of the MS fragmentation patterns of each of the isomers showed fragment ions at *m/z* 315 and 289, corresponding to vinylepicatechin and epicatechin, respectively, resulting from cleavage of the ethyl bridge, as classically described for these molecules (Saucier *et al*, 1997). An additional fragment at *m/z* 453, arising from a retro Diels Alder fragmentation of one of the epicatechin units, was also observed.

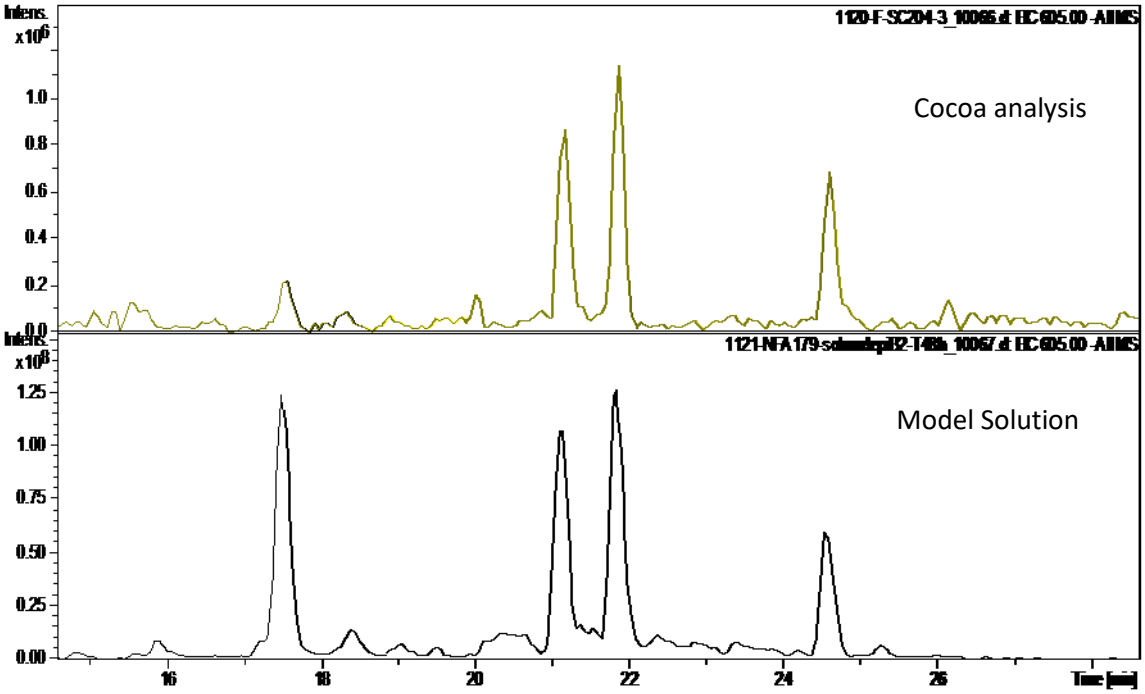


Figure 2: Extracted ion chromatograms recorded at m/z 605 after UPLC-ESI-MS analysis of the cocoa extract (top) and of the model solution (bottom).

Comparison of the extracted ion chromatograms recorded at *m/z* 893 for a cocoa extract and for the model solution containing procyanidin B2, epicatechin and acetaldehyde is presented in Figure 3.

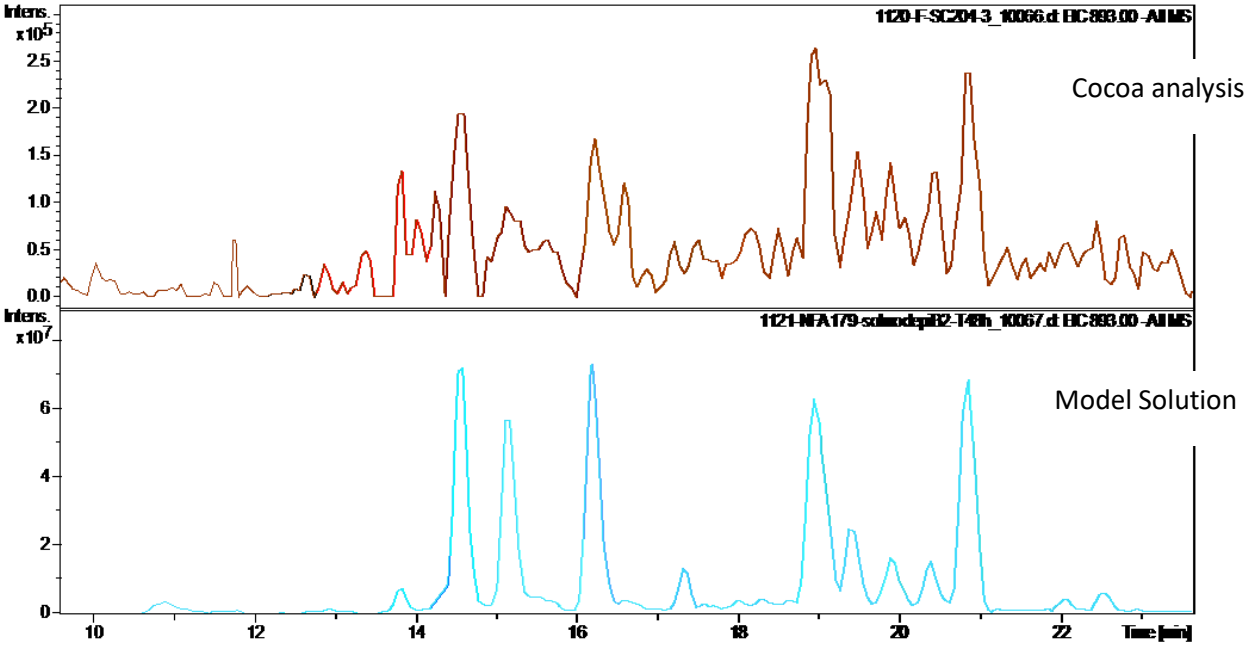


Figure 3: Extracted ion chromatograms recorded at m/z 893 after UPLC-ESI-MS analysis of the cocoa extract (top) and of the model solution (bottom).

Numerous isomers were detected in the model solution, confirming that acetaldehyde, epicatechin, and procyanidin B2 can react together to form ethyl-bridged flavanol trimers. The large number of isomers results from the diversity of positions (2, *i.e*. C6 and C8 for epicatechin; 3 for procyanidin B2, as the C8 position of the lower epicatechin unit is involved in the interflavanic bond) and the presence of R and S enantiomers for each of the resulting product. MS fragmentation yielded fragment ions at *m/z* 603 and *m/z* 577, corresponding to vinyl procyanidin dimer B2 and procyanidin dimer B2, formed by cleavage of the ethyl bridge (loss of epicatechin, -290 a.m.u and of ethyl-epicatechin, -316 a.m.u), in agreement with the postulated structures. Another fragment at *m/z* 451 corresponds to RDA fragmentation (loss of 152 u.m.a) from the fragment at *m/z* 603.

Extraction of the signal at *m/z* 893 in cocoa samples yielded a similar profile with the major isomers eluted at 14.5, 16.2 min, 19.1 min and 20.8 min, confirming that these signals correspond to ethyl-bridged epicatechin –procyanidin dimer adducts which had never been characterized.

Role of ethyl-bridged flavanol oligomers in the discrimination of chocolate sensory poles

Chemometrics analysis was performed on the polyphenol composition data obtained by UPLC-ESI-IT-MS analysis of 16 cocoa samples distributed in the 4 sensory poles. The average spectra recorded along the first 24min of the elution profile were used as fingerprint of cocoa polyphenol composition (Bruker Compass, DataAnalysis, version 4.1). A list of *m/z* signals (around 900 different *m/z* values ranging from 110 to 1300) was established and the corresponding intensities were recorded for each of the triplicate extracts prepared from the 16 cocoa samples. A matrix of intensities for each m/z value and each sample was thus built and analysed by partial least square discriminant analysis (PLS-DA).

Projection of the cocoa samples on the first two axes, representing 78% and 13% respectively of the sample variability, showed good reproducibility (as most triplicate samples were grouped) and good discrimination of the sensory poles (Figure 4A). Projection of the variables on the first two axes (Figure 4B) indicates that only a few of them, i.e. those showing the highest loadings, were involved in the

discrimination. Based on this assumption, the distance of each variable to the graph centre was calculated and used to select the 50 most relevant variables for sensory pole discrimination.

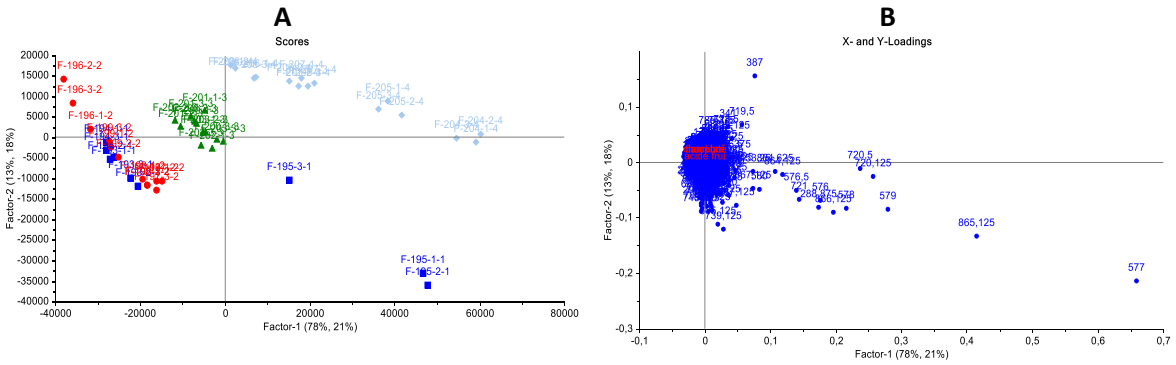


Figure 4 A: projection of the cocoa samples (with different colors representing the different sensory poles) and B: loadings of the variables on the first two axes obtained after the PLS-DA of cocoa bean polyphenol extracts.

The signals with the highest loadings could be attributed to flavan-3-ols monomers and oligomers (up to the hexamer) and their derivatives. In particular, the ethyl-linked dimers and trimers were ranked 9th and 14th, respectively, after elimination of redundant signals such as isotopic peaks, indicating that they play an important role in the discrimination of chocolate sensory poles.

It is worth noting that ethyl-linked epicatechin oligomers have been reported to be as astringent as procyanidins of similar chain length and taste more bitter (Vidal *et al*, 2004).

Conclusion

Our strategy involving a combination of mass spectrometry analysis and model solution studies allowed identification of ethyl-bridged flavanol dimers and trimers in cocoa. The former were already known in other fermented foods while the latter had never been reported. Their formation, resulting from condensation reactions of flavanols with acetaldehyde, is expected to depend on the composition of the unfermented beans but also, as acetaldehyde is produced by various microorganisms, on the fermentation conditions and flora involved.

Chemometrics analysis of cocoa polyphenol extracts demonstrated that these molecules play an important role in the discrimination of chocolate sensory poles from cocoa composition. Future studies

will aim at determining the fate of these molecules in chocolate processing and their contribution to cocoa and chocolate taste, especially bitterness and astringency.

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