Isolation and characterization of major diterpenes from *C. canephora* roasted coffee oil

Elena Guercia,[a] Federico Berti,[b] Luciano Navarini,[a,c] Nicola Demitri,[d] Cristina Forzato[b,*]

[a]*Aromalab, illycaffè S.p.A., AREA Science Park, Padriciano 99, 34012 Trieste, Italy.*
[b]*Dipartimento di Scienze Chimiche e Farmaceutiche, Università degli Studi di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy*
[c]*illycaffè S.p.A., via Flavia 110, 34147 Trieste, Italy*
[d]*Elettra – Sincrotrone Trieste, S.S. 14 Km 163.5 in Area Science Park, 34149 Basovizza – Trieste, Italy*

Abstract

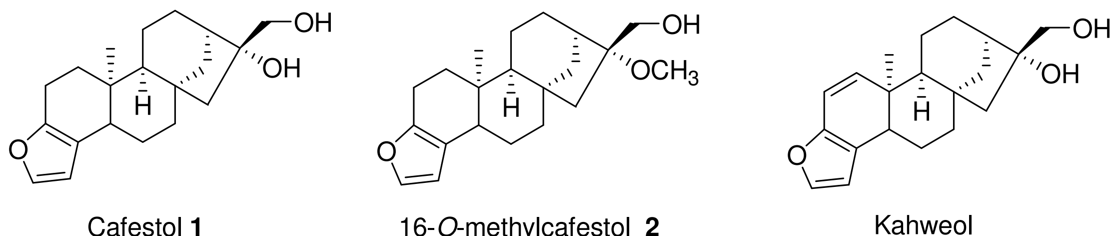
A simple laboratory procedure of isolation of pure cafestol and 16-*O*-methylcafestol together with β-sitosterol from coffee is proposed. Cafestol and 16-*O*-methylcafestol have been exhaustively characterized through 1D and 2D ^{1}H-, ^{13}C-NMR, CD and X-Ray diffraction. For the first time molecular structure of cafestol is reported and assignment of the absolute configuration is unequivocally given exploiting anomalous scattering of a brominated derivative.

Keywords: coffee oil, cafestol, 16-O-methylcafestol, absolute configuration, circular dichroism, sitosterol

Organic solvent extraction of coffee beans produces the so called coffee oil, a lipid fraction composed principally of triacylglicerols (75%), esters of diterpenes alcohols and fatty acids, free diterpenes alcohols, esters of sterols and fatty acids, sterols, tocopherols, phosphatides and triptamines derivatives ranging from 0.04% (tocopherols) to 18.5% (esters of diterpens alcohols).[1]

[*] Corresponding author: Cristina Forzato, e-mail: cforzato@units.it, phone: +390405583921

Saponification of coffee oil gives origin to the unsaponifiable fraction which is mainly constituted by diterpenes and sterols in minor amount although they are present in the coffee beans mainly esterified with different fatty acids. The three most important diterpenes are cafestol **1**, 16-*O*-methylcafestol (16OMC) **2** and kahweol which belong to the kaurene family but the most important one is probably 16OMC **2** since it is present only in *Coffea canephora* (Robusta) and so it can be considered a molecular marker capable of detecting the lack of authenticity of the product when it is commercialized as *Coffea arabica* (Arabica) 100%.



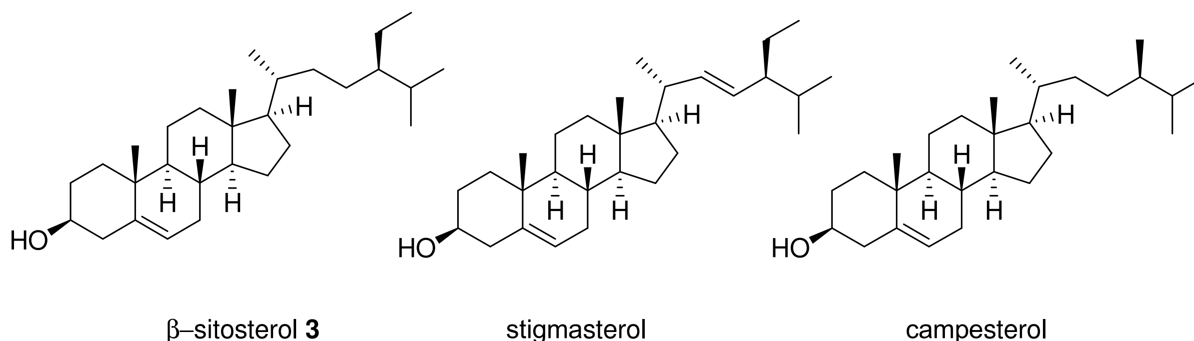
Cafestol **1** 16-*O*-methylcafestol **2** Kahweol

The difference in price of the two species, with the Robusta cheaper with respect to Arabica, stimulated the researchers in finding new rapid methodologies to distinguish them. This is confirmed by the appearance in the literature of very recent articles regarding the identification of these compounds in coffee by ^{1}H NMR spectroscopy.[2]

Since the availability on the trade market of cafestol and 16OMC is uncommon and they are quite expensive (especially 16OMC), the extraction of these compounds from ground roasted coffee is preferable. From a synthetic point of view, only two syntheses are proposed in the literature for these compounds but are rather complicated and with a very low yield.[3] Besides diterpenes, about 5% of sterols are also present in the coffee oil, which are in both free form (around 40%) and esterified form (around 60%). Coffee contains an amount of sterols that is typical for other seed oils as well.[4] In addition to 4-desmethylsterols, various 4-methyl- and 4,4-dimethylsterols have been identified. The desmethylsterols represent 90% of the total sterol fraction.

The amounts of single sterols in the two coffee species are significantly different, except for cholesterol and this fact induced researchers to study the coffee sterolic fraction in order to differentiate Arabica from Robusta.[5] However, because of their varying natural contents, they are qualified for determining Robusta in roasted coffee blends only from 20% onwards. Usually, after the extraction of the coffee oil, the lipids are saponified and the sterols present in the unsaponifiable fraction are separated by TLC, converted into trimethyl silyl (TMS) derivative and analyzed by gas chromatography (GC) with flame ionization detection (FID).

The main sterol is β-sitosterol **3**, which belongs to the desmethylsterols family, at about 50%, followed by stigmasterol and campesterol.



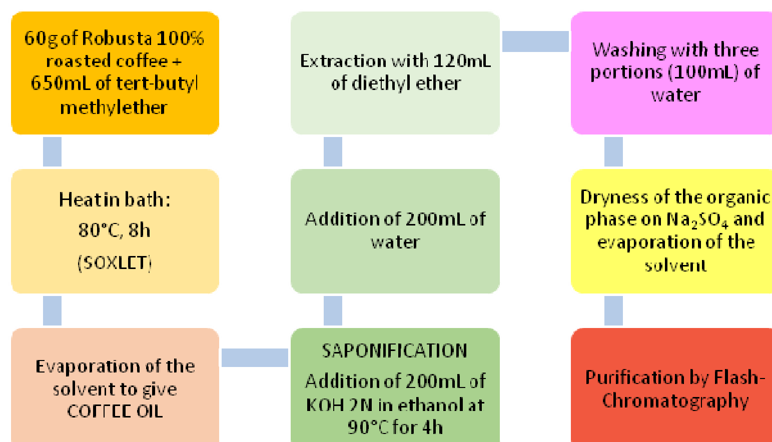
β–sitosterol **3** stigmasterol campesterol

Different approaches are reported in the literature for the extraction of unsaponifiable fractions in food matrices.[6] Some of them describe the use of preliminary extraction of lipid as a first step (for instance the Soxhlet method) before saponification,[7] while others suggest direct saponification without pre-extraction.[8] Direct saponification, either cold (DCS) or hot (DHS), has been described as the most rapid and efficient alternative for extracting unsaponifiable compounds in different food matrices, because it is faster and lower amounts of solvent are required.

An official and validated method of AOAC (Association of Official Analytical Chemists), called DIN Method 10779, has been optimized for the determination of 16OMC content of roasted coffee and it uses HPLC as the final step to purify the compound. This method is appropriate for concentrations from 50mg/Kg to 300mg/Kg of coffee but it is rather time consuming and requires solvents with HPLC purity. Recently a microwave-assisted approach[9] or a supercritical fluid coextraction applied to spent coffee grounds[10] have been considered, indicating that new solutions are under consideration in order to isolate these compounds in a simpler way.

The aim of the present work is to provide a simple method to extract cafestol **1** and 16OMC **2** as pure compounds with low cost equipment in order to use them as standards for further analysis. Pure β-sitosterol can be also obtained from this process. All isolated compounds have been fully characterized.

Extraction of diterpens cafestol **1** and 16OMC **2** was based on DIN method modified by using diethyl ether instead of *tert*-butylmethyl ether (*t*-BME) as the extraction solvent of the unsaponifiable fraction and by performing the purification by column chromatography instead of HPLC. This method permits to obtain cafestol and 16OMC in high yield with a very high degree of purity. The general procedure is illustrated in Scheme 1.



Scheme 1. Scheme of the procedure optimized for the extraction of diterpenes from roasted coffee powder.

Direct solvent extraction in a Soxhlet apparatus was performed on 60 g of commercial roasted and ground 100% *C. canephora*. The coffee oil Soxhlet extraction was carried out over 8 hours by using *t*-BME according to the DIN method. The solvent was removed under reduced pressure and the residue was weighted. 19 different extraction runs have been performed leading to an average of 7.4g of coffee oil. The average extraction yield, expressed as wt.% = 100 x $w_{oil}/w_{coffee\ sample}$, was 13%.

Since cafestol **1** and 16OMC **2** are present in coffee oil both as free diterpenes and diterpenes esterified with different fatty acids, a saponification is needed to obtain diterpenes as free compounds. Accordingly, coffee oil was treated with a potassium hydroxide (2N ethanolic solution) and the resulting mixture was refluxed for 4 hours at 90° C. The free diterpene alcohols were then extracted with diethyl ether and the separated organic phase was washed three times with 100 mL of distilled water. After anhydrous sodium sulfate treatment and solvent evaporation, an average of 0.46g unsaponifiable fraction was obtained.

This fraction was purified by flash–chromatography by using a gradient elution with petroleum ether/ethyl acetate 8/2 to 1/1 on silica gel.

Compounds having $R_f > 0.32$ have been collected in a single fraction which has been subjected to further purification by flash-chromatography while compounds at $R_f = 0.15$ (cafestol) and $R_f = 0.32$ (16OMC) have been collected separately.

A total amount of 0.511g of cafestol **1** and 0.574g of 16OMC **2** could be obtained in a yield equal to 5.8% and 6.6% of the unsaponifiable matter, respectively.

The characterization of **1** and **2** by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy was performed both in DMSO-d_6 and in $CDCl_3$ whereas chirooptical properties were investigated by optical rotatory power and circular dichroism. (See supporting information for ^{1}H NMR spectra of **1** and **2** both in $CDCl_3$ and DMSO-d_6, figures S1, S2, S3 and S4).

Although NMR data for these compounds were already reported in the literature[11] the assignment of the chemical shifts of protons at C-5 and C-9 has been revised. In particular, 1D NOESY and 1DzTOCSY NMR experiments (see supporting information figure S12 and S13 respectively) performed on cafestol disclosed the correct assignment of the signal at 2.31 ppm to proton H-5, while the signal at 1.31-1.13 to proton H-9. 1D NOESY experiments were particularly useful for the negative Overhauser effects on the resonance of H-5 and the furan

proton H-18 (figure S12 of supporting information). The correct assignment was reported in the literature for the corresponding esters confirming our data.[2b]

It is interesting to observe a different behavior of the diastereotopic protons H-17 of 16OMC by examing ^{1}H-NMR spectra obtained in $CDCl_3$ and in DMSO-d_6. While in $CDCl_3$ these protons appear as a singlet, in DMSO-d_6 the signal is a typical AB part of an ABX system (Figure 1).

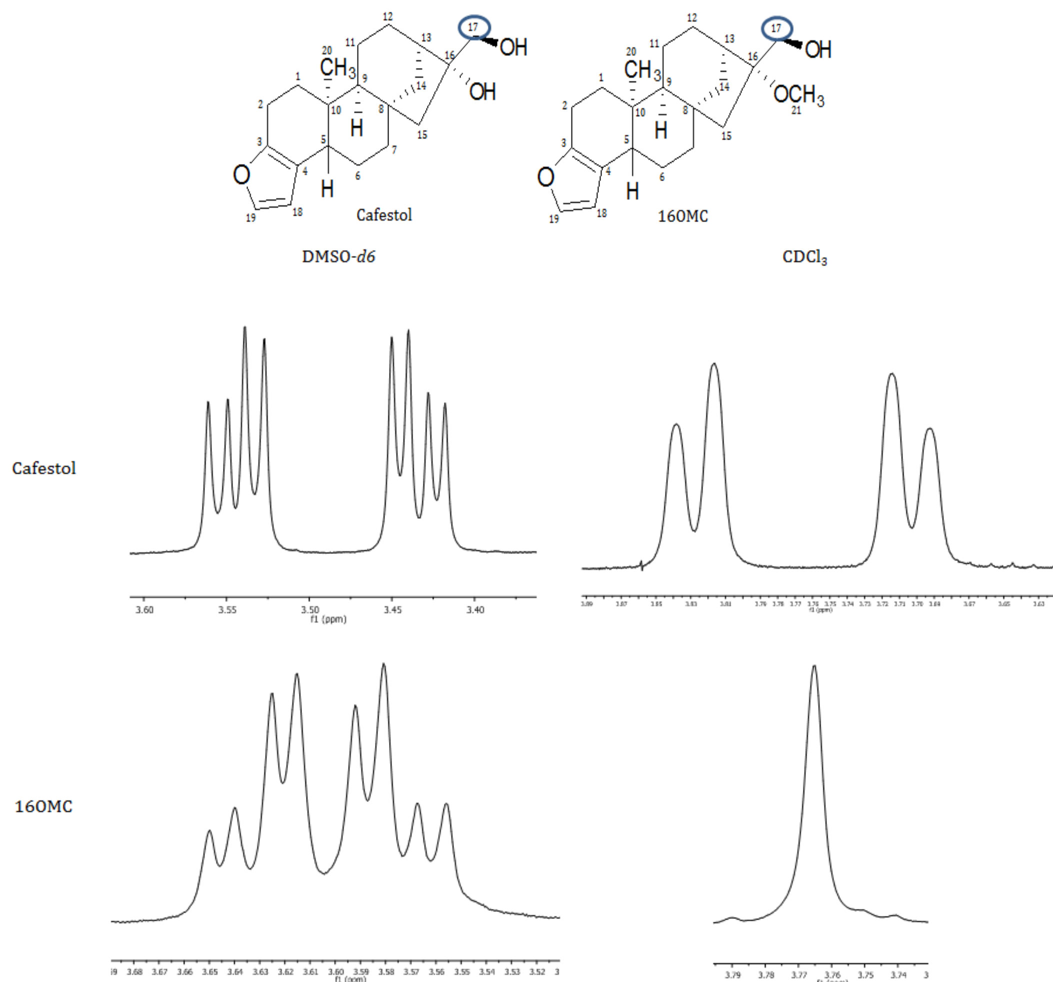


Figure 1. Comparison of the signals of protons at C-17 of cafestol **1** and 16OMC **2** in $CDCl_3$ and DMSO-d_6

This finding is due to rapid or slow conformational conversion, as rotation of the whole group along the C-C bond is significantly faster than NMR relaxation time at room temperature in the case of 16OMC, while it might be slower in cafestol due to strong intramolecular hydrogen bond which may "freeze" the system in a pseudocyclic conformation. In DMSO-d_6, diastereotopic protons of the 17-methylene are AB part of an ABX system both in cafestol and 16OMC because the solvent viscosity could influence the relaxation time, and therefore it could slow down the rotation of the whole group along the C-C bond of 16OMC, where intramolecular hydrogen bonds are absent.

By performing the experiments at higher temperatures (40, 55, 70 °C) for 16OMC in DMSO-d_6 (Figure 2), it is possible to speed up the rotation of protons 17 leading to a conversion from an AB part of an ABX system to an AB system.

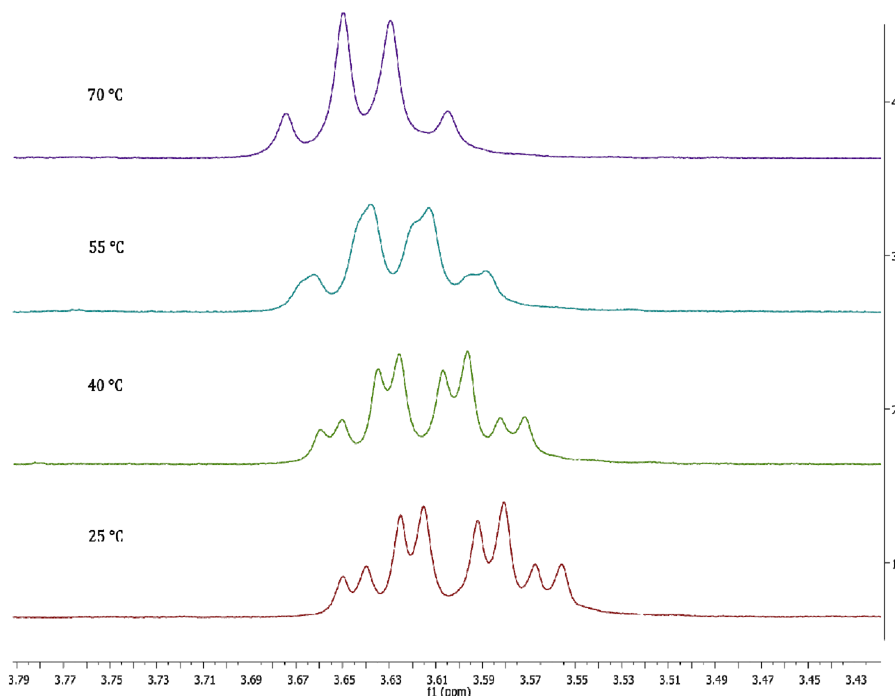


Figure 2. Comparison of the signals of the 17-methylene of cafestol **1** and 16OMC **2** DMSO-d_6 at different temperatures.

Despite 16OMC structure has been already reported in the literature (data collected at 93 K),[12] no crystallographic data has been published for pristine cafestol so far.
Cafestol crystalline solid samples, suitable for X-ray analysis, were obtained from pure cafestol fractions using ligroin solutions, and few drops of acetone as precipitant.
Cafestol crystallizes with two molecules in the Asymmetric Unit (ASU) (Figure 3), in a monoclinic unit cell ($P\ 2_1$ space group) with the following cell dimensions: a = 11.7240(22) Å, b = 7.1420(4) Å, c = 21.4270 (7) Å and β = 101.790(7)° ($V_{cell/Z}$ = 1756/4 = 439 Å^3). (Figure 3)
Unit cell and crystal packing differs substantially from 16OMC structure: "unmethylated" cafestol oxygens act as both H-bond donor and acceptors, trapping water molecules in the crystal packing; 16OMC structure instead shows a more compact packing ($V_{cell/Z}$ = 431 Å^3), lacking solvent molecules.
Full crystal data and structure refinement statistics are reported in the supporting information Table S1.

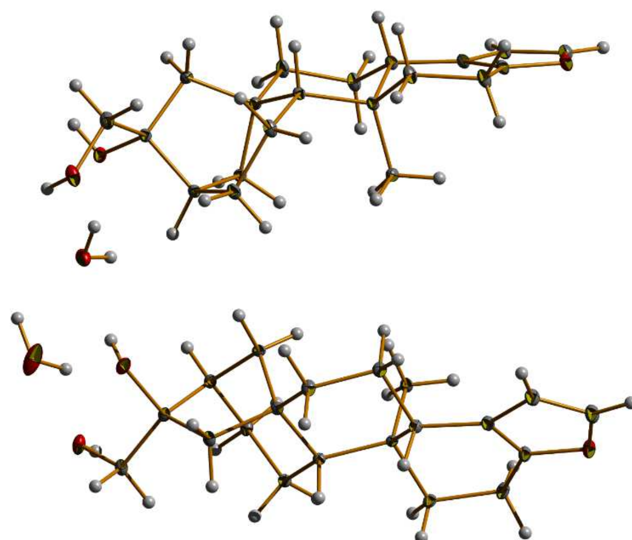
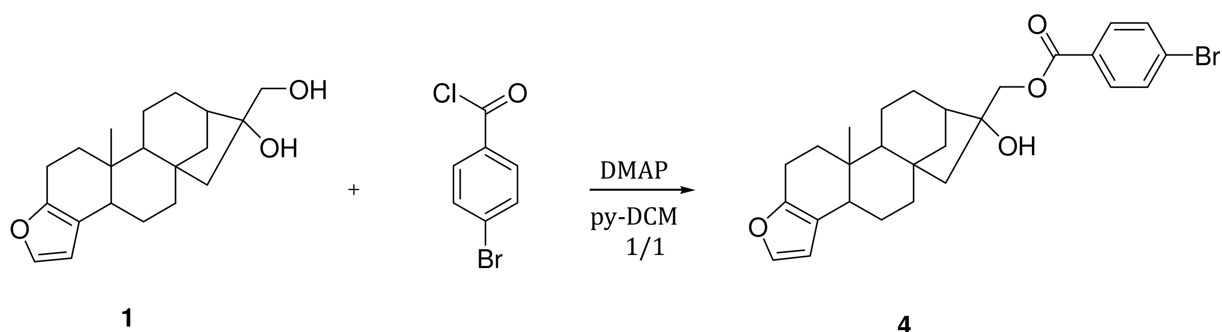


Figure 3. Ellipsoid representation of cafestol crystals (**1**) ASU content (50% probability). Two crystallographically independent molecules are present, linked by bridging water molecules.

Cafestol stereochemistry was tentatively assigned in 1958 by Djerassi[13] and in a further study in 1960[14] but only in 1962, Scott et al. definitely assigned the absolute configuration.[15] It was principally based on the analysis of the optical rotatory dispersion curves but although the use of empirical rules, such as the octant rule or the comparison of circular dichroism spectra or optical rotatory dispersion curves with similar compounds is sometime possible, unambiguous assignment using X-ray diffraction is preferable.
A brominated derivative **4** was synthetized using a derivatization approach that doesn't affect the stereochemistry of the molecule. The addition of a bromine atom permits to collect diffraction data that reveal chirality, exploiting strong anomalous scattering contribution of the bromine atoms.
The synthesis was achieved following a literature procedure for similar compounds.[16] (Scheme 2)



Scheme 2. Synthesis of the brominated derivative of cafestol **4**

Brominated cafestol **4** crystals, suitable for X-ray analysis, were obtained by slow solvent evaporation from an acetone solution, at 4°C. The crystals show thin needle-like habits. The

brominated derivative exhibited high sensitivity to radiation damage, so data from different crystals have been used. The structural model obtained for the cafestol bromine derivative confirms the known stereochemistry (Figure 4).

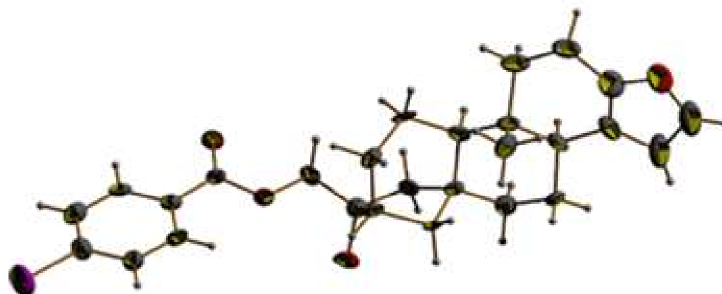


Figure 4. X-ray molecular structure of *p*-bromobenzoyl cafestol **4** (50% probability ellipsoids). Final refined Flack parameter 0.041(14)[17] confirms the reliability of the stereochemical configuration shown.

The absolute configuration is so established as 5*S*, 8*S*, 9*R*, 10*S*, 13*R*, 16*R*.
CD spectra for both cafestol and 16OMC are also reported in this work for the first time. (Figure 5)
Since they both have positive Cotton effect the absolute stereocenter configurations for the two compounds must be the same.
This is to confirm that the biosynthesis of 16OMC derives from a methylation reaction of cafestol catalyzed by a SAM methyl transferase since they are both present in *Coffea canephora*.

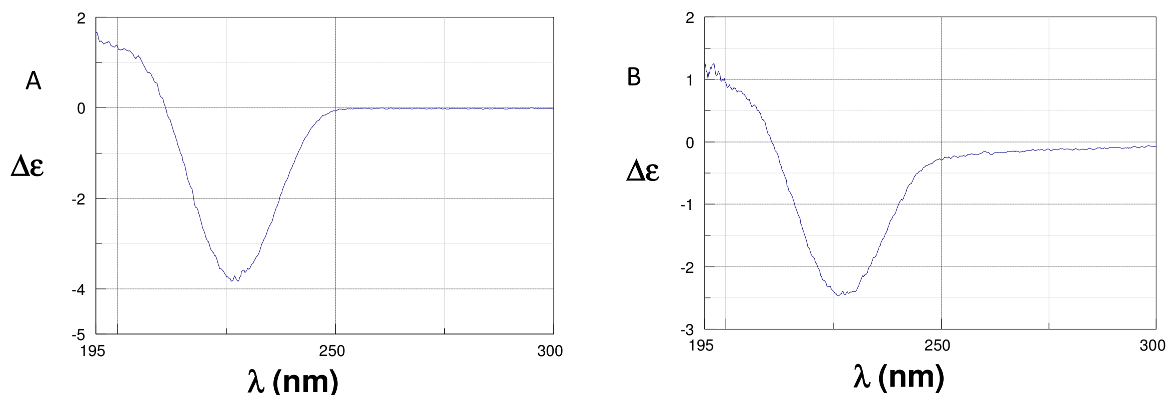


Figure 5. Circular dichroism spectra of cafestol **1** (A) and 16OMC **2** (B) in methanol

The single fraction with compounds having $R_f > 0.32$ obtained from the previous column chromatography has been purified by a second column chromatography using petroleum ether/ethyl acetate 8/2 as the mobile phase. Different fractions were collected and one of them led to the isolation of β-sitosterol which was further recrystallized from ethyl acetate to obtain **3**

as colourless crystals. All obtained spectroscopic data are in accordance with the literature although some discrepancies have been observed in the assignment of all signals in ^{13}C-NMR spectrum. These discrepancies could be derived in part from a different number assignment of the carbons at the side chain, while for carbons of the ring part (where the numbering is the same) the authors report a different chemical shift for C-7. In particular, Yang A. et al.[18] attributed the signal at 21.07ppm at C-7 while Ragasa C. Y.[19] and Chang Y.-C.[20] assigned the signal at 31.88ppm and 31.6ppm respectively to C-7. In the other collected fractions, we could recognize other sterols such as stigmasterol and another sterol (although in admixture with β-sitosterol) with a methylene group on the side chain with a signal at 4.67ppm at ^{1}H-NMR and a signal at 105.9ppm in the ^{13}C-NMR, so it was not possible to characterize them completely.
Crystals of β-sitosterol **3** in thin needle-like habits have been achieved by slow solvent evaporation from an acetone solution at 4°C and identity was confirmed by X-ray structural analysis. For β-sitosterol, only one water molecule links the steroid molecules (Figure 6), due to the lower number of free hydroxyl groups available. The molecules pack in a similar alternating, layered pattern, along the longest axis. As with other steroids, double layers can be identified with polar groups on outer hydrated surfaces (parallel to *ab* face), while hydrophobic portions remain confined in between. This packing is equivalent to those available in literature for (22*E*)-24-ethyl-24-methylcholesta-5,22-dien-3b-ol methanol solvate hemihydrate[21] and stigmasterol hemihydrate,[22] but is not found in previously published β-sitosterol structures.

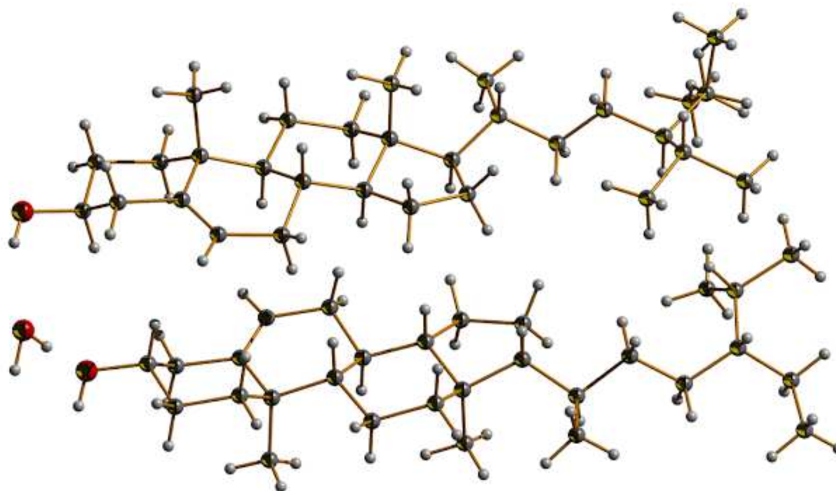


Figure 6. Ellipsoid representation of β-sitosterol crystals **3** ASU content (50% probability). Two crystallographically independent molecules are present, linked by a bridging water molecule.

In summary, this work propose an easy route to isolate cafestol and 16-*O*-methylcafestol in high yield (> 5% of unsaponifiable matter) with very high purity. In view of its simple and cheap steps, the described method could be considered a practical choice for the extraction of diterpenes from spent coffee grounds which represent a promising source not yet adequately exploited.

NMR characterization for both cafestol and 16OMC has been carried out in two different solvents ($CDCl_3$ and DMSO-d_6) and all signals could be assigned leading to a revision of the literature data for H-5 and H-9 of cafestol.
The molecular structure of cafestol has been studied for the first time by using X-ray diffraction with XRD1 beamline of the Elettra Synchrotron, and through a brominated derivative its absolute configuration as 5*S*,8*S*,9*R*,10*S*,13*R*,16*R* has been definitely assigned.
An interesting side-product, as a result of the present diterpenes isolation process, has been obtained since β-sitosterol in pure form can be easily recovered. Crystallographic data of this coffee sterol showed a packing pattern not previously observed for this compound. Finally, NMR characterization led to the revision of the assignment of ^{13}C-NMR spectrum.

Experimental section

Materials
Commercial coffee blends of *Coffea canephora* 100% (Robusta) were used for all samples preparations. Geographical origin is unknown. All solvents used were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used as received.

Oil extraction
60 g of ground roasted Robusta 100% coffee were weighted in an extraction thimble and placed in a 500 mL Soxhlet apparatus. The extraction was carried out using 650 mL of *tert*-butyl methyl ether (*t*-BME) as the solvent at a water bath temperature of 80°C, with four siphons taking place per hour for eight hours. After extraction, the solvent was evaporated under vacuum and the oil was weighted (on average 7-8 g of oil, yield 12-13%).

Hydrolysis of coffee oil
Coffee oil was saponified with 200 mL of an ethanolic potassium hydroxide solution 2N in a water bath at a temperature of 90°C for 4 hours. The reaction mixture was diluted with 200 mL of water and extracted with 120 ml of diethyl ether. The organic phase was washed with water (3 times, each using 50 mL) and dried on anhydrous sodium sulfate. The solvent was evaporated under vacuum and the residue was purified by flash chromatography.

Diterpenes purification
Cafestol **1** and 16-*O*-methylcafestol **2** were purified from 0.46 g (on average) of unsaponifiable fraction obtained with the procedure described in section *2.3* by column chromatography ("flash chromatography"). The mobile phase used was petroleum ether/ethyl acetate 8/2 → 1/1 v/v (gradient elution). The fractions collected were grouped by similarity according to their thin layer chromatography (TLC) profile. The solvents were evaporated under vacuum and the isolated diterpenes were obtained: on average 0.051 g of cafestol and 0.057 g of 16OMC. Fractions with higher R_f were put all together (about 350 mg) and purified later by a second flash chromatography to achieve sterols.

*Cafestol **1***: white solid (ligroin); m.p. = 132-135°C; $[\alpha]_D^{20}$ = -119 (c = 0.06 , CH_3OH); CD: $[\theta]_{228}$ = - 12636 (CH_3OH); UV (CH_3OH): λ_{max} (log ε) 223 (3.76) nm; IR (nujol) ν_{max} 3582, 3385, 2925, 2852, 1702, 1629, 1453, 1104, 1069, 1010 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.24 (1H, d,

J=1.8 Hz, H_{19}), 6.21 (1H, d, J=1.8 Hz, H_{18}), 3.84-3.69 (2H, AB system, J= 11.00 Hz, H_{17}), 2.63-2.61 (2H, dd, J=5.8, 2.8 Hz, H_2), 2.29-2.25 (1H, dq, J=12.7, 2.4 Hz, H_5), 2.07-2.03 (3H, m, H_{13}, H_1,H_{14}), 1.96 (1H, s, OH), 1.92 (1H, s, OH), 1.83-1.79 (1H, ddd, J=13.00, 6.4, 3.2 Hz, H_6), 1.74-1.48 (10H, m, H_6, H_7, H_{11}, H_{12}, H_{14}, H_{15}), 1.27-1.21 (1H, dt, J=12.4, 9.4 Hz, H_1), 1.18 (1H, d, J=7.6 Hz, H_9), 0.84 (3H, s, H_{20}); ^{13}C NMR ($CDCl_3$, 125.4 MHz) δ 148.89 (C, C_3), 140.73 (CH, C_{19}), 120.28 (C, C_4), 108.46 (CH, C_{18}), 82.05 (C, C_{16}), 66.52 (CH_2, C_{17}), 53.60 (CH_2, C_{15}), 52.26 (CH, C_9), 45.61 (CH, C_{13}), 44.84 (C, C_8), 44.41 (CH, C_5), 41.03 (CH_2, C_7), 38.80 (C, C_{10}), 38.35 (CH_2, C_{14}), 35.90 (CH_2, C_1), 26.22 (CH_2, C_{12}), 23.26 (CH_2, C_6), 20.79 (CH_2, C_2), 19.18 (CH_2, C_{11}), 13.49 (CH_3, C_{20}); ^1H NMR (DMSO-d_6, 500 MHz) δ 7.39 (1H, d, J=1.7 Hz, H_{19}), 6.29 (1H, d, J=1.8 H_{18}), 4.35 (1H, t, J=5.4 Hz, OH), 3.89 (1H, s, OH), 3.56-3.42 (2H, AB part of an ABX system, J= 5.3 Hz, H_{17}), 2.55 (2H, dd, J=7.9 Hz, H_2), 2.21-2.18 (1H, dd, J=12.7, 2.3 Hz, H_5), 2.03-1.99 (1H, m, H_1), 1.91 (1H, d, J=3.4 Hz, H_{13}), 1.86 (1H, d, J=11.2 Hz, H_{14}), 1.79-1.75 (1H, ddd, J=12.8, 5.7, 3.3 Hz, H_6), 1.65-1.54 (6H, m, H_7, H_{11}, H_{12}, H_{14}), 1.51-1.48 (1H, dd, J=14.1, 1.1 Hz, H_{15}), 1.42-1.39 (1H, dd, J=12.8, 3.5 Hz, H_6), 1.37 (1H, br, H_{12}), 1.35-1.32 (1H, d, J=14.1 Hz, H_{15}), 1.21-1.15 (1H, dd, J=21.8, 9.5 Hz, H_1), 1.12 (1H, d, J=5.6 Hz, H_9), 0.75 (3H, s, H_{20}); ^{13}C NMR (DMSO-d_6, 125.4 MHz) δ 148.03 (C, C_3), 140.88 (CH, C_{19}), 119.90 (C, C_4), 108.51 (CH, C_{18}), 80.56 (C, C_{16}), 65.31 (CH_2, C_{17}), 53.09 (CH_2, C_{15}), 51.63 (CH, C_9), 44.68 (CH, C_{13}), 43.98 (C, C_8), 43.65 (CH, C_5), 40.70 (CH_2, C_7), 38.11 (C, C_{10}), 37.80 (CH_2, C_{14}), 35.10 (CH_2, C_1), 25.69 (CH_2, C_{12}), 22.74 (CH_2, C_6), 20.16 (CH_2, C_2), 18.62 (CH_2, C_{11}), 13.19 (CH_3, C_{20}); ESI$^+$-MS: $[M+Na]^+$ = 339 m/z; $[M+H]^+$ = 317 m/z.

*16-O-methylcafestol **2***: white solid; m.p. 174°C; $[\alpha]_D^{20}$ = -102 (c = 0.13 , CH_3OH); CD: $[\theta]_{228}$ = -8133 (CH_3OH); UV (CH_3OH): λ_{max} (log ε) 223 (3.63) nm; IR (nujol): 3583, 3384, 2913, 2852, 1702, 1629, 1453, 1104, 1069, 1010 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.24 (1H, br, H_{19}), 6.20 (1H, d, J=1.7 Hz, H_{18}), 3.77 (2H, s, H_{17}), 3.17 (3H, s, H_{21}), 2.62 (2H, dt, J=5.8, 2.8 Hz, H_2), 2.27 (1H, dd, J=12.7, 2.4 Hz, H_5), 2.22 (1H, d, J=2.9, H_{13}), 2.05 (1H, dt, J=13.2, 4.1 Hz, H_1), 1.99 (1H, d, J=11.6 Hz, H_{14}), 1.83-1.78 (1H, ddd, J=12.9, 6.2, 3.1, H_6), 1.71 (1H, d, J=7.0 Hz, H_{11}), 1.67-1.42 (8H, m, H_6, H_7, H_{11}, H_{12}, H_{14}, H_{15}) 1.43 (1H, dd, J=14.6, 1.9 Hz, H_{15}), 1.29-1.22 (1H, m, H_1), 1.19 (1H, d, J=7.6 Hz, H_9), 0.82 (3H, s, H_{20}); ^{13}C NMR ($CDCl_3$, 125.4 MHz) δ 148.91 (C, C_3), 140.72 (CH, C_{19}), 120.28 (C, C_4), 108.45 (CH, C_{18}), 87.20 (C, C_{16}), 60.64 (CH_2, C_{17}), 52.28 (CH, C_9), 49.26 (CH_2, C_{15}), 49.10 (CH_3, C_{21}), 44.56 (C, C_8), 44.41 (CH, C_5), 41.62 (CH, C_{13}), 41.16 (CH_2, C_7), 38.84 (C, C_{10}), 37.96 (CH_2, C_{14}), 35.93 (CH_2, C_1), 25.88 (CH_2, C_{12}), 23.27 (CH_2, C_6), 20.80 (CH_2, C_2), 19.31 (CH_2, C_{11}), 13.46 (CH_3, C_{20}); ^1H NMR (DMSO-d_6, 500 MHz) δ 7.39 (1H, br, H_{19}), 6.29 (1H, d, J=1.7 Hz, H_{18}), 4.30-4.28 (1H, t, J=5.2 Hz, OH), 3.65-3.56 (2H, AB part of an ABX system, J= 5.4, H_{17}), 3.07 (3H, s, H_{21}), 2.55 (2H, d, J=8.3 Hz, H_2), 2.22-2.19 (1H, dd, J=12.6, 1.7, H_5), 2.12 (1H, d, J=2.5, H_{13}), 2.02 (1H, m, H_1), 1.85 (1H, d, J=11.3 Hz, H_{14}), 1.77 (1H, dd, J=12.9, 2.7 Hz, H_6), 1.65-1.58 (4H, m, H_7, H_{11}, H_{12}), 1.51-1.34 (6H, m, H_6, H_7, H_{12}, H_{14}, H_{15}), 1.19 (1H, m, H_1), 1.16 (1H, d, J=4.5, H_9), 0.75 (3H, s, H_{20}); ^{13}C NMR (DMSO-d_6, 125.4 MHz) δ 148.03 (C, C_3), 140.89 (CH, C_{19}), 119.88 (C, C_4), 108.5 (CH, C_{18}), 86.23 (C, C_{16}), 59.63 (CH_2, C_{17}), 51.59 (CH, C_9), 49.19 (CH_2, C_{15}), 48.73 (CH_3, C_{21}), 43.75 (CH, C_5), 43.58 (C, C_8), 41.12 (CH, C_{13}), 40.81 (CH_2, C_7), 38.13 (CH_2, C_{14}), 37.76 (C, C_{10}), 35.10 (CH_2, C_1), 25.58 (CH_2, C_{12}), 22.73 (CH_2, C_6), 20.15 (CH_2, C_2), 18.68 (CH_2, C_{11}), 13.15 (CH_3, C_{20}); ESI$^+$-MS: $[M+Na]^+$ = 353 m/z, $[M+H]^+$ = 331 m/z.

β-sitosterol purification
β-sitosterol **3** was purified from about 350 mg of unsaponifiable fraction obtained with procedure described in section 2.4 by flash chromatography. The mobile phase used was petroleum ether/ethyl acetate 8/2. Isolated β-sitosterol was obtained as colorless crystals (51.3 mg, 12.38 mmol). We also obtained other mixed fractions of different sterols present in unsaponifiable matter.

All spectroscopic data are in accordance with those reported in the literature.[18, 19, 20]

Cafestol crystallization
If cafestol presented traces of impurities, observed in the NMR spectra, a crystallization was performed following the Ferrari method[23] with some modifications. The crystallization was carried out at reflux for 5 hours using few mL of ligroin (boiling range 80°-120° C) and few drops of acetone. The crystals obtained are yellow needle-like.

X-ray diffraction
Crystals of cafestol **1**, its brominated derivative **1Br** and β-sitosterol **4**, suitable for diffraction experiments, have been obtained by slow solvent evaporation from an acetone solution, at 4°C. All crystals showed similar thin needle-like habits (with average dimensions of 0.2·0.03·0.03 mm^3). Complete datasets have been collected at a monochromatic wavelength of 0.700 Å through the rotating crystal method, using a Pilatus 2M detector, at the XRD1 beamline of the Elettra Synchrotron (Trieste, Italy).[24] The crystals were dipped in N-paratone and mounted on the goniometer head with a nylon loop. All the diffraction experiments were conducted at 100 K, using a nitrogen stream supplied through an Oxford Cryostream 700. Sine brominated cafestol samples shown high sensitivity to radiation damage, data from different crystals have been merged to obtain a complete set.
The diffraction data were indexed, integrated and scaled using XDS.[25] The structures were solved by direct methods using Sir2014,[26] Fourier analyzed and refined by the full-matrix least-squares based on F^2 implemented in SHELXL-2014.[27] Coot program has been used for modeling.[28] In the final refinement, all non-hydrogen atoms with full occupancy, were treated anisotropically and the hydrogen atoms were included at calculated positions with isotropic $U_{factors} = 1.2 \cdot U_{eq}$ or $U_{factors} = 1.5 \cdot U_{eq}$ for methyl groups (U_{eq} being the equivalent isotropic thermal factor of the bonded non hydrogen atom)..
The structure of brominated derivative **1Br** exhibits extensive disorder solvent inside crystal channels. The contribution of this region to the scattering was estimated as ca. 80 electrons/cell, in a volume of 395 $Å^3$ and it was removed with the SQUEEZE routine of PLATON.[29] Empirical absorption correction has also been applied as implemented in the XABS2 program.[30] The structural characterization of the cafestol bromine derivative allowed unambiguous stereochemistry assignment of cafestol chiral carbons, confirming the absolute configuration reported in literature for 16OMC.[12] β-sitosterol stereocentres configurations are in agreement

with previously deposited structures for this molecule[31,32] (data collected at 100 K and r.t.). Essential crystal and refinement data are reported in the ESI (Table S1).

Synthesis of p-bromobenzoyl ester of cafestol

To a solution of cafestol (45.1 mg, 0.143 mmol) in 2 mL of pyridine-dichloromethane (1:1 v/v) were added *p*-bromobenzoyl chloride (157 mg, 0.175 mmol) and 4-(dimethylamino)pyridine (DMAP) (18 mg, 0,155 mmol) in one portion. After stirring at room temperature for 4 hours, the mixture was filtered on a paper filter (to eliminate the white solid) and the solution was diluted with EtOAc. The extract was washed successively with aqueous HCl (5%) and brine, dried and concentrated under vacuum to give a residue, which was then purified by flash chromatography with $CHCl_3$/MeOH – 50/1 to afford *p*-bromobenzoyl ester of cafestol.

white solid, m.p. 125-128°C; $[\alpha]_D^{20}$ = -84.0 (c 0.13, $CHCl_3$); IR (nujol) ν_{max} 3583, 2923, 1718, 1589, 1452, 1270, 1117, 1012; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.25 (1H, d, J=1.6 Hz, H_{19}), 7.92-7.88 (2H, m, H_{24},H_{26}), 7.62-7.58 (2H, m, H_{23}, H_{27}), 6.21 (1H, d, J=1.8 Hz, H_{18}), 4.53 (2H, AB system, J=11.4 Hz, H_{17}), 2.65-2.60 (2H, m, H_2), 2.28 (1H, dd, J=12.7, 2.5 Hz, H_5), 2.10-2.03 (3H, m, H_1, H_{13}, H_{14}), 1.96 (1H, s, OH), 1.86-1.79 (1H, m, H_6), 1.74-1.48 (10H, m, H_6, H_7, H_{11}, H_{12}, H_{14}, H_{15}), 1.29-1.21 (m2H,, H1, H_9), 0.85 (3H, s, H_{20}); ^{13}C NMR (CDCl3, 125.4 MHz) δ 165.9 (C, C_{21}), 149.8 (C, C_3), 140.1 (CH, C_{19}), 132.1 (C, C_{27},C_{23}), 131.5 (C, C_{24},C_{26}), 129.1 (C, C_{22}), 127.4 (C, C_{25}), 120.28 (C, C_4), 108.9 (CH, C_{18}), 79.06 (C, C_{16}), 70.01 (CH_2, C_{17}), 53.60 (CH_2, C_{15}), 52. 6 (CH, C_9), 45.61 (CH, C_{13}), 44.84 (C, C_8), 44.41 (CH, C_5), 41.03 (CH_2, C_7), 38.80 (C, C_{10}), 38.35 (CH_2, C_{14}), 35.90 (CH_2, C_1), 26.22 (CH_2, C_{12}), 23.26 (CH_2, C_6), 20.79 (CH_2, C_2), 19.18 (CH_2, C_{11}), 13.49 (CH_3, C_{20}); ESI^+-MS: $[M+H]^+$ = 499 m/z, $[M+Na]^+$ = 521 m/z.

Acknowledgments

Authors acknowledge the University of Trieste for the financial support.

Supplementary material

CCDC-1434207, 1434208 and 1434206 contain the supplementary crystallographic data for compounds **1**, **3** and **4**. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

References

1. Ferrari, M., Ravera, F., De Angelis, E., Suggi Liverani, F., Navarini, L. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2010**, 365, 79-82.

2. a) Monakhova, Y. B., Ruge, W., Kuballa, T., Ilse, M., Winkelmann, O., Diehl, B., Thomas, F., Lachenmeier, D. W. *Food Chemistry*, **2015**, *182*, 178-184; b) Schievano, E., Finotello, C., De Angelis, E., Mammi, S., Navarini, L. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 12309-12314; c) D'Amelio, N., De Angelis, E., Navarini, L., Schievano, E., Mammi, S. *Talanta* **2013**, *110*, 118-127.

3. a) Zhu, L. Luo, J., Hong, R. *Organic Letters* **2014**, *16*, 2162-5; b) Corey, E. J., Wess, G., Xiang, Y. B., Singh, A. K. *Journal of the American Chemical Society,* **1987**, *109*, 4717-4718.

4. Phillips, K. M., Ruggio, D. M., Toivo, J. I., Swank, M. A., Simpkins, A. H. *Journal of Food Composition and Analysis,* **2002**, 123-142.

5. a) Cizkova, H., Soukupova, V., Voldrich, M., Sevcik, R. *Journal of Food and Nutrition Research*, **2007**, *46*, 28-34; b) Speer, K., Kolling-Speer, I. *Braz. J. Plant. Physiol*., **2006**, *18*, 201-216; c) Kamm, W., Dionisi, F., Fay, L.-B., Hischenhuber, C., Xchmarr, H.-G., Engel, K.-H. *Am. Oil. Chem. Soc*., **2002**, *79*, 1109-1113; d) Carrera, F., Leon-Camacho, M., Pablos, F., Gonzales, A. G. *Anal. Chim. Acta*, **1998**, *370*, 131-139.
6. Dias, R. C. E., de Faria, A. F., Mercadante, A. Z., Bragagnolo, N. Benassi, M. *J. Braz. Chem. Soc.*, **2013**, *24*, 492-499.
7. a) Rubayiza, A. B., Meurens, M. *Journal of Agricultural and Food Chemistry*, **2005**, *53*, 4654-9; b) Kölling-Speer, I., Strohschneider, S., Speer, K. *Journal of High Resolution Chromatography*, **1999**, *22*, 43-46; c) Pettitt, B. C. *Journal of Agricultural and Food Chemistry,* **1987**, *35,* 549-551.
8. a) Roos, B., van der Weg, G., Urgert, R., van de Bovenkamp, P. Charrier, A., Katan, M. B. *Journal of Agricultural and Food Chemistry*, 1997, *35*, 549-551; b) de Souza, R. M. N., Benassi, M. T. *Journal of the Brazilian Chemical Society*, **2012**, *23*, 1347-1354.
9. Tsukui, A., Santos Junior, H. M., Oigman, S. S., de Souza, R. O. M. A., Bizzo, H. R., Rezende, C. M. *Food Chemistry*, **2014**, *164*, 266-271.
10. Barbosa, H. M. A., de Melo M. M. R., Coimbra, M. A., Passos, C. P., Silva, C. M. *The Journal of Supercritical Fluids*, **2014**, *85*, 165-172.
11. Scharnhop, H., Winterhalter, P. *Journal of Food Composition and Analysis*, **2009**, *22*, 233-237.
12. Liao, X.-L., Chen, X.-Z., Yu, K.-B., Li, G.-Y. *Acta Crystallographica*, **2010**, *E66*, o760.
13. Djerassi, C., Cais, M., Mitsher, L. A. *Journal of the American Chemical Society*, **1958**, *80*, 247-248.
14. Finnegan, R. A., Djerassi, C. *Journal of the American Chemical Society*, **1960**, *82*, 4342-4344.
15. a) Scott, A. I., McCapra, F., Comer, F., Sutherland, S. A., Young, D. W., Sim, G. A., Ferguson, G. *Tetrahedron*, **1964**, *20*, 1339-1358; b) Scott, A. I., Sim, G. A., Ferguson, G. Young, D. W., McCapra, F. *Journal of the American Chemical Society*, **1962**, 3197-3199.
16. Nishimura, K., Hitotsuyanagi, Y., Sugeta, N., Sakakura, K. I. Fujita, K., Fukaya, H., Aoyagi, Y., Hasuda, T., Kinoshita, T., He, D. H., Otsuka, H., Takeda, Y., Takeya, K. *Tetrahedron*, **2006**, *62*, 1512-1519.
17. Parsons, S., Flack, H. D., Wagner, T. *Acta Crystallographica Section B*, **2013**, *69(3)*, 249-259.
18. Yang, A., Li, H., Liu, J., Guo, W., Wu, R. *Advanced Material Research*, **2013**, *634-638*, 905-908.
19. Ragasa, C. Y., Buluran, A. I., Mandia, E. H., Shen, C.-C. *Der Pharma Chemica*, **2014**, *6*(6), 418-422.
20. Chang, Y.-C., Chang, F.-R., Wu, Y.-C. *Journal of the Chinese Chemical Society*, **2000**, *47*, 373-380.
21. Echigo, S., Castellanos, L., Duque, C., Uekusa, H., Hara, N., and Fujimoto, Y. *Journal of the Brazilian Chemical Society*, **2011**, *22*, 997–1004.
22. Jiang, R. W., Ma S.-C., Pui-Hay But P., Mak T. C. W. *Journal of Natural Products*, **2001**, *64(10)*, 1266–1272.
23. Ferrari, G. *Società Chimica Italiana*, **1989**, *7*, 134-7.
24. Lausi A., Polentarutti M., Onesti S., Plaisier J. R., Busetto E., Bais G., Barba L., Cassetta A., Campi G., Lamba D., Pifferi A., Mande S. C., Sarma D. D., Sharma S. M., Paolucci G. *The European Physical Journal Plus*, **2015**, *130(43)*, 1-8.
25. Kabsch W. *Acta Crystallographica Section D,* **2010**, *66(2)*, 125–132.
26. Burla M. C., Caliandro R., Carrozzini B., Cascarano G. L., Cuocci C., Giacovazzo C., Mallamo M., Mazzone A., Polidori G. *Journal of Applied Crystallography*, **2015**, *48(1)*, 306-309.
27. Sheldrick G. M. *Acta Crystallographica Section A*, **2008**, *64(1)*, 112-122.
28. Emsley P., Cowtan K. *Acta Crystallographica Section D,* **2004**, *60(12)*, 2126–2132.
29. Spek A. L. *Acta Crystallographica Section D,* **2009**, *65(2)*, 148–155.
30. Parkin S., Moezzi B., Hope H. *Journal of Applied Crystallography*, **1995**, *28(1)*, 53-56.
31. Wu X., Chao Z., Wang C., Sun W., Zhang G. *Chinese Journal of Structural Chemistry*, **2014**, *33*, 801-806.
32. a) Moreno-Calvo, E., Temelli, F., Cordoba, A., Masciocchi, N., Veciana, J., Ventosa, N. *Crystal Growth and Design*, **2014**, *14(1)*, 58-68; b) Argay G., Kalman A., Vladimirov S., Zivanov-Stakic D., Ribar, B. *Zeitschrift für Kristallographie - Crystalline Materials*, **1996**, *211*, 725-727.