Rapid and Simultaneous Analysis of 16-*O*-Methylcafestol and Sterols as Markers for Assessment of Green Coffee Bean Authenticity by On-line LC–GC

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ABSTRACT: An on-line LC–GC method for analysis of 16-*O*-methylcafestol (16-OMC) and total sterols in oils obtained from green beans of *Coffea canephora* (Robusta) and *C. arabica* (Arabica) has been developed. HPLC pre-separation of the transesterified lipids allows quantification of the content of 16-OMC. The fraction containing the sterols is transferred to on-line GC analysis. The results obtained for Arabica and Robusta samples were in good agreement with literature data obtained by classical analytical procedures. The method allows rapid and simultaneous determination of two markers (16-OMC and Δ^5-avenasterol) proposed for authenticity assessment of coffee beans and overcomes time-consuming sample pretreatment steps.

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KEY WORDS: Authenticity, Δ^5-avenasterol, *Coffea arabica, Coffea robusta,* coffee, diterpenes, 16-*O*-methylcafestol, on-line LC–GC, sterols.

The two coffee species *Coffea canephora* and *C. arabica* (commonly called Robusta and Arabica) are of major importance for commercial coffee production. Arabica coffees are generally considered superior in quality. This leads to price differences on the market and the potential for adulteration or misrepresentation by a dishonest trader. It is important to have appropriate methods for the discrimination of the two coffee species, and chemical analysis of different components of coffee beans is often applied as a useful tool.

Analytical approaches described in the literature for the discrimination of Arabica and Robusta coffees comprise the investigation of volatile compounds (1) and alkaloids (2); the use of IR spectroscopy (3); or DNA analysis *via* polymerase chain reaction (4). Lipid constitutents, especially minor components such as the diterpene 16-*O*-methylcafestol (16-OMC) (5–8) or sterols (9–12) also have been described as useful analytical parameters.

The use of 16-OMC as a marker component is based on its exclusive presence in Robusta coffee (5,13) and its stability during roasting of coffee beans or deodorization of coffee oils (6,8). The amount of 16-OMC is usually determined by extraction of the lipid fraction, saponification, liquid–liquid extraction, and subsequent analysis by RP-HPLC (14). Variations of this method use column chromatography on silica gel rather than liquid–liquid extraction for cleanup of the extracts (15,16) or direct HPLC analysis of the saponified material (17). Lipid extraction has been avoided by direct saponification of the ground coffee beans, extraction of the unsaponifiable matter, derivatization, and subsequent GC analysis (18).

Within the class of sterols, Δ^5-avenasterol is another marker proposed for discrimination of Arabica and Robusta (9–12). Its analysis is usually based on the official methods developed for determining total sterols in animal and vegetable fats and oils (19–21). Such analyses involve saponification of the lipids, extraction of the unsaponifiables, pre-separation by TLC, derivatization, and subsequent GC analysis. On-line coupling of HPLC and capillary GC (LC–GC) (22–24) can be a useful alternative to this tedious procedure. This technique has been successfully applied to the analysis of sterols in plant-derived oils, such as olive oil (22–26), and was shown to yield results comparable to the official method (23,24).

The objective of this study was to establish such an LC–GC methodology for the investigation of sterols in coffee oil and to extend this approach to the analysis of 16-OMC. This should result in a rapid and simultaneous determination of two marker substances suitable for the differentiation of Robusta and Arabica coffees.

MATERIAL AND METHODS

Materials. Green beans of *C. canephora* (Robusta) and *C. arabica* (Arabica) were obtained from Africa (Uganda, Togo) and Latin America (Brazil, Costa Rica), respectively. The beans were ground using a laboratory mill (Laboratory mill 3303, Falling Number AB, Huddinge, Sweden). During grinding the beans were chilled with solid carbon dioxide to avoid pastiness. The ground material was stored under nitrogen at –20°C. For the evaluation of the method, an industrial coffee oil, obtained in-house, was used without further treatment. Extra virgin olive oil was obtained from Olio Sasso (Nestlé Italiana, Milano, Italy).

Chemicals. Stigmasterol (96%), β-sitosterol (98%), campesterol (65%), 5α-cholestan-3β-ol (95%), and *tert*-butyl methyl ether for LC (gradient grade) were purchased from Sigma (Buchs, Switzerland). 16-OMC (97%) was synthesized as

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described previously (13). 2-Propanol, *n*-hexane (gradient grade), water (LiChrosolv grade), sodium methylate in methanol (30%), and citric acid monohydrate were purchased from Merck Eurolab (Dietikon, Switzerland). *N,O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA; 99% puriss.) and trimethylchlorosilane (99% puriss.) were obtained from Fluka (Buchs, Switzerland).

Sample preparation. About 15 g of ground green coffee beans were extracted with *tert*-butyl methyl ether for 8 h using the Büchi extraction system (B-811; Büchi, Flawil, Switzerland). The solvent was evaporated with nitrogen, and the residue was dried at 105°C. About 100 mg of the coffee oil obtained was weighed into a centrifuge tube containing 100 µL of a solution of 5α-cholestan-3β-ol in *n*-hexane (2 mg/mL). One milliliter of a sodium methylate solution (13 mL of 30% sodium methylate in methanol and 27 mL methanol made up to 100 mL with *tert*-butyl methyl ether) was added. The mixture was homogenized by vortexing. After 20 min at room temperature, 1 mL of water and 15 mL of *n*-hexane were added, and the mixture was gently shaken. The bottom phase was removed by means of a Pasteur pipette and 1 mL of water containing 1 mg/mL citric acid was added; this washing step was repeated. The *n*-hexane phase was analyzed by on-line LC–GC.

On-line LC–GC. The analysis was performed using a fully automated on-line LC–GC instrument (Dualchrom 3000; C.E. Instruments, ThermoQuest, Rodano, Italy). The HPLC part of this instrument included two 20-mL syringe pumps (Phoenix 20; C.E. Instruments), one used as the master pump for eluent delivery and the other as slave pump for backflushing the HPLC column. Detection was performed with a variable wavelength detector (Micro UVIS 20; Linear Instruments, Reno, NV). The separation column was thermostated using a column oven (Jetstream Plus; Advanced Separation Technologies Inc., Whippany, NJ). The GC was equipped with a fully automated interface valve system. A loop-type interface with a 750 µL sample loop and a solvent vapor exit was used for the transfer of the fraction of interest from LC. An FID was used for detection. All components were controlled by the Dualchrom software. Data aquisistion was performed by the Chromcard Software (C.E. Instruments).

LC–GC conditions. HPLC pre-separation of the transesterified oil was performed with a silica gel column (HPLC Hypersil, 2.1 × 100 mm, 60 Å, 5 µm particle size; Agilent, Geneva, Switzerland) using a mixture of 0.8% 2-propanol in *n*-hexane as eluent. The column was kept at 15°C, the flow rate was set to 200 µL/min, and UV detection was at 205 nm. Backflush of the column was performed with *tert*-butyl methyl ether. The latter was delivered by the slave pump at a flow rate of 200 µL/min during backflush and at 10 µL/min during standby. The backflush was started after 16 min and returned to standby 5 min later.

GC separation was performed on a 25 m × 0.25 mm i.d. fused-silica capillary coated with a film thickness of 0.4 µm (CP-Sil8CB; Stehelin, Basel, Switzerland), connected in series with an uncoated phenyldimethyl silylated fused-silica capillary (2 m × 0.25 mm i.d.) and a pre-column (1 m × 0.25 mm i.d.) coated with the same stationary phase as the analytical column. An early solvent vapor exit (0.2 m × 0.25 mm i.d. fused silica) was installed between the coated pre-column and the separation column *via* a Y-piece press-fit connector (BGB Analytik; Anwil, Switzerland). Hydrogen was used as carrier gas at a flow rate of 2.0 mL/min, measured at 120°C. The carrier gas inlet pressure behind the flow regulator was set at 200 kPa.

The transfer of the sterol fraction (750 µL) started 9.5 min after injection of the sample (5 µL) and occurred by concurrent eluent evaporation at 120°C. With a delay of 40 s on the reduction of the inlet pressure by 40 kPa at the end of the transfer, the solvent vapor exit was switched to a restrictor (1 m × 0.05 mm i.d. fused silica), leaving a small purge flow during analysis. After holding the transfer temperature of 120°C for 5 min, the column temperature was programmed to 220°C at 40°C/min, and then to 300°C at 5°C/min, which was held for 15 min. The FID was set to 320°C. The transfer of the 16-OMC fraction (750 µL) started 13.5 min after injection and was performed using the conditions described for the sterol fraction.

GC–MS. GC–MS analyses were performed on a Finnigan SSQ 7000 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) working in EI mode at 70 eV. 16-OMC fractions obtained after pre-separation by HPLC were collected manually, and the eluent was dried under a stream of nitrogen. Trimethylsilylation was performed using a mixture of BSTFA/TMCS (4/1, vol/vol) and heating for 30 min at 60°C. Samples were introduced *via* a gas chromatograph (Hewlett-Packard 5890; Agilent) equipped with an HP-7673 autosampler using the following conditions: cold on-column injection, fused-silica capillary column DB-5 (J&W Scientific, Folsom, CA) 30 m × 0.32 mm i.d., film thickness 0.25 µm. The carrier gas used was helium at a constant inlet pressure of 69 kPa. The temperature program was 60°C (1 min) programmed to 200°C (20 min) at 30°C/min, then to 250°C (10 min) at 20°C/min and finally to 300°C (20 min) at 3°C/min. The transfer line and the source were kept at 250 and 180°C, respectively. The analysis of 16-OMC was carried out in full-scan mode at unit resolution from 20 to 500 Da. Data were processed using the MassLib v8.6 software package (MSP Friedli, Koeniz, Switzerland).

Quantification. The amounts of sterols were determined by comparing the GC peak areas to those obtained for the internal standard 5α-cholestan-3β-ol (378 µg/g coffee oil). For the calculation, relative response factors of 1.0 were used.

16-OMC was quantified by using an external calibration curve (concentration range: 0.6–153.9 µg/mL in *tert*-butyl methyl ether; $r^2 = 0.999$).

Recovery of 16-OMC. The recovery of 16-OMC was evaluated by spiking an industrial coffee oil with a known amount of the reference compound (1.87 mg/g oil) and comparing the result to that obtained for the unspiked coffee oil.

RESULTS AND DISCUSSION

Method development. LC–GC analysis of coffee oil was based on the following steps: (i) transesterification of the oil, (ii) pre-separation of the fractions of interest from other lipid constituents by HPLC, and (iii) on-line transfer to GC.

Results obtained for coffee oil from Robusta are shown in Figure 1. In accordance with the procedure described for olive oil (23), normal-phase HPLC on silica gel was used to separate the sterol fraction (**I**) from the rest of the transmethylated sample (Fig. 1A). Robusta coffee oil showed an additional, well-separated peak (**II**), which was also cut out and transferred on-line to the gas chromatograph.

GC analysis of fraction **I** confirmed the transfer of the sterols *via* HPLC. Campesterol (1), stigmasterol (2), and β-sitosterol (4) were identified by analysis of the respective reference compounds. Clerosterol (3) and Δ^5-avenasterol (5) were assigned on the basis of results obtained from extra virgin oil analyzed under comparable conditions (23,24). The pattern obtained by this procedure is in accordance with previously reported distributions of total sterols in Robusta coffee determined by the classical procedures (9–12,27,28).

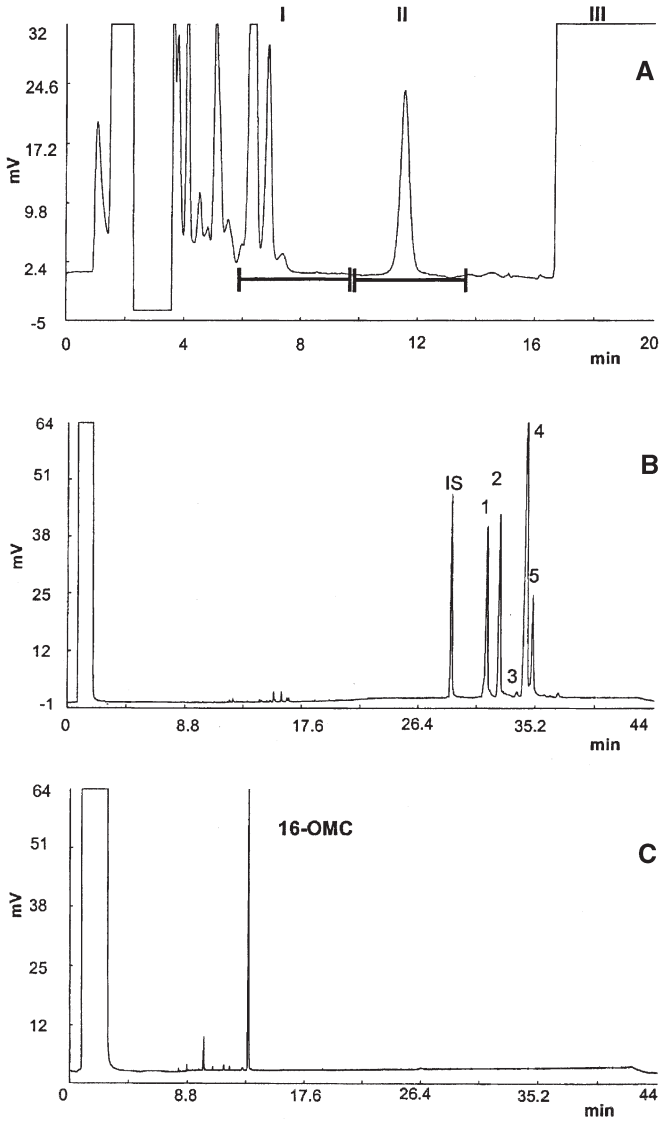

FIG. 1. Analysis of a Robusta coffee sample (Togo). (A) Separation on normal-phase LC; transferred fractions: (**I**) sterols, (**II**) 16-*O*-methylcafestol (16-OMC); backflush (**III**) with *tert*-butyl methyl ether (MTBE); (B) GC separation of fraction **I**: (internal standard; IS) 5α-cholestane-3β-ol, (1) campesterol, (2) stigmasterol, (3) clerosterol, (4) β-sitosterol, (5) Δ^5-avenasterol; (C) GC chromatogram of fraction **II**; for conditions see Materials and Methods section.

Precision data on the on-line transfer of sterols from transesterified oils on the basis of the same procedure as described in this study have been reported, and good comparability with the classical saponification method has been demonstrated (23,24).

GC analysis revealed a high degree of purity of the HPLC fraction (**II**) (Fig. 1C). Subjection of the respective reference compound to the on-line LC–GC procedure demonstrated the transferred lipid constituent to be 16-OMC. Unambiguous confirmation of the identity of this peak was achieved by repeated manual sampling of HPLC fraction **II**, silylation, and subsequent GC–MS analysis. The mass spectrum obtained was identical to previously reported data (13).

If two individual transfers are performed, the described procedure allows the on-line LC–GC analysis of both total sterols and 16-OMC. In principle, "scanning" of an HPLC run is possible using "stopped flow" conditions and has been described for the analysis of essential oils (29).

Considering the excellent HPLC separation of 16-OMC from the other components of the transesterified oil, the possibility of quantifying this compound directly in the HPLC pre-separation step and thus avoiding a second transfer was further investigated. The repeatability of the analysis of 16-OMC by HPLC was evaluated with an industrial coffee oil; sixfold sample preparation and analysis resulted in a mean content of 16-OMC of 1.48 mg/g and a repeatability SD of 0.07 mg/g. A recovery of 96.2% (SD: 3.3%; $n = 3$) was determined by spiking an industrial coffee oil with 16-OMC (1.87 mg/g). The detection limit was determined as 0.01 mg/kg coffee oil.

Application of the LC–GC method to the analysis of green coffee beans. Robusta and Arabica coffee beans of different geographical origin were analyzed according to the described LC–GC procedure. Examples of the sequence of chromatograms obtained for Arabica coffees are shown in Figure 2 for the sample from Brazil.

The absence of 16-OMC in Arabica coffee oil demonstrated in the HPLC run (Fig. 2A) and confirmed by the on-line GC analysis (Fig. 2C) underscores the role of this constituent as a marker to discriminate Arabica and Robusta species (5–8). The concentrations of 16-OMC determined in the Robusta samples from Togo and Uganda *via* quantification in the HPLC step were 0.69 and 0.67 g/kg coffee beans, respectively. These levels are within the range reported in the literature (0.6–1.3 g/kg) (6).

The analysis of the sterol fractions (Table 1) confirmed the reported differences in the Δ^5-avenasterol contents of Arabica and Robusta coffees. The relative percentage amounts determined in Arabica (2.5 and 2.6%) and Robusta (10.2 and 10.8%) are in excellent agreement with the literature (9,10,12). The ratios between the average contents of β-sitosterol and Δ^5-avenasterol in Robusta (4.8) and Arabica (21.4) show good accordance with the respective ranges (4.2–4.5 for Robusta; 17.2–23.5 for Arabica) calculated from data previously determined in green and roasted coffee beans by the classical analytical procedures (9,11,12).

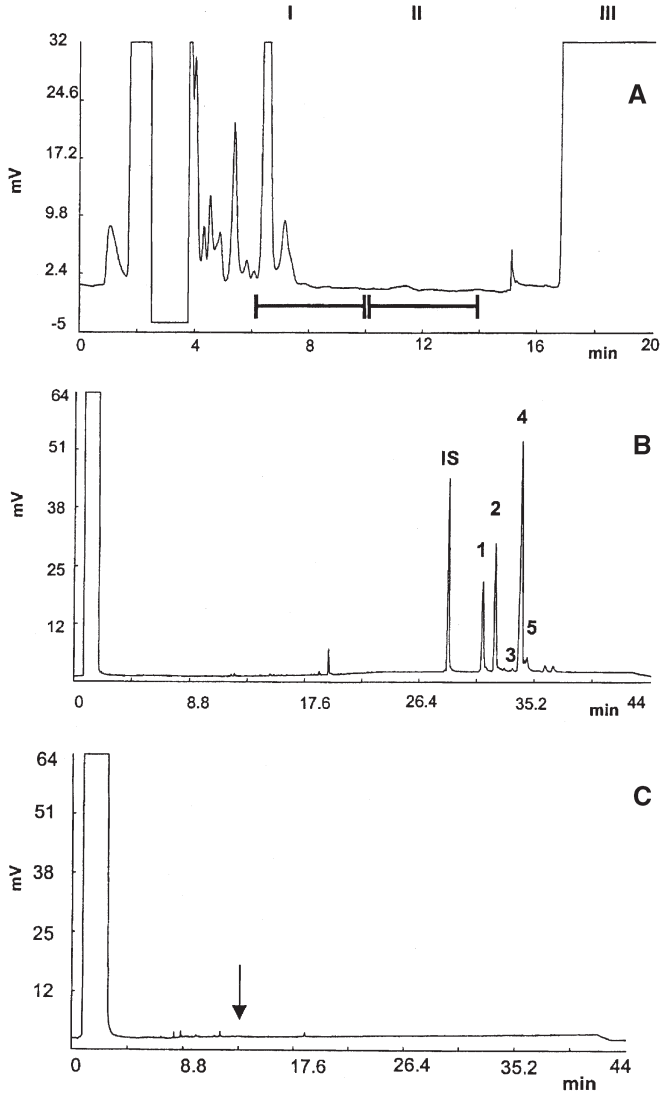

FIG. 2. Analysis of an Arabica coffee sample (Brazil). (A) Separation on normal-phase LC; transferred fractions: (**I**) sterols, (**II**) 16-OMC; backflush (**III**) with MTBE; (B) GC separation of fraction **I**: (IS) 5α-cholestane-3β-ol, (1) campesterol, (2) stigmasterol, (3) clerosterol, (4) β-sitosterol, (5) Δ^5-avenasterol; (C) GC chromatogram of fraction **II**; the expected retention time of 16-OMC is indicated by an arrow. For conditions see Materials and Methods section. For abbreviations see Figure 1.

Outlook. The methodology described offers the possibility to screen two markers simultaneously and thus to increase confidence in the conclusions drawn in terms of authenticity assessment of green coffee beans. The rapid and convenient on-line LC–GC procedure fulfills major prerequisites to be applied as high-throughput analysis.

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TABLE 1
Distribution of Sterols Determined *via* LC–GC in Oils from Arabica and Robusta Green Coffee Beans

	Robusta				Arabica			
	Togo		Uganda		Costa Rica		Brazil	
	mg/kg	rel. %	mg/kg	rel. %	mg/kg	rel. %	mg/kg	rel. %
Campesterol	306	19.4	341	19.9	244	18.4	260	18.6
Stigmasterol	296	18.8	328	19.2	318	24.0	332	23.7
Clerosterol	9	0.6	10	0.6	8	0.6	6	0.4
β-Sitosterol	796	50.4	855	50.0	719	54.3	765	54.7
Δ^5-Avenasterol	171	10.8	175	10.2	35	2.6	35	2.5
Total	1578		1709		1324		1398	

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