Coffee Metabolomics: A New Approach to Defining Quality Markers

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Overview

Purpose: To establish a methodology for metabolome analysis in roasted coffee

Methods: Metabolite profiling and fragmentation were both performed on a high resolution, high mass accuracy platform. The mass spectrometer was mass calibrated prior to starting the sequence of injections. All data was acquired using external calibration, and positive ionization. mode

Results: Masses were measured with high, sub-ppm to max 2 ppm accuracy, leading to identifications based on elemental composition analysis. Metabolite profiles were acquired at high resolution (>60 000) and following chromatographic alignment and peak detection, statistical analysis was performed to discriminate metabolites which may serve as markers for roasting characteristics. Further identifications were carried out via resonance excitation CID experiments. Results obtained on a hybrid system from metabolite profiling and identification experiments provide evidence that the strategies selected can be successfully applied in the LC-MS based detection and identification of metabolites in plant beverages.

Introduction

Metabolomics provides sensitive and specific tools for the assessment of quality markers of coffee (1-2). The neuro-stimulating effects of coffee beans are well documented, along with effects on blood pressure, and protection against oxidative stress (3-4). Recently, a sensitive method has been published for the quantification of coffee chlorogenic acid metabolites from plasma samples by LC-ESI-MS/MS (5). Green coffee beans of the two main varieties, Coffea arabica (Arabica) and Coffea canephora (Robusta) have been characterized by mass spectrometric analyses. However, coffee roasting and products obtained by further technological treatments, including the final consumed beverage, need to be profiled. Herewith, results from metabolite profiling and identification experiments in light and medium roasted coffee samples will be presented.

Methods

Sample preparation:
Green *Arabica* coffee was roasted in a gas roaster for 8 min. Roasting temperatures were 280 °C and 285 °C to produce light- or medium-roasted coffee. Roasted coffee was ground on a Ditting grinder at an average size of 600 µm. Coffee extraction was conducted on a bench-scale Dionex extractor ASE200, using water at 100 °C. The extraction procedure was applied to 3 g of coffee and was repeated 20 times.
The extracts were freeze dried. 25 mg of freeze dried sample were weighed in a 5 mL flask and dissolved in 5 mL methanol/water (50/50). The sample was filtered on 0.2 µm Whatman FP 30 and kept on dry ice prior to the LCMS analysis. Before injection (10 µL) into the mass spectrometer, samples were vortexed and centrifuged (5000 RPM). Samples of light and medium roasted varieties were analyzed by a minimum of three technical replicates each.
Chromatography: Separations were performed on Hypersil Gold™ columns, 100 x 2.1 mm, 1.9 µm particles (Thermo Fisher Scientific, Runcorn, UK). An Accela 600 pump was used (Thermo Fisher Scientific, San Jose, CA). The mobile phase separation ran from 90-50% A over 44 min, then 50-5% over 5 min, isocratic conditions maintained for a further 5 min prior to a quick (0.1 min) return to 90% solvent A (water, 0.1% formic acid) where the column was equilibrated for 4.9 min. Solvent B was methanol with 0.1% formic acid. The pump was operated at 300 µL/min.
Mass spectrometry: MS detection was carried out using a Thermo Scientific LTQ Orbitrap XL hybrid mass spectrometer in positive mode using a high resolution full MS (m/z 300-800, 60000 resolving power) followed by three high resolution MS^n (30000 resolving power). MS^n precursor selection was done in data dependent operation mode where the most intense ion of the previous scan was selected for fragmentation (see figure 2). A normalized collision energy of 35% for CID was used. In further experiments an inclusion list was added to the method for validation of identification.
Data processing: Alignment, peak detection and metabolite identification based on elemental composition (SIEVE™ software with ChemSpider™ interface) were followed by statistical analysis (SIMCA-P™ software). CID was particularly useful for metabolite identification based on spectral library searches (Mass Frontier™ software), with accurate MS^n complemented by Data Dependent Analysis (DDA) experiments and inclusion list-driven MS^n for metabolite structural determination.

Results

Good chromatographic performance was obtained employing methanol in the mobile phase and 44 min gradients, whereby hundreds of components were profiled and separation of isobaric compounds was achieved. In conjunction with robust calibration values obtained via external calibration, metabolites were measured with high mass accuracy, leading to strongly suggestive identifications made by elemental composition software. PCA along with OPLS-DA analyses were used to distinguished components of interest and potential markers, which were identified employing a mix of accurate mass and MS^n strategies, searches in spectral libraries and fragment ion searches (FISH), where identifications of substructures are used as a starting point to "re-assemble" fragment ions for reconstruction of the precursor masses.
Notably, among them chlorogenic acid components were identified, some of which can be used as quality markers. However, further analyses and systematic studies are needed to establish direct correlations between composition (fingerprint), individual compounds, and quality attributes such as taste, color, and health benefits. Mass errors registered were well within instrument specifications, with data acquired on the LTQ Orbitrap XL™ instrument showing results across multiple acquisitions with mass accuracy better than 1 ppm.

FIGURE 1. Metabolomics workflow representing the sequence of events involved in metabolite biomarker discovery (1-4) and progressing towards development of routine quantitative measurement.

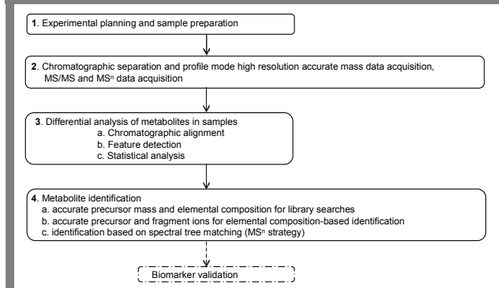
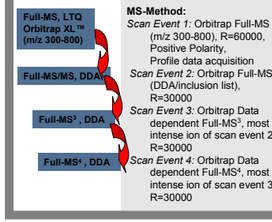


FIGURE 2. MS acquisition set-up used for metabolite profiling and metabolite identification.



MS-Method:
Scan Event 1: Orbitrap Full-MS (m/z 300-800), R=60000, Positive Polarity, Profile data acquisition
Scan Event 2: Orbitrap Full-MS^2 (DDA/inclusion list), R=30000
Scan Event 3: Orbitrap Data dependent Full-MS^3, most intense ion of scan event 2, R=30000
Scan Event 4: Orbitrap Data dependent Full-MS^4, most intense ion of scan event 3, R=30000

FIGURE 3. Basepeak chromatograms (m/z 300-800) of (A) light roasted coffee and (B) medium roasted coffee. Chlorogenic acid peaks marked.

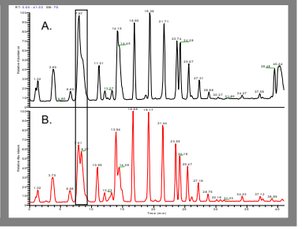


FIGURE 4. EICs of important coffee components. (A) Coumaroylquinic acid, (B) Chlorogenic acid, (C) Feruloylquinic acid.

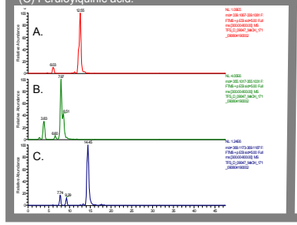


FIGURE 5. EIC of chlorogenic acid in (A) light roasted coffee and (B) medium roasted coffee. (C) Software calculated mass error for chlorogenic acid.

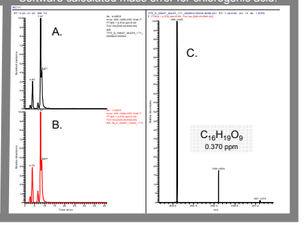
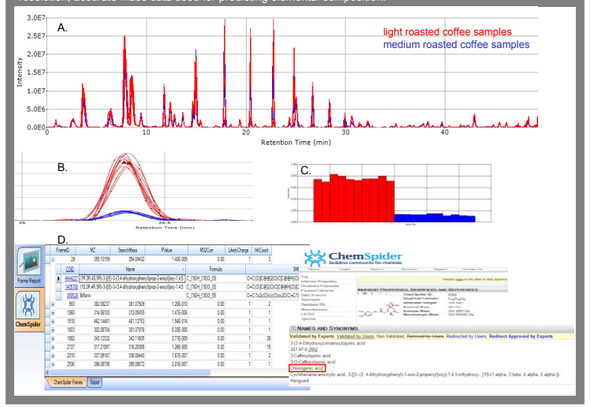


FIGURE 6. (A) Chromatographic alignment and (B) framing in SIEVE software, (C) integrated intensities of component in sample groups, (D) metabolite identification in ChemSpider search engine based on high resolution, accurate mass data used for predicting elemental composition.



High resolution, accurate mass strategies were useful for robust metabolite profiling and for preliminary metabolite identification based on elemental composition. To limit the number of false positive matches, mass tolerance for searches in ChemSpider was set to 0.001Da. The tolerance for searches based on MS^n strategies and identifications in Mass Frontier software was set to 10 ppm.
Statistical analysis was used to define the number of metabolites that may be significant as quality markers for coffee. PCA in SIMCA-P software was used to find trends and outliers, the latter to be eliminated from further discriminating analysis where potential biomarker candidates are distinguished among metabolites.

FIGURE 7. Statistical analysis (A, B) followed by decision which metabolites should be structurally identified. (C) Fragmentation example of Chlorogenic acid.

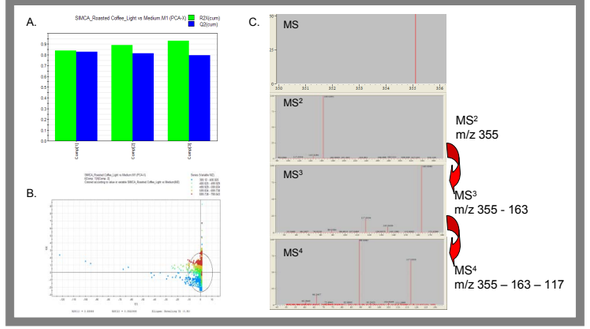
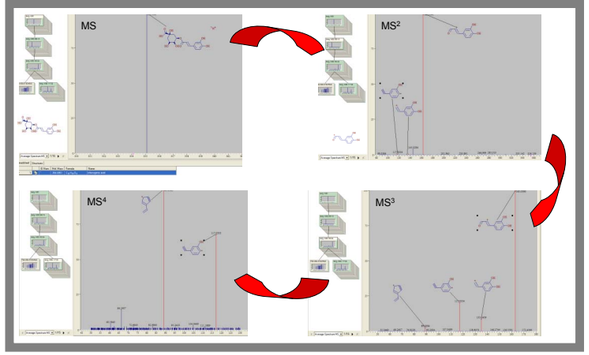


FIGURE 8. Metabolite identification (chlorogenic acid) in Mass Frontier software, based on MS^n fragmentation and spectral library searches.



Conclusions

- **Sample preparation for global metabolite discovery can be performed efficiently, with hundreds of features profiled using SIEVE software**
- **Chromatographic conditions employed 44 min gradients, resulting in peak widths of under 30 s for standard HPLC and abundant components; such conditions were optimal for both metabolite profiling and for metabolite identification experiments**
- **Resolution of 60 000 was used for metabolite fingerprinting, enhancing discrimination of components as well as metabolite identification based on accurate mass measurements, for components identified in publicly available databases and compound libraries.**
- **Metabolite identification, a crucial component in metabolomics experiments, was performed using two approaches:**
  1. **Accurate mass determination generating elemental composition within a narrow mass tolerance window (0.001Da) for identification based on accurate precursor masses**
  2. **MS/MS and MS^n product ion data matching (10 ppm) against theoretical fragmentation patterns derived with Mass Frontier software, where standards were available, or supporting *de novo* interpretation**
- **Resonance excitation CID experiments performed on the LTQ Orbitrap XL generated high-quality spectra, useful for fragmentation-based compound identification strategies.**

References

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