Technical note | 002891

Metabolomics

Shades of fine dark chocolate: polyphenol metabolomics and molecular networking to enlighten the brown from the black

Authors

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Keywords

Dark chocolates, metabolomics, feature-based molecular networking, polyphenol metabolomics, phenolic compounds, discriminating compounds, Orbitrap Exploris 480 mass spectrometer, Compound Discoverer software

Goal

To evaluate the chemical composition of black and brown dark chocolates to reveal compounds that discriminate between the different colored chocolates and to propose potential markers of those chocolates.

Introduction

Chocolate, one of the most loved foods in the world, traditionally comes in three types: dark, milk, and white. Consumption of dark chocolate, specifically, has been linked with several health benefits, which has caught the attention of researchers. Among the biological effects of chocolate, the most extensive studies have focused on the antioxidant, anti-inflammatory, cardiovascular, and metabolic effects, which are mainly associated with phenolic compounds.[1,2]

Derived from the seed kernels of the *Theobroma cacao* L. (Malvaceae) tree,[3] cocoa beans are initially processed by farmers through different stages, including fermenting and drying. Next, they are processed by manufacturers through roasting, alkalization, and conching to obtain chocolate liquor, cocoa powder, and butter—all of which are ingredients in chocolate and other finished products. The chemical composition of chocolate depends on several variables related to the raw material, formulation, and processing.

High-quality dark chocolates (70% cocoa content) can have shades from light to dark brown color. These color differences may be related to different chemical profiles. Color variations are related in part to the phenolic compounds, whose oxidation products contribute to the formation of colored compounds,[4,5] and also to Maillard reaction products that correspond to brown polymeric compounds.[6,7]

More knowledge is needed to better understand the color diversity and phenolic profiles of dark chocolate. In this work, we conducted a non-targeted metabolomics study based on ultra-high performance liquid chromatography – high-resolution mass spectrometry/mass spectrometry (UHPLC-HRMS/MS) experiments, as well as univariate, multivariate, and molecular networking analyses.

Experimental

Chocolate samples

Thirty-seven fine chocolate samples (70% cocoa), manufactured from commercial cocoa beans by a standardized process in 2019 and 2020, were provided by Valrhona SA, Tain l'Hermitage, France.

Color classification

After melting the chocolate bars in triplicate in an oven at 45° C, color analysis was performed by CIELAB colorimetry. The color parameters that describe the color perceived by the observer were expressed as: Lightness (L* = 0 black to L* = 100 white), a* (a* < 0 green to a* > 0 red) and b* (b* < 0 blue to b* > 0 yellow). The chocolate L*, a*, and b* values were used for the reconstruction of the chocolate colors with Adobe™ Photoshop™ software. A sensory analysis (by six evaluators) classified the color images into three groups with black, brown, and intermediate chocolates. This last group was eliminated from further analysis, and eight dark black samples and eight light brown samples were selected (Figure 1).

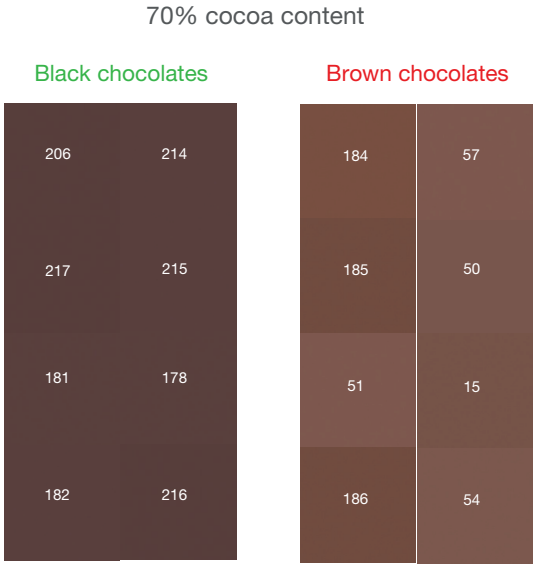

Figure 1. Color images of black and brown chocolate groups obtained from their L*, a*, b* color parameters by Adobe Photoshop software

Extraction of chocolate samples

To perform extraction, the chocolate was cut into small pieces and frozen with liquid nitrogen. Once frozen, the sample was ground in liquid nitrogen, and approximately 360 mg of chocolate powder was transferred to test tubes and melted in an ultrasonic bath (40° C). The samples were first defatted with hexane. The residues were then extracted with acetone/water/acetic acid (70/28/2, v/v). After centrifugation, the supernatant was evaporated and the dry material suspended in methanol/water (80/20) before injection.

LC-MS analysis

The black and brown chocolates were analyzed using a Thermo Scientific™ Vanquish™ UHPLC system. The column used was a 1.8 m, 100 mm x 1.0 mm ID column. Samples of 0.5 µL each were injected, and the mobile phase was eluted with a constant flow rate of 220 µL/min. Eluent A was 1% formic acid in water, and eluent B was acetonitrile/water/formic acid (80/19/1).

The UHPLC system was hyphenated with a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer. The samples were injected in two series of analyses. The first (S1) was performed to obtain HRMS spectra using the full scan mode and a resolution set to 240 k. For identification purposes, a single quality control sample was injected with a resolution set to 480 k. The second series (S2) sought to obtain HRMS and HRMS/MS^2 spectra from two scan events (one using the full scan mode with 60 k resolution, and the second using data-dependent acquisition mode to select the three most intense precursor icon from the first scan to perform the HRMS/MS^2 experiments set to 30 k resolution).

Chemometrics

The HRMS data (S1 series) were processed, and the resulting dataset of compounds (*m/z* x Rt x areas) was analyzed by principal components analysis (PCA) (Figure 2) and by a volcano plot for discriminating compound selection (Figure 3). The HRMS/MS^2 data (S2 series) were used to build molecular networks establishing the relationships among compounds (Figure 5). Both series were processed using Thermo Scientific™ Compound Discoverer™ 3.3 SP2 software.

The LC-MS compound abundances were used to estimate the relative ion abundance of each compound in each class of samples (Figures 4 and 5).

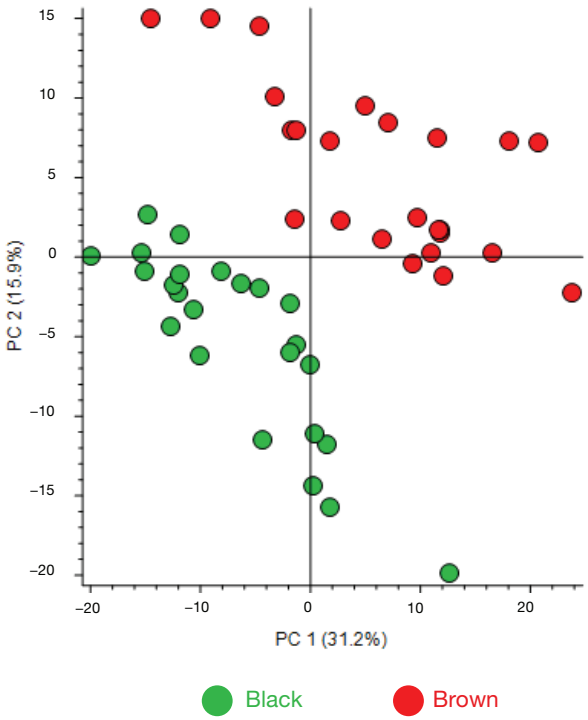

Figure 2. Score plot of principal component analysis from the UHPLC–HRMS features of the black and brown chocolates

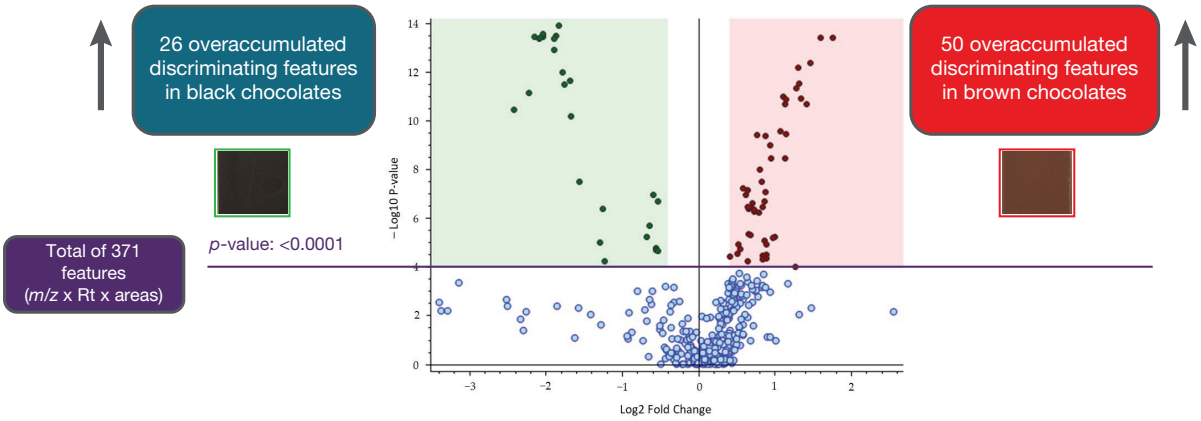

Figure 3. Volcano plot comparing black versus brown chocolates. Green and red dots represent over-accumulated discriminating compounds for black and brown chocolates, respectively, and blue dots indicate features that were not highly discriminating for brown and black chocolates.

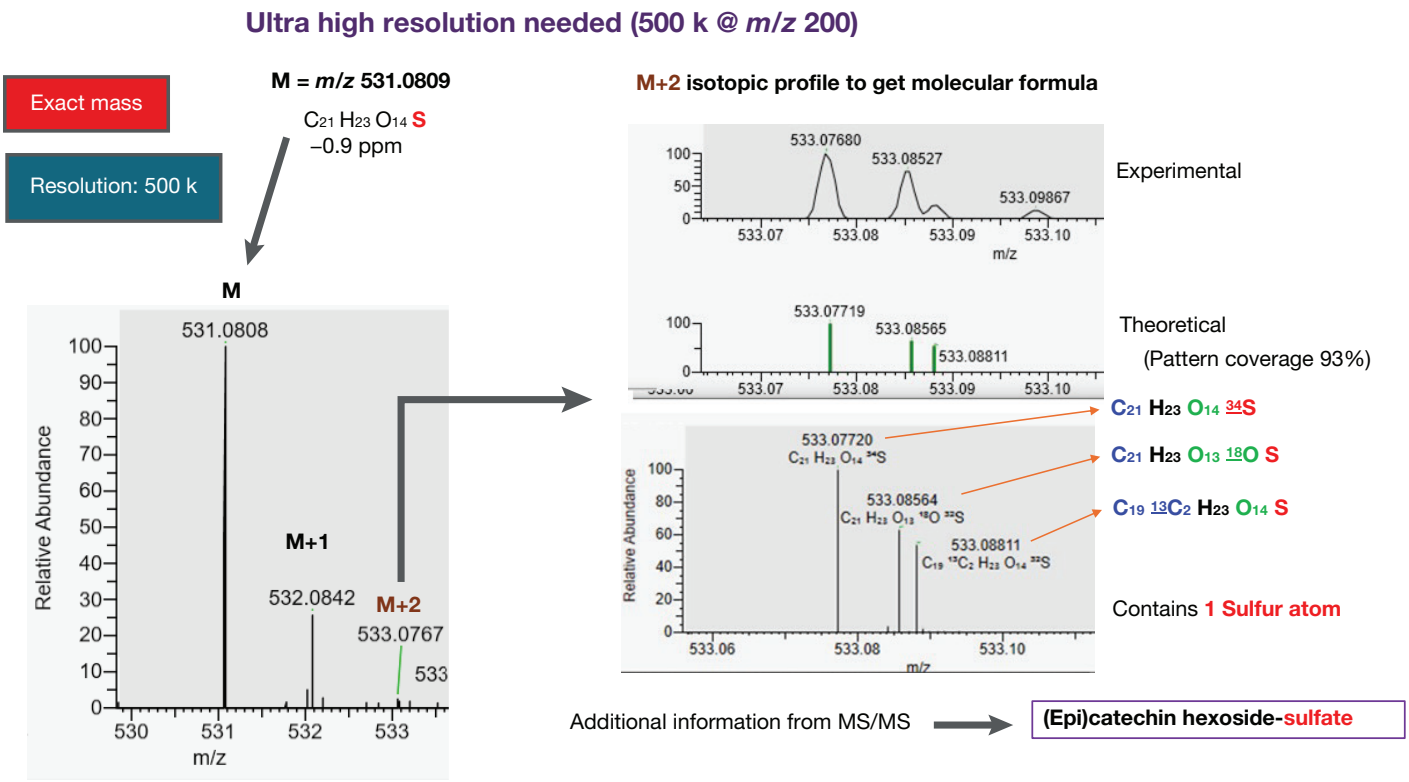

Figure 4. Example of the importance of ultra-high-resolution mass spectrometry for compound annotation

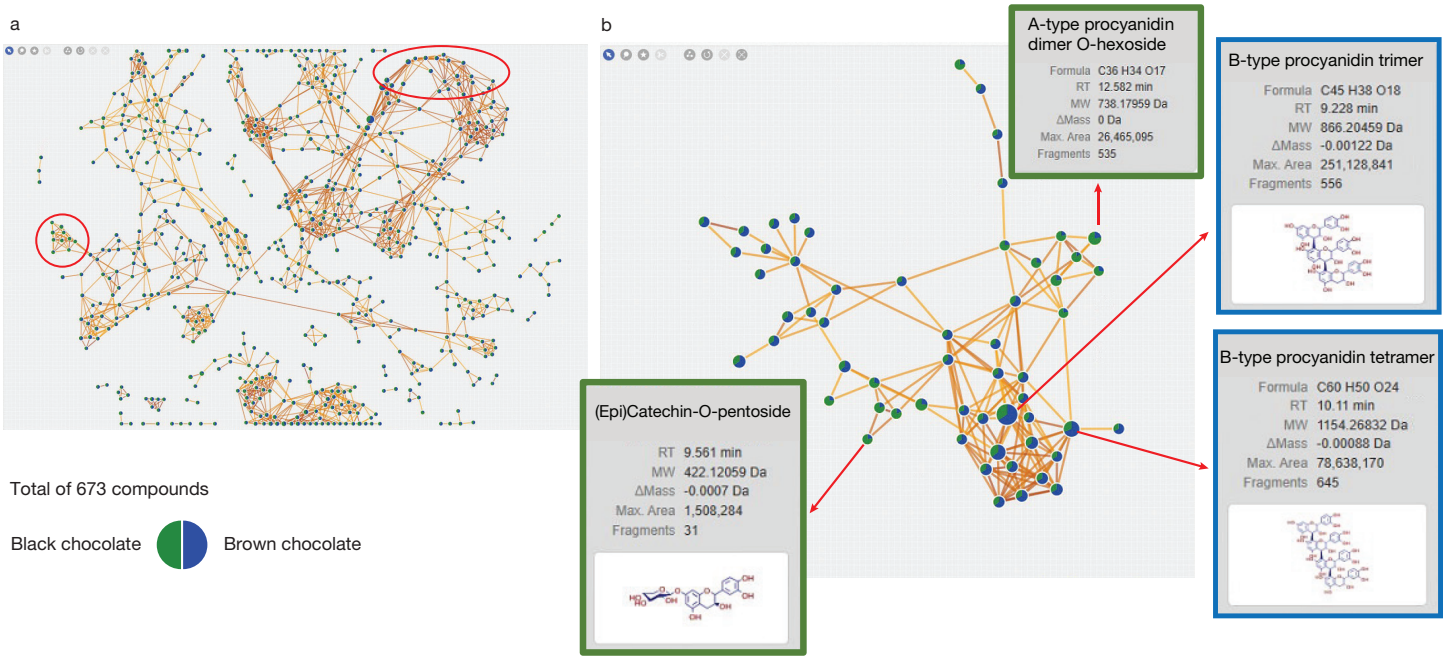

Figure 5. Molecular networking. (a) Compound-based molecular networks of compounds of black and brown chocolates. Pie charts represent the relative abundance of each feature in the black and brown chocolates. It is noteworthy that this representation highlights some highly similar compounds (from the same cluster) that differentiate between black and brown chocolates. (b) Focus on the discriminating compounds selected from the volcano plot.

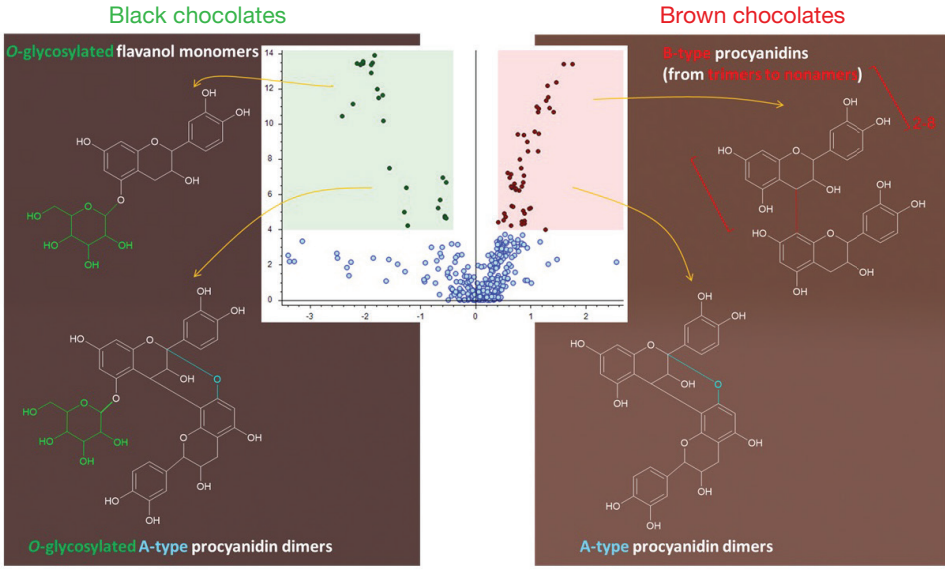

Figure 6. Mass spectrometry-based untargeted metabolomics identifying discriminating metabolites for black and brown dark chocolates

Results

The results of this work[10] revealed 26 discriminating compounds for black chocolates, of which 16 were tentatively annotated, and 50 discriminating compounds for brown chocolates (38 annotated). Compound annotation was based on predicted molecular formulae, fragmentation patterns, comparison to our internal database of complex polyphenols (from PFP Polyphenol analysis facility) and to literature.

The discriminating compounds from the volcano plot (Figure 3) were highlighted on the molecular networks (Figure 5a). The compounds were annotated by comparing correlated compounds in the same molecular network (Figure 5b).

For the black chocolates, the compounds were mainly glycosylated A-type procyanidin dimers and trimers (seven compounds) and O-glycosylated flavan-3-ol monomers (six compounds), but also one oxidized A-type procyanidin dimer, one phenolic acid, and one amino acid derivative. For the brown chocolates, the discriminating compounds corresponded mainly to larger non-glycosylated B-type procyanidins with degrees of polymerization between 3 and 9 (twenty-seven compounds), along with C-glycosylated B-type procyanidins (one dimer and one trimer), two A-type procyanidin dimers, two dehydrodicatechins B, one phenolic acid, and two amino acid derivatives. See Figure 6. The discriminating compounds found in this work can potentially be markers of these types of chocolates.

Conclusion

This study increases the knowledge on the chemical diversity of dark chocolates by providing new information about the phenolic profiles of black and brown chocolates.

The work was made possible with the Orbitrap Exploris 480 mass spectrometer due to its high resolving power. The Orbitrap Exploris 480 system offers the highest resolution at the low *m/z* range. This exceptional resolution enables identification of the isotopes and the ability to assign elemental composition for the polyphenols.

The main families of discriminating compounds for black chocolates were glycosylated flavanols with a low degree of polymerization. For brown chocolates, we found non-glycosylated flavanols including compounds with a higher degree of polymerization from dimers to nonamers. In addition, we found other phenolics and some amino acid derivatives in black (protocatechuic acid and methoxytyrosine) and brown (syringic acid hexoside, dehydrodicatechin B, epicatechin hexoside-sulfate, phenylalanylphenylalanine and feruloyl aspartic acid) chocolates.

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