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Homostachydrine (pipecolic acid betaine) as authentication marker of roasted blends of *Coffea arabica* and *Coffea canephora* (*Robusta*) beans

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ABSTRACT

The occurrence of pipecolic acid betaine (homostachydrine) and its biosynthetic precursor *N*-methylpipecolic acid was detected for the first time in green coffee beans of Robusta and Arabica species. The analyses were conducted by HPLC–ESI tandem mass spectrometry and the metabolites identified by product ion spectra and comparison with authentic standards. *N*-methylpipecolic acid was found at similar levels in green coffee beans of Robusta and Arabica, whereas a noticeable difference of homostachydrine content was observed between the two green coffee bean species. Interestingly, homostachydrine content was found to be unaffected by coffee bean roasting treatment because of a noticeable heat stability, a feature that makes this compound a candidate marker to determine the content of Robusta and Arabica species in roasted coffee blends. To this end, a number of certified pure Arabica and Robusta green beans were analyzed for their homostachydrine content. Results showed that homostachydrine content was 1.5 ± 0.5 mg/kg in Arabica beans and 31.0 ± 10.0 mg/kg in Robusta beans. Finally, to further support the suitability of homostachydrine as quality marker of roasted blends of Arabica and Robusta coffee beans, commercial samples of roasted ground coffee blends were analyzed and the correspondence between the derived percentages of Arabica and Robusta beans with those declared on packages by manufacturers was verified.

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1. Introduction

The genus *Coffea* L. comprises more than one hundred species but only about 25 produce fruits with commercial value. Among these, 4 are used by industry to produce the coffee used for the preparation of the most popular beverage in the world after tea. *Coffea arabica* L. and *Coffea canephora* P. (known as Robusta) are the species most used in the preparation of commercial blends of roasted coffee. *C. arabica* L. represents about 70% of the world production and is the most appreciated species by the consumer for superior flavor and lower caffeine content than Robusta. However, the latter is more productive and resistant to diseases and also suitable for the cultivation in lowlands. For all these reasons, the Arabica species has commercial values consistently higher than Robusta. The other two coffee species used by industry, although in much less amount, are the Liberica, grown mainly in Liberia and Côte d'Ivoire, and the Excelsa. These species are grown mainly to meet local consumption (Davis, Govaerts, Bridson, & Stoffelen, 2006; Tafani, 2013). Food and beverage adulteration, which become a major issue in the era of market globalization, involves many different edible products, including coffee (Toci, Farah, Pezza, & Pezza, 2015), and the assessment of their authenticity is of paramount importance for quality and economical reasons. Therefore, various chemical components, such as sterols, volatile components, and metals (Frega, Bocci, & Lercker, 1994; Krivan, Barth, & Morales, 1993; Lercker, Frega, Bocci, & Rodriguez-Estrada, 1994; Murota, 1993), devised to discriminate among different coffee species, are mainly utilized as indicators of beans of Robusta species in Arabica coffee lots. Presently, the occurrence of Robusta beans into roasted coffee is mainly detected by measuring the concentration of 16-O-methylcafestol (16-OMC), a specific pentacyclic diterpene alcohols derived from cafestol. This metabolite, identified and isolated from Robusta coffee beans

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(Speer & Mischnick, 1989; Speer & Mischnick-Lubbecke, 1989), is absent in those of Arabica species (Frega et al., 1994; Trouche, Derbesy, & Estienne, 1997). In addition, being stable during the roasting process, 16-OMC has been proposed as an appropriate marker for assessing the presence of Robusta in commercial blends of roasted coffee (Speer & Kölling-Speer, 2006; Speer, Tewis, & Montag, 1991). Based on these studies, a HPLC method was validated for the determination of 16-OMC in roasted coffee blends by the German Institute for Standardization (DIN 10779 (1999)). Unfortunately, the dosage of this specific marker requires a long and cumbersome analytical procedure. Actually, the samples require initial Soxhlet extraction with organic solvents, like tert-butyl methyl ether, petroleum ether or hexane for many hours followed by saponification with KOH in ethanol. Finally, the extract is analyzed by HPLC with UV detection. Recently, as an alternative to the above method, an interesting approach has been proposed in which the detection of the presence of Robusta species is achieved directly in coffee extracts by rapid quantitative determination of 16-esterified OMC through high resolution proton nuclear magnetic resonance spectroscopy (Schievano, Finotello, De Angelis, Mammi, & Navarini, 2014).

In the course of our studies on betaines in plants, we started an investigation on the characterization of these important metabolites, ubiquitous in the vegetal world, also in Robusta and Arabica beans. Betaines play important roles in the plant physiological mechanisms of accumulation and adaptation (Rhodes & Hanson, 1993), taking part to the defensive response toward biotic and abiotic stresses. As plants synthesize betaines through biosynthetic patterns peculiar of the specific genus, we sought to investigate whether *Coffea* genus shows particular expression of such metabolites. In this paper, we report that substantial differences between Arabica and Robusta species in betaine expression exist. Interestingly, these differences allowed the development of a rapid test to quantify the percentage of Robusta and Arabica species in roasted coffee blends.

2. Materials and methods

2.1. Chemicals

Pipecolic acid, 1-methylpiperidine-2-carboxylic acid hydrochloride (1,2-*N*-methylpipecolic acid) and *N*-methyl-L-proline were from Sigma–Aldrich (Milan, Italy). *N,N*-Dimethyl-L-proline (stachydrine) and 4-hydroxy-L-prolinebetaine (betonicine) were purchased from Extrasynthese (Genay, France). Pipecolic acid betaine was synthesized and purified as described by Servillo et al. (2012). The purified product tested by HPLC analysis showed a purity of 98%. Milli-Q water was used for all the preparations of solutions and standards. The solution of 0.1% formic acid in water used for HPLC–ESI-MS/MS analyses was from Sigma–Aldrich (Milan, Italy).

2.2. Coffee samples

2.2.1. Authentic green coffee bean samples

Arabica and Robusta authentic monovarietal green coffee beans of certified geographical origin were provided by Caffè Salimbene Company (Naples, Italy). Seven Arabica coffee bean samples were from Ethiopia, Brazil, Colombia and Uganda. Three Robusta coffee bean samples were from Uganda, India and Java. Four different lots of each sample were analyzed.

2.2.2. Commercial roasted coffee samples

A total of 28 packages of commercial roasted and ground coffee beans were purchased in local markets for successive analyses. They were: (A) 4 samples labeled as 100% Arabica; (B) 4 samples labeled as containing 20% Robusta and 80% Arabica by weight; (C) 4 samples labeled as containing 30% Robusta and 70% Arabica by weight; (D) 4 samples labeled as containing 40% Robusta and 60% Arabica by weight; (E) 4 samples labeled as containing 50% Robusta and 50% Arabica by weight; (F) 4 samples labeled as containing 60% Robusta and 40% Arabica by weight; (G) 4 samples labeled as containing 80% Robusta and 20% Arabica by weight.

2.3. Sample preparation and metabolite extraction

2.3.1. Green coffee extracts

Monovarietal green coffee beans of certified geographical origin were finely ground in a mortar and the powder homogenized in a mixer with 0.1% formic acid in the ratio 1:50 (w/w). Homogenates were kept under constant stirring for 1 h and then centrifuged at 18,000*g* for 30 min. The clarified extracts were filtered through 0.45 μm Millipore filters and stored frozen at −20 °C until used for the determinations.

2.3.2. Roasted coffee extracts

A proper amount of roasted coffee powder accurately weighed was dissolved 1:50 w:V with 0.1% formic, the suspension was kept under constant stirring for 1 h and then centrifuged at 18,000*g* for 30 min. The supernatant solution was clarified by filtration through 0.45 μm Millipore filters and immediately used for analytical determinations.

2.4. Determination of thermostability

Amounts of Robusta coffee powdered green beans (500 mg) were weighted in watch glasses and heated in an oven at 190 ± 5 °C and 210 ± 5 °C for 10, 20 and 30 min. After thermal treatment, the samples were left for 1 h in a desiccator and, then, extracted and analyzed for the residual compound determinations.

2.5. LC-ESI-MS/MS analysis

HPLC–ESI-MS/MS analyses were performed with an Agilent 1100 series liquid chromatograph using a Supelco Discovery C8 column, 250 × 3.0 mm, particle size 5 μm. The chromatography was conducted isocratically with 0.1% formic acid in water at flow rate of 100 μL/min. Volumes of 10 μl of standard solutions or samples were injected. The mass spectrometer Agilent MSD SL quadrupole ion trap, in positive multiple reaction monitoring (MRM) mode, was operated utilizing nitrogen as the nebulizing and drying gas. The instrumental conditions were as follows: nebulizer pressure, 30 psi; drying temperature, 350 °C; drying gas 7 l/min. The ion charge control (ICC) was applied with target set at 30,000 and maximum accumulation time at 20 ms. Each extract was analysed in triplicate and the mean concentration of each compound was calculated and expressed in mg/kg of product. Standard stock solutions of each analyte were prepared at 2 mg/L. Additional calibration levels (0.2, 0.1, 0.05, 0.02, 0.002 and 0.001 mg/L) were prepared by serial dilution with water containing 0.1% formic acid. The calibration curves were build using these standard solutions. The linear regression analysis was carried out by plotting the peak areas of the monitored fragment ions versus the concentrations of the analyte standard solutions. The linearity of the instrumental response was assessed by correlation coefficients (r^2) > 0.99 for all analytes. Betaines and related compounds were identified on the basis of their retention times and MS^2 fragmentation patters. Compound quantification was generally obtained by comparison of the peak area of its most intense MS^2 fragment with the respective calibration curve built with standard solutions. The following MS^2 transitions were used for quantifying compounds:

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