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Green coffee oil analysis by high-resolution nuclear magnetic resonance spectroscopy

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ABSTRACT

In this work, we show how an extensive and fast quantification of the main components in green coffee oil can be achieved by NMR, with minimal sample manipulation and use of organic solvents. The approach is based on the integration of characteristic NMR signals, selected because of their similar relaxation properties and because they fall in similar spectral regions, which minimizes offset effects. Quantification of glycerides, together with their fatty acid components (oleic, linoleic, linolenic and saturated) and minor species (caffeine, cafestol, kahweol and 16-O-methylcafestol), is achieved in less than 1 h making use of ^{1}H and ^{13}C spectroscopy. The compositional data obtained are in reasonable agreement with classical chromatographic analyses.

1. Introduction

Lipids are among the most important components of green coffee beans from a quantitative point of view. In the mature coffee endosperm, they are present in the form of oil bodies functioning as an energy reserve, mobilized during germination [1]. The two commercially relevant coffee species, *Coffea arabica* Linn. (Arabica) and *Coffea canephora* Pierre ex Froehner var. robusta (Robusta), contain between 7% and 17% of lipids (w/w dry matter), the average lipid content of green Arabica being significantly higher (15%) than that of green Robusta (10%) [2–4]. Other coffee species, such as *C. heterocalyx* and *C. salvatrix*, may contain lipids up to 20–30% [5].

In terms of chemical composition, the coffee lipid fraction, also known as coffee oil, is extremely complex; it is mainly constituted by triacylglycerols with a fatty acids profile similar to that of common edible vegetable oils. However, the relatively large unsaponifiable fraction (up to 18.5% w/w) [6,7] as well as the presence of caffeine, makes coffee oil a peculiar product if compared with common edible vegetable oils.

The unsaponifiable fraction is rich in diterpenes, contains sterols, tocopherols, phosphatides and waxes (tryptamine derivatives) and is very important, together with fatty acid composition, as a chemical marker for authentication and traceability purposes [4,8–11]. The diterpenes cafestol and kahweol are important constituents of the unsaponifiable fraction and their biological activity has been studied. Both desirable and undesirable physiological actions of these diterpenes have been reported. In addition to anticarcinogenic, antioxidant, anti-inflammatory properties and hepatoprotective effects, a hypercolesterolemic activity (particularly for cafestol) has been reported [12].

Thanks to its skin moisturizing properties, green coffee oil has been used in the cosmetics industry and it has been shown to offer a good potential in sunscreen applications [5]. Very recently, coffee oil from defective green coffee beans has been suggested as a possible raw material for biodiesel production [13].

In spite of the important role played by coffee lipids in bean biology, human physiology, and industrial applications, studies focused on comprehensive chemical characterizations of these coffee components on several green coffee oil samples are still rather scarce. One drawback in getting qualitative and quantitative data on coffee oil could be the high content of unsaponifiable matter, and thus, the necessity to resort to different experimental approaches based on classical analytical instrumentation (CG, HPLC), which can be relatively time-consuming, laborious and tedious. Also, methods that rely on lipid hydrolysis followed by derivatization only provide limited insight in actual lipid composition. High-resolution Nuclear Magnetic Resonance (NMR) spectroscopy has the considerable advantage that it can yield information simultaneously in a rapid and non-destructive way

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on small amounts of sample. This technique offers the possibility to quickly perform routine analysis with minimal (if any) manipulation of the sample, leading to more detailed insights into the actual intact lipid composition that cannot be obtained by classical methods. For this reason, NMR is used in combination or in place of such methods in lipidomics studies [14–18].

In the past decade, high-resolution NMR has been widely and successfully applied to the analysis of edible vegetable oils and particularly extra-virgin olive oil [19–29]. NMR has been used for several analytical purposes, such as the determination of fatty acid profile, the identification and verification of olive oils, the monitoring of stability and geographical origin, and the assessment of correlation with sensory attributes [27,30–38]. Furthermore, quantification of lipids and other species in oil has been proposed by ^{31}P NMR [39–41]. Even if some NMR data on coffee components are present in the literature [42–45], as far as we know, coffee oil has not yet been the subject of high-resolution NMR investigations. Wei et al. [46] recently studied green coffee bean extracts, but their study did not include a quantification of the oil components.

In the present work, we explore the potential of NMR in the analysis and possibly in the quantification of green coffee oil components. This study is the prerequisite for the development of specific analytical methods. Both ^{1}H and ^{13}C spectra were examined and assigned through two dimensional experiments, showing different analytical possibilities. ^{1}H NMR spectroscopy proved to be a useful tool for the quick and sensitive determination of diterpenes. We also exploited the intrinsically high resolution of the ^{13}C NMR spectrum to quantitatively characterize the coffee oil composition. Specifically, fatty acids, glycerides and other minor components were determined by ^{13}C NMR in several green coffee oils from different geographical origins and botanical species. Despite the difficulties in the use of ^{13}C NMR for quantitative purposes, careful choice of sequence parameters and spectral integration regions yielded an unexpectedly fair agreement with classical analytical methods (GC, HPLC).

2. Materials and methods

2.1. Green coffee beans and sample preparation

Four different samples of green *Coffea arabica* L. (Arabica) beans (geographical origin: Brazil, Kenya, Colombia, and Guatemala) and one sample of *Coffea canephora* Pierre ex Froehner *var. robusta* (Robusta) (geographical origin: India) from commercial lots were used. Identification of the samples along the text will refer to their geographical origin or to the different botanical species (e.g. Robusta vs. Arabica).

The green coffee beans were ground after contact with liquid N_2 by using a batch mill (Ika M20) to pass a 600 μm sieve (Giuliani). Coffee oil was isolated by means of Soxhlet extractions. In details, 10 g of ground coffee samples were extracted with 130 mL of *n*-pentane (Sigma Aldrich) for 4 h at the solvent boiling point, siphoning five times per hour. The extract was paper filtered and dried over anhydrous sodium sulfate. The solvent was removed by reduced pressure evaporation (Buchi, Rotavapor R114) and the residue was dried to constant weight to obtain the coffee oil.

Extra-virgin olive oil (used for comparison) was purchased at a local market.

2.2. NMR

All experiments were carried out at 300 K on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer equipped with a two channel BBI probe. Samples were prepared adding 60 μL of d_6-DMSO to 600 μL of pure oil. The residual DMSO signal was used for chemical shift referencing imposing its ^{1}H resonance at 2.5 ppm and its ^{13}C signal at 40 ppm. The assignment of glycerides was preliminarily attempted by comparing ^{13}C spectra of oils diluted in DMSO with that of each methylated fatty acid component in the same experimental conditions (e.g. oil vs. methyl oleate or methyl linoleate or methyl palmitate etc.). The procedure was not successful as the peaks (whose chemical shift is well known to change significantly by dilution in organic solvents) were not found in the same position even though the oil and the standards were diluted to similar concentrations. Unambiguous hydrogen and carbon resonance assignment was then achieved through a series of 2D spectra, namely 2D-NOESY, 2D-COSY, 2D-ROESY, 2D-^{1}H,^{1}H-TOCSY, 2D-$_{1}$H,$_{13}$C-HSQC, 2D-^{1}H,^{13}C-HMBC, and 2D-$_{1}$H,$_{1}$H-TOCSY-$_{1}$H,$_{13}$C-HSQC. These experiments were used also for the assignment of diterpenes, whose NMR data have been published in methanol and chloroform [43,45]. The assignment of lipids was confirmed by addition of few μL of each methylated fatty acid component to the oil samples, causing an increase in intensity of all related fatty chain peaks.

^{13}C NMR spectra were recorded with a relatively long relaxation delay (15 s) to ensure complete recovery of the magnetization. ^{1}H decoupling was used only during acquisition, to avoid differential NOE effects on the integrals of carbons with different mobilities and/or number of attached protons. Given the concentration of the samples, relatively few scans (128) were necessary to obtain a good signal to noise ratio.

For quantification, samples were prepared by adding 60 μL of d_6-DMSO to 500 μL of pure oil and 100 μL of methyl linoleate (Sigma-Aldrich) used as an internal standard. The measured molar concentrations were corrected for dilution and converted to g/kg taking into account the measured density (0.916 g/mL).

Triglyceride dynamics was monitored by ^{13}C T_1 relaxation, which provides an estimation of the motional correlation time τ_c of molecules. Carbon relaxation times were measured with the inversion recovery pulse sequence. ^{1}H decoupling was used only during acquisition, to avoid cross-correlation contributions to relaxation rates *R*.

In the case of carbons directly attached to protons, the longitudinal relaxation rate is mainly due to dipolar interaction (R^{dip}) given by [47]:

$$R_1^{dip} = \frac{n}{10}\frac{\gamma_H^2\gamma_C^2\hbar^2}{r^6}\left(\frac{\mu_0}{4\pi}\right)^2\left\{\frac{\tau_c}{(1+(\omega_H-\omega_C)^2\tau_c^2)}+\frac{3\tau_c}{1+\omega_C^2\tau_c^2}+\frac{6\tau_c}{(1+(\omega_H+\omega_C)^2\tau_c^2)}\right\} \quad (1)$$

where ω_H and ω_C are the ^{1}H and ^{13}C Larmor frequencies, respectively, τ_c is the correlation time modulating the dipolar interaction, μ_0 is the permeability of vacuum, γ_H and γ_C are the ^{1}H and ^{13}C gyromagnetic ratios, respectively, *r* is the carbon–proton distance and *n* is the number of attached protons.

For carbon atoms not directly attached to protons (such as carbonyl carbons), the main relaxation mechanism is Chemical Shift Anisotropy (CSA) [47]:

$$R_1^{CSA} = \frac{2}{5}\left(\frac{\omega_C\Delta\sigma}{\sqrt{3}}\right)^2\left\{\frac{\tau_c}{1+\omega_C^2\tau_c^2}\right\} \quad (2)$$

where $\Delta\sigma$ is the chemical shift tensor anisotropy in ppm (we used 148 ppm [48]) whose value is needed to extract the correlation time τ_c from Eq. (2).

The values of τ_c were extracted numerically by plotting Eqs. (1) and (2) as a function of the motional correlation time. Out of the two possible solutions, the one with a lower τ_c was chosen as physically more reasonable.

2.3. Analysis of total fatty acids by GC

10 mg of oil was dissolved in 1 mL of n-hexane (Sigma-Aldrich). Then, a 10% methanolic BF_3 solution (Sigma-Aldrich, 3 mL) was used as derivatizing reagent for 45 minutes in boiling water. Vials were added with 2 mL of n-hexane and washed with Milli-Q water (Millipore) at room temperature. The supernatant solvent was separated by centrifugation and heptadecanoic acid methyl ester (Sigma-Aldrich) was added as internal standard. Heptadecanoic acid is a minor constituent of the coffee lipid fraction, but in view of its very low content [3] it has already been used as an internal standard in similar studies [49].

The fatty acid methyl esters were injected in an Agilent Technologies 6890 N gas chromatograph equipped with a flame ionization detector (FID). The analytical conditions employed were as follows. Injector: Splitless at a temperature of 200 °C; initial temperature of 170 °C followed by a temperature increase at a rate of 3 °C/min up to 250 °C; FID temperature of 250 °C (air flow, 450 mL/min; hydrogen flow, 40 mL/min). An HP-WAX 60 m × 0.25 mm × 0.25 μm column was employed. Peak identification was carried out by comparison of the retention times with those for the respective fatty acid methyl ester standards.

2.4. Diterpenes analysis

Kahweol, cafestol and 16-O-methylcafestol were determined according to DIN method 10779 with the separation of the diterpenes by HPLC (DIN 10779, 1999) in an accredited lab according to ISO 17025 [50]. According to this method, the standard deviation of the measured values is within the range 9.8–11.6% [50]. High-performance liquid chromatography (HPLC) was carried out with Agilent instrumentation under the following experimental conditions: mobile phase: 60/40 acetonitrile/water; volume injected: 20 μL; flow rate: 1 mL/min; and wavelength: 220 nm.

The calibration curves were built with three single standard solutions, one for each diterpene. The content of kahweol, cafestol and 16-O-methylcafestol was expressed as g/kg of oil. All standards and solvents were purchased from Sigma-Aldrich.

3. Results and discussion

Most NMR studies reported in the literature on olive oil, one of the most investigated among edible oils, are performed on samples diluted in deuterated solvents with the aim of improving spectrum quality. To avoid sample manipulations and to increase the intensities of the signals from minor components, we decided to analyze the oil samples without dilution (only small amounts of deuterated DMSO and optionally methyl linoleate were added as lock and quantification reference, respectively). This resulted in a larger peak linewidth (Fig. 1), but reduced acquisition time of experimental data. For routine applications, this approach would avoid the need of substantial amounts of organic solvents and would provide NMR data in short times (less than 1 h). The high signal to noise ratio allowed us to use a long relaxation delay resulting in more reliable peak integrals and to avoid the use of relaxation agents for quantification [46].

3.1. Spectra analysis of green coffee oil as compared to olive oil

3.1.1. ^{1}H NMR spectra

The ^{1}H NMR spectrum of Arabica green coffee oil is shown in Fig. 1 (top) together with that of commercial extra-virgin olive oil shown for comparison. The two spectra are quite similar at a first glance; however, as is well known, linoleate is more abundant in coffee oil while oleate predominates in olive oil. This difference is evident when considering the intensities of the peaks due to bis-allylic and allylic methylenes (at 2.81 ppm and 2.09 ppm, respectively, and indicated by ∗ and ∗∗ in the figure). Differences in linolenic acid content are also visible in the methyl region, although not so apparent due to the contribution of saturated chains to the oleic acid peak. The spectrum of coffee oil in chloroform is also shown (Fig. 1, bottom). Although the peaks are clearly sharper, the information that can be obtained is similar.

A more detailed analysis reveals the presence of significant amounts of additional species in coffee oil, namely kahweol, cafestol, and caffeine. Panels A and B in Fig. 1 show how the peaks of these species are very well resolved and possibly useful for quantification, provided an internal standard, such as methyl linoleate, is added (see further in the text). A clear example of the analytical potential of ^{1}H NMR in green coffee oil is the discrimination between Arabica and Robusta in the region of diterpenes (Fig. 2). The figure clearly shows that the signals attributed to kahweol almost disappear in the Robusta sample, whereas a methyl signal at 3.13 ppm reveals the presence of 16-O-methyl-cafestol, a typical marker for coffee authenticity applications [51].

In principle, the addition of methyl linoleate as an internal reference should allow the quantification of these species. In practice, although the values that we found are within the ranges reported in the literature, the agreement with the DIN method [50] is limited. The ^{1}H signals of methyl linoleate are affected by partial overlap with other resonances and there might be problems of radiation damping because of the high concentration of the analytes. Nevertheless, a better choice of experimental conditions and of an appropriate standard may lead to the development of a suitable NMR method.

As far as caffeine is concerned, the average content determined in the examined samples is in good agreement with the caffeine concentration in green coffee oil reported in the literature (2.1–2.7 g/kg oil) [52,53]. It is well known that the amount of caffeine extracted with coffee oil is very small [54,55]. As a consequence, the values we obtained are affected by large errors because of the limited signal to noise ratio of the peaks analyzed. In spite of the inadequate level of confidence in the quantitative determination of caffeine, our results show that it is possible to ascertain the presence of this analyte in green coffee oil. This might be of interest in the cosmetic industry, where coffee oil is becoming of increasing importance. There is no standardized method to determine caffeine in coffee oil, and the advantage of NMR over other techniques is that no sample manipulations are necessary.

3.1.2. ^{13}C NMR spectra; fatty acids and glycerides

In the analysis of vegetable oils, ^{13}C NMR has been widely used because of its intrinsically higher resolution compared to ^{1}H NMR [14,19,22–24]. The ^{13}C spectrum of green coffee oil is shown in Fig. 3 together with that of olive oil; each region of the spectrum can be analyzed to provide valuable information.

a) Carbonyl region (Fig. 3, top left).
   In this region, the relative amounts of free and esterified fatty acids can be obtained. The carboxyl carbon appears at higher ppm values followed by the carbonyl carbons linked to the α (1,3) and β (2) position of the glyceryl moiety, respectively [19].
   The percentage of free fatty acid with respect to the esterified forms determines the acidity of the oil, giving an indication, in the case of green coffee oil, of its quality in terms of aging and storage conditions [56,57]. The free acidity (FFA %) reported

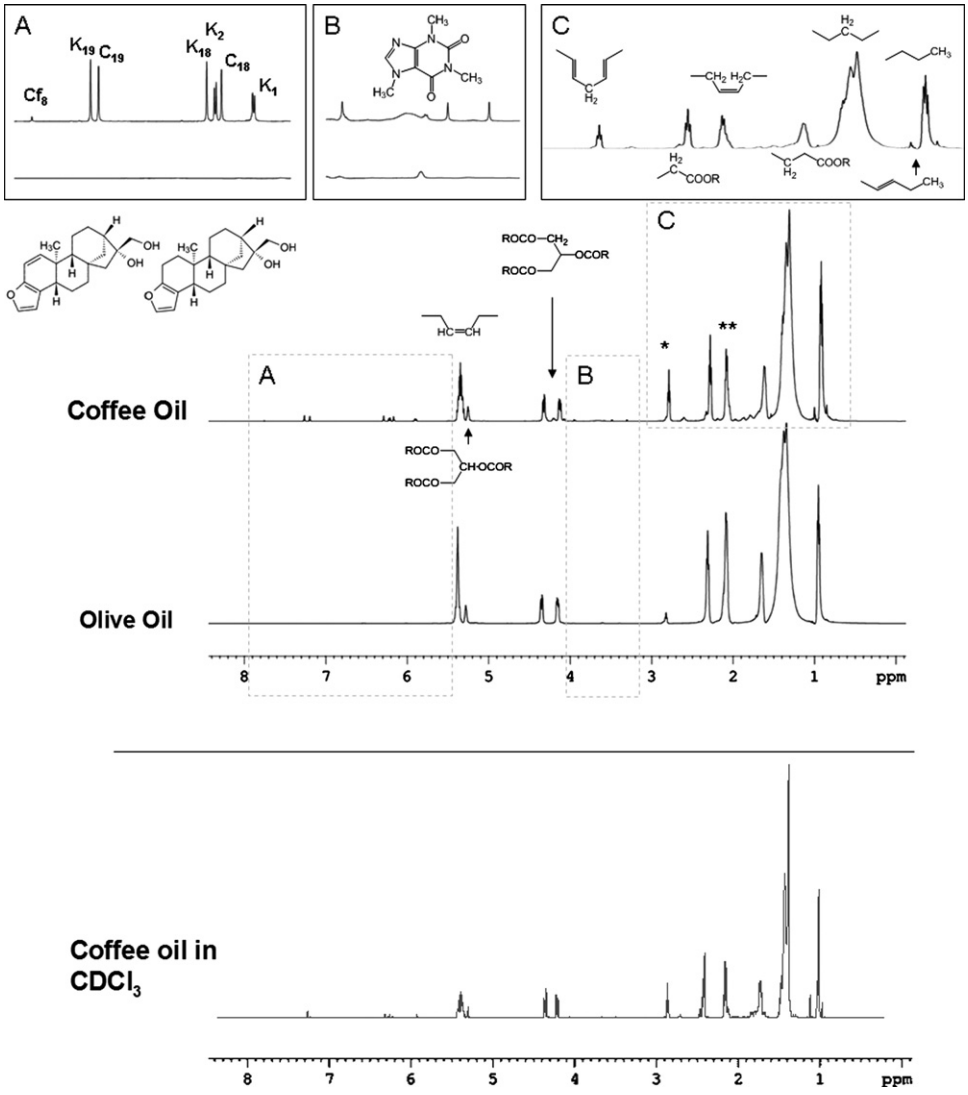

Fig. 1. (top) ^{1}H NMR spectra of green Arabica coffee oil from Brasil and commercial extra-virgin olive oil. Panels A, B and C are magnifications of diterpenes (K indicates kahweol, C indicates cafestol), caffeine (Cf) and fatty acids regions of the top spectrum. One and two asterisks indicate bis-allylic and allylic protons, respectively; **(bottom)** ^{1}H NMR spectrum of coffee oil dissolved in $CDCl_3$.

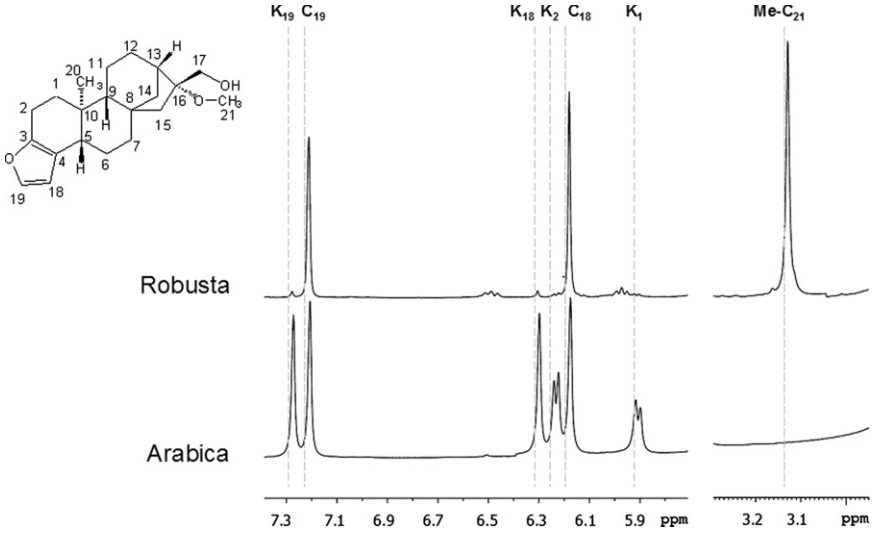

Fig. 2. Selected diterpene signals from the ^{1}H NMR spectra of green Robusta coffee oil from India (top) and Arabica from Guatemala (bottom). The near absence of kahweol and the presence of 16-O-methylcafestol is apparent in Robusta. K indicates kahweol and C indicates cafestol. The molecular structure of 16-O-methylcafestol is shown.

in Table 1, can be calculated as

$$free\ acidity = \frac{A}{A+B+C+D+E} \quad (3)$$

where capital letters refer to the integral labels in Fig. 3. The values obtained for our samples, ranging from 0.9% to 2.0% for Arabica, are in good agreement with the few data reported in the literature [6,7].

The use of pure oil samples did not allow the discrimination among the carbonyl carbons of different esterified fatty acids as in the case of chloroform solutions [19]. However, the α carbonyl of 1,3-diglycerides is visible (indicated as α′ in Fig. 3). With respect to an olive oil ^{13}C spectrum, one additional peak at 172.7 ppm is apparent in the spectrum of coffee oil, which was unequivocally assigned to the fatty acid carbonyl group esterified with kahweol or cafestol.

Table 1
Major lipid classes in green coffee oil as determined by ^{13}C NMR (w/w %). FFA=free fatty acids, TAG=triacylglycerols, DTE=diterpene esters, DAG=1,3-diacylglycerols.

Sample	FFA	TAG	DTE	DAG
Brasil	1.6	81.1	17.3	0.5
Kenya	1.4	84.3	14.5	0.4
Colombia	1.9	83.5	14.4	0.8
Guatemala	1.5	81.1	17.5	0.3
Robusta (India)	2.9	90.9	5.1	1.8

In principle, the same region of the spectrum allows the quantification of triglycerides (TAG), diglycerides (DAG) and diterpene esters mainly derived from cafestol and kahweol (DTE).

$$I_{TAG} = \frac{D}{2} = E$$
$$I_{DAG} = C$$
$$I_{DTE} = B \quad (4)$$

where *D*, *E*, *C*, and *B* are the integrals of the corresponding regions in Fig. 3.

However, given the low amounts of DAG and DTE, their integrals (*B* and *C* in Fig. 3) are affected by large errors, also caused by partial overlap with the signal of TAG (*D* in Fig. 3). Moreover, this region could contain carbonyl signals from sterols. For these reasons, the amounts of TAG, DAG and DTE reported in Table 1 are based on the glyceryl region (see further in the text).

b) Olefinic region (Fig. 3, top center)

This region can be conveniently used to quantify unsaturated fatty acids. The relative amounts of linolenic (Ln), linoleic (L) and oleic (O) acid can be determined. Because $O_{9,10}$ overlap with signals from both linoleic and linolenic acid, the quantities of oleic, linoleic and linolenic species can be determined as follows:

$$I_O = \frac{G-H+2F}{2} = \frac{R-2Q-F}{2}$$
$$I_L = \frac{H-3F}{2} = S-2F = Q$$
$$I_{Ln} = F \quad (5)$$

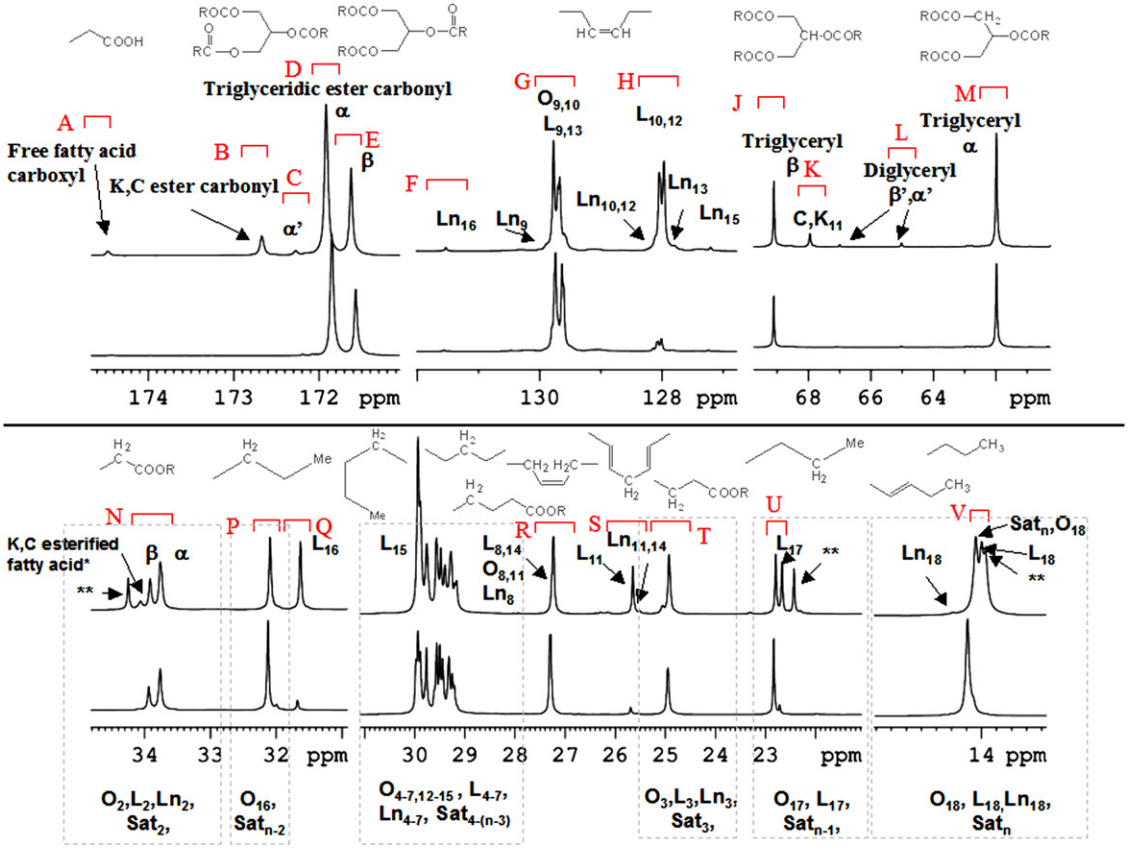

Fig. 3. ^{13}C NMR spectrum of green coffee oil (upper spectrum); the spectrum of olive oil is also reported for comparison (lower spectrum). Integrals used for quantification in the equations are displayed in red and labeled with capital letters. Functional groups resonating in various spectral regions are graphically displayed in light gray indicating with "C" only the carbon of interest. Saturated fatty acid resonances are indicated with "Sat" while those assigned to oleic, linoleic, linolenic, kahweol and cafestol are indicated by O, L, Ln, K and C, respectively. α and β positions in triglycerides and diglycerides (α′ and β′) are also indicated. The subscripts refer to the carbon position, with *n* referring to the last position in the fatty acid carbon chain. Peaks marked with ** arise from residual n-pentane, used for extraction. These peaks disappear with time due to evaporation of the solvent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

where G, H, F, R, S, and Q are the integrals of the regions indicated in Fig. 3. The use of peaks resonating in the same region of the spectrum and a long relaxation delay reduce errors from unequal irradiation and relaxation losses. The error on the absolute value of the integral for oleic and linoleic species is further reduced using the average of the values calculated in different ways in Eq. (5). The values obtained are reported in Table 4.

Some resonances arising from kahweol, cafestol (or 16-O-methylcafestol), usually present in coffee oil, fall in the olefinic region. Their quantification will be discussed further in the text.

c) Glyceryl region (Fig. 3, top right)

The region between 60 and 72 ppm allows the determination of the relative amounts of triglycerides, diglycerides (1,2 and 1,3), monoglycerides [19] and esterified kahweol and cafestol. Symmetrical esters (2-monoacyl, 1,3-diacyl, and triacyl) give rise to two signals at 1:2 intensity ratio, at variance with the asymmetrical esters, which give three separate signals of equal intensity. The amounts of TAG, DAG, and DTE can be quantified using the following integrals (see Fig. 3):

$$I_{TAG} = \frac{M}{2} = J$$
$$I_{DAG} = \frac{L}{2}$$
$$I_{DTE} = K \quad (6)$$

In our spectra, we found significant amounts of triglycerides, 1,3-diglycerides, and esterified kahweol and cafestol as reported in Table 1; monoglycerides and 1,2-diglycerids are not concentrated enough to be quantified.

d) Aliphatic region (Fig. 3, bottom)

This region contains aliphatic carbons of glycerides and fatty acids. Specifically, C2 carbons can be used to distinguish the type of ester bond (α and β position or esterification with kahweol or cafestol). When the sample is dissolved in $CDCl_3$ (and not analyzed "as is"), this region also provides information on the kind of fatty acid involved [19,25]. As for the carbons in $n-2$ position (with n=chain length), linoleic acid gives a peak at 31.6 ppm, well separated from all the others and labeled Q in Fig. 3.

Other peaks in this region can be used to quantify the total amount of saturated fatty acids. In fact, regardless of the chain length, carbons in positions 2, 3, $n-2$, $n-1$ and n resonate at characteristic chemical shifts. Unfortunately, the values of the integrals are affected by the presence of unsaturated fatty acids whose integral (estimated from the olefinic region as explained above) should be subtracted. Given the different mobilities, positions in the spectrum, and number of protons attached, the integral of the peaks quantifying the unsaturated species may not be rigorously comparable with that of the aliphatic carbons and this contributes to a larger error in the determination of saturated species. The values obtained from carbons in position 3 (R in Fig. 3) yielded the best results for quantification.

$$I_{Sat} = R - I_O - I_L - I_{Ln} \quad (7)$$

3.1.3. ^{13}C NMR spectra; diterpenes

Cafestol and kahweol contents in Arabica green beans are about 0.3–0.9% and 0.1–1.0% dry matter basis, respectively, showing great variability (particularly for kahweol), depending on variety and geographical origin [4,58,59]. Assuming an average oil content of green Arabica coffee beans close to 15% w/w [4], cafestol and kahweol contents are about 20–60 and 6.7–66.7 g/kg coffee oil, respectively. Robusta green beans contain less cafestol (0.1–0.7%), traces of kahweol ($<0.01\%$) and 16-O-methylcafestol in the range 0.08–0.25% [2]. Assuming an average oil content of green Robusta coffee beans close to 10% w/w [4], the three diterpenes account for 10–70 g (cafestol), less than 1 g (kahweol) and 8–25 g (16-O-methylcafestol) per kg of coffee oil. Robusta green beans contain trace amounts of 16-O-methylkahweol ($<0.003\%$) [2], which cannot be detected under the present NMR experimental conditions.

Besides being clearly visible in the ^1H spectrum, diterpenes can be quantified also by ^{13}C NMR. The full resonance assignment in pure coffee oil is reported in Fig. 4 (assignment of kahweol in CD_3OD has been reported elsewhere [45]). The peaks in Fig. 4 are well resolved for quantification; specifically, good results were obtained by using the average of the integrals relative to carbons in positions 3 and 4 (around 149 and 121 ppm, respectively).

These results are reported in Table 2 and compared with the DIN method [50]. In both cases, the diterpene concentrations are within the ranges reported in the literature. The agreement between the two sets of data is only partial. This discrepancy can be ascribed, at least in part, to the saponification and post-saponification steps of the DIN method, which are particularly critical from an experimental point of view. Moreover, although the DIN method has been already used to determine diterpenes in green coffee [2], the method was originally developed to analyze roasted coffee. Clearly, a more extensive investigation is required to define an NMR procedure that could flank or take the place of the DIN method, which is much more labor intensive and subject to low reproducibility and repeatability [50]. Nevertheless, our study underscores the interesting possibilities of the NMR approach to the simultaneous quantification of several components in such a complex mixture as green coffee oil.

3.2. Green coffee oil quantitative analysis by ^{13}C NMR

The use of ^{13}C spectra for quantification of species is hampered by many factors that influence the integrals of the peaks:

- The large spectral window does not allow a uniform irradiation of the signals even using short pulses ($\sim$10 μs). In cases where the 90° pulse is calibrated near the frequency of irradiation, peaks far away from the center of the spectrum (carbonyls and methyls) tend to display different intensities (generally less intense).
- The transverse relaxation rate T_2 of each atom within one molecule influences its peak line-width and consequently its measured integral. The relaxation times of different carbon atoms are affected by different factors (specifically, number of attached protons for atoms whose relaxation is dominated by the dipolar mechanism or chemical shift anisotropy for carbons not bound to protons, such as carbonyls). In addition, molecular motions modulating the relaxation rates are very different within triglyceride molecules. Table 3 shows the correlation times modulating the motion of each carbon, calculated from the longitudinal relaxation times reported in the same table (measured as described in the experimental part). The carbonyl carbons are the most restrained atoms in the motion (long correlation times), followed by the "glycerol" carbons. As we move along the chain, motion becomes faster (shorter correlation times) even though regions with unsaturations display lower mobility (probably because of steric hindrance introduced by double bonds).
- Different relaxation times affect the integral of the peaks not only in terms of T_2 line broadening, but also because of incomplete recovery of the magnetization during the delay between

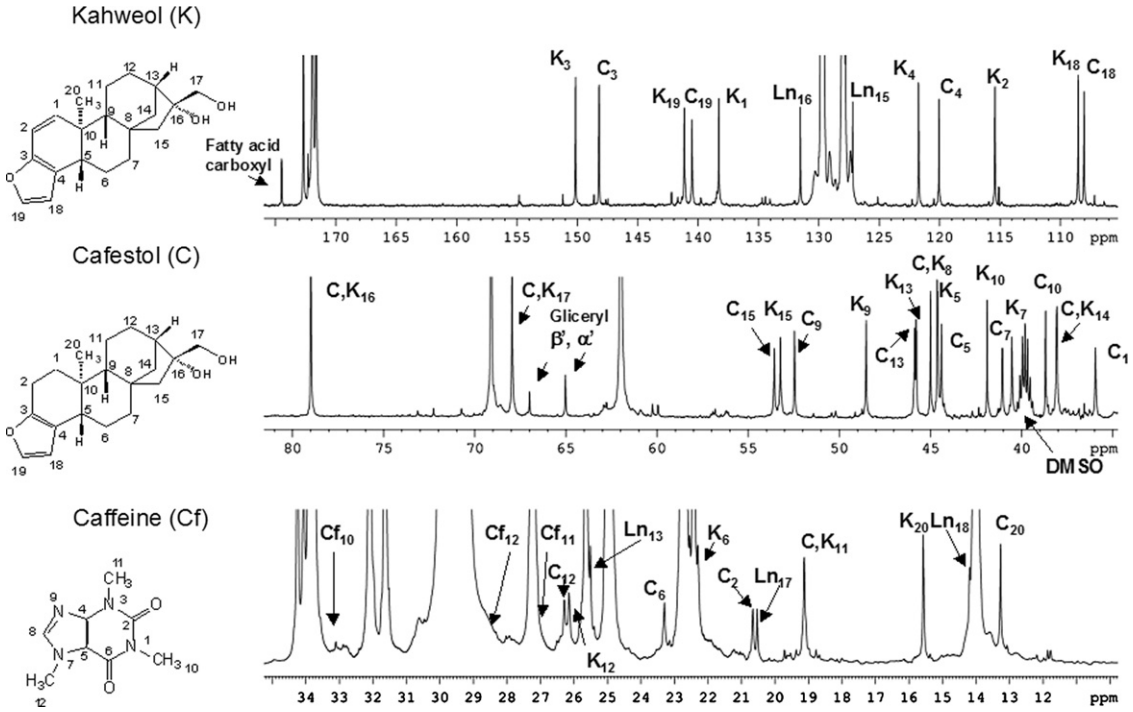

Fig. 4. ^{13}C NMR assignment of kahweol (K) and cafestol (C) in Arabica green coffee oil. Few signals from other components, such as linolenic acid (Ln) and caffeine (Cf), are also labeled.

Table 2
Minor components in green coffee oil as determined by ^{13}C NMR. Diterpenes as determined using the DIN method [50] are shown for comparison.

Sample	Kahweol		Cafestol		16-O-Methylcafestol	
	DIN (g/kg oil)	NMR (g/kg oil)	DIN (g/kg oil)	NMR (g/kg oil)	DIN (g/kg oil)	NMR (g/kg oil)
Brasile	26.9	29.5	35.3	28.9	0	0
Kenya	17.8	24.8	28.6	28.7	0	0
Colombia	10.0	25.6	17.7	28.4	0	0
Guatemala	36.3	28.8	57	32.9	0	0
Robusta (India)	0.4	0.5	8.8	14.6	6.0	9.1

consecutive scans (due to longitudinal relaxation time T_1). In the case of diluted samples displaying long relaxation times, it is generally advisable to use a short relaxation delay and achieve a steady state, in which the loss of magnetization is fairly well compensated by a larger number of scans. However, in such conditions, different relaxation times influence the integral of the signal in different ways. For this reason, relaxation agents (e.g., paramagnetic species) are sometimes added to the sample [46]. Alternatively, the delay between subsequent scans should be long enough to recover all the magnetization (as we actually did in the present work).

The NOE effect exerted by protons on carbons is another source of large errors in comparing signal integrals, as carbons with different numbers of attached hydrogens are expected to behave differently. Inverse gated decoupling allows one to minimize such effects [46,60].

To avoid all these sources of errors, peaks for quantification were chosen, where possible, in similar spectral regions (to minimize offset irradiation effects) and arising from similar types of carbons (to minimize differences introduced by T_2 relaxation).

Table 3
^{13}C longitudinal relaxation rates (s^{-1}) displayed by coffee oil glycerides and motional correlation times as derived from ^{13}C longitudinal relaxation rates (see Experimental section). Since smaller values indicate increased mobility, the motion tends to be faster moving from the glyceryl moiety toward the methyl fatty acid ends, with slower motion at the level of the insaturations.

Carbon	**^{13}C longitudinal relaxation rate (s^{-1})**	**Correlation time (ps)**
CO α	1.44	260.0
CO β	1.10	360.0
C=c	0.77	68.8
CH β	0.38	184.0
CH_2 α	0.22	142.0
C_2	0.35	76.0
C_{n-2}	1.00	25.2
C_{n-2}(L)	1.18	21.2
$(CH_2)_n$	0.58	44.4
C-c=c	0.60	42.8
c=c–C–c=c	0.58	44.0
C_3	0.41	63.2
C_{n-1}	1.35	18.4
C_{n-1}(L)	1.48	16.8
CH_3	2.74	6.4

Furthermore, we recorded our spectra keeping the relaxation delay long and decoupling the protons only during acquisition (to avoid T_1 and NOE effects).

To estimate the performance of the quantification, we added a small amount of methyl linoleate (100 μL in 500 μL of oil and 60 μL of d_6-DMSO), which should provide a good internal standard because of its similar chemical nature. Its methyl ester carbon falls in an isolated region at 50.9 ppm, and its integral I^{ref} can be taken as an internal standard for molar quantification of other species in the oil provided all integrals used for quantification were subtracted of I^{ref}, in case of overlap with any of the other methyl linoleate signals. The concentrations obtained should be also corrected for the dilution factors (due to the

Table 4
Percent fatty acid composition as determined by GC and NMR (w/w %) in green Arabica and Robusta (India) coffee oils. NMR data for commercial extra-virgin olive oil are also reported for comparison. Data in parenthesis were obtained using GC data for a more precise calculation of $\overline{MW}$ in Eq. (9), distinguishing the relative contributions from distinct saturated fatty acids.

Fatty acid	India		Brasil		Kenya		Guatemala		Colombia		Olive
	GC (w/w %)	NMR (w/w %)	GC (w/w %)	NMR (w/w %)	GC (w/w %)	NMR (w/w %)	GC (w/w %)	NMR (w/w %)	GC (w/w %)	NMR (w/w %)	NMR (w/w %)
C14	0.08	–	0.05	–	0.08	–	0.06	–	0.07	–	–
C16	28.29	–	27.60	–	31.20	–	30.94	–	31.98	–	–
C18	7.93	–	9.01	–	8.31	–	7.57	–	7.04	–	–
C20	4.45	–	4.05	–	3.15	–	3.16	–	2.83	–	–
C22	0.80	–	1.12	–	0.91	–	0.86	–	0.64	–	–
C18:1	11.85	10.1 (9.9)	9.21	9.3 (9.1)	7.74	8.2 (8.1)	7.66	8.1 (8.0)	7.31	7.1 (7.0)	62.4 (63.2)
C18:2	45.66	48.3 (47.4)	47.47	49.5 (48.5)	47.1	46.5 (45.7)	48.15	48.3 (47.5)	48.71	50.7(49.6)	17.7 (17.6)
C18:3	0.93	1.1 (1.1)	1.48	1.7 (1.7)	1.51	1.7 (1.7)	1.60	1.6 (1.5)	1.42	1.5 (1.4)	0.9 (0.9)
Tot Sat	40.4	41.5 (41.6)	39.4	41.7 (40.5)	43.4	43.5 (44.4)	39.8	42.5 (40.1)	40.6	42.5 (41.7)	17.8 (18.1)

addition of small amounts of the standard and the deuterated DMSO for lock) to obtain the concentration in the oil.

The percentage w/w composition can be obtained from the ^{13}C NMR spectrum even in the absence of an internal reference (Tables 1 and 4), provided a good estimate of the total integral due to lipids is given, that is the sum of the integrals of one type of carbon belonging to different lipid species (e.g., all carbonyls or all methyl carbons from unsaturated and saturated fatty acids and glycerides). Since this number is different depending on the nature of the chosen carbon type, we used the average of the values obtained from C_2 and C_3 carbons, which yielded the most reliable results (see further in the text). Neglecting the contributions of minor components (molecules other than glycerides, diterpene esters, and free fatty acids) the percent weight of the *ith* species is given by

$$i(w/w,\%) = \frac{I_i}{\bar{I}}\frac{MW_i}{\overline{MW}}100 \tag{8}$$

where $\overline{MW}$ is the integral-weighted average of molecular weight of the lipid species. $\overline{MW}$ is given by:

$$\overline{MW} = \sum_i \frac{I_i}{\bar{I}} MW_i \approx \frac{I_{FFA}}{\bar{I}}\overline{MW_{FFA}} + \frac{I_{TAG}}{\bar{I}}\overline{MW_{TAG}} + \frac{I_{DAG}}{\bar{I}}\overline{MW_{DAG}} + \frac{I_{DTE}}{\bar{I}}\overline{MW_{DTE}} \tag{9}$$

and

$$\begin{aligned}
\overline{MW}_{FFA} &\approx \frac{1}{\bar{I}}(I_O MW_O + I_L MW_L + I_{Ln} MW_{Ln} + I_P MW_P)\\
\overline{MW}_{TAG} &\approx 3\overline{MW}_{FFA} - 3\times 18.0 + 92.1\\
\overline{MW}_{DAG} &\approx 2\overline{MW}_{FFA} - 2\times 18.0 + 92.1\\
\overline{MW}_{DTE} &\approx \frac{I_K}{I_C+I_K}\left[\overline{MW}_{FFA} - 18.0 + 314.4\right]\\
&\quad + \frac{I_C}{I_C+I_K}\left[\overline{MW}_{FFA} - 18.0 + 316.4\right]
\end{aligned} \tag{10}$$

where

$$I_{FFA} = A$$
$$I_{TAG} = \frac{(J + M/2)}{2}$$
$$I_{DAG} = \frac{L}{2}$$
$$I_{DTE} = K$$

In the equations, the subscripts O (Oleic), L (linoleic), Ln (Linolenic), P (Palmitic), C (cafestol), K (Kahweol), FFA (Free Fatty Acids), TAG (Triglycerides), DAG (Diglicerides), and DTE (Diterpenes) indicate the type of molecule while the letters *A*, *J*, *M*, *L*, and *K* refer to the integrals shown in Fig. 3. The value of $\overline{MW}$ cannot be determined precisely, as NMR is not able to distinguish among different saturated fatty acids. However, multiplying the total integral of saturated fatty acids for the molecular weight of palmitic acid as an average introduces negligible errors in the calculation of $\overline{MW}$ at least when dealing with coffee and olive oils. This is demonstrated in Table 4, which reports the quantification of the species using either the values of $\overline{MW}$ calculated in this way or the exact weighted average based on the saturated fatty acid composition obtained by GC.

The use of Eq. 8 for quantification requires also a reliable value of $\bar{I}$. In principle, the value can be obtained from the integrals of different types of carbons, calculated in the following regions: carbonyl (from 171.5 ppm to 174.5 ppm, $A+B+C+D+E$ in Fig. 3), C2 (from 33.4 ppm to 34.3 ppm, *N* in Fig. 3), C3 (from 24.5 ppm to 25.2 ppm, *T* in Fig. 3), C_{n-2} (from 31.3 to 32.3 ppm, $P+Q+F$ in Fig. 3), and methyl (from 13.7 ppm to 14.4 ppm, *V* in Fig. 3). However, due to uneven irradiation and relaxation properties, the carbonyl and methyl regions give significantly different values from the others; in addition, the C_{n-2} region contains contributions from unsaturated fatty acids. For these reasons, the results obtained using the average of the integrals in the C2 and C3 regions are the most reliable:

$$\bar{I} = \frac{N+T}{2} \tag{11}$$

Tables 2 and 4 report the quantification of species using Eqs. (8)–(11). The procedure did not make use of an internal standard for quantification. The results are in remarkable agreement with the time-consuming standard procedure by means of gas chromatography.

Total fatty acid composition of oil from green coffee beans has been the subject of several studies and reviews [3]. The GC profile reported in Table 4 is characterized by the presence of palmitic acid (27.60–31.98%) and linoleic acid (45.66–48.71%) as dominating components, stearic (7.04–7.93%) and oleic acids (7.31–11.85%) in lower and almost equal amounts, arachidic acid (2.83–4.45%) and linolenic acid (0.93–1.60%) in even lower amounts, and quantities of myristic and behenic acids close to or less than 1% each. Our data are in excellent agreement with literature data [3]. The quantities of unsaturated fatty acids determined by NMR are in very good agreement with those determined by GC: the average difference is about 6%, with the largest discrepancy found for Robusta coffee beans. However, our NMR approach does not permit the quantitative determination of each saturated fatty acid present in the sample. Only the total saturated fatty acid amount can be compared with data obtained by the classical GC method (Table 4). In the case of olive oil, palmitic acid is the dominant saturated fatty acid [61] and the remaining saturated fatty acids amount to about 2–3% [62–64].

Unfortunately, the saturated fatty acid profile of green coffee oil is more complex and in this regard the NMR approach cannot be considered as informative as GC methods.

The estimation of global tri-, di-glycerides and esterified diterpenes (mono-glycerides were not visible in the spectrum) reported in Table 1 was obtained comparing integrals in the glyceryl region as follows:

$$TAG,DAG,DTE(w/w,\%) = \frac{I_{TAG,DAG,DTE}\overline{MW}_{TAG,DAG,DTE}}{I_{TAG}\overline{MW}_{TAG}+I_{DAG}\overline{MW}_{DAG}+I_{DTE}\overline{MW}_{DTE}+I_{FFA}\overline{MW}_{FFA}}100 \quad (12)$$

So far, very scarce data on lipid class composition of green coffee oil have been reported in the literature. Kaufman and Hamsagar [65] found that the most abundant class in oil samples was the triacylglycerols (TAG) accounting for 75.2%, but the origin of green coffee was not clearly specified. Diterpene esters, DTE, were the second most abundant class (18.5%), followed by sterol esters (1.4%); unidentified components were 4.2% of the total. Nikolova-Damyanova et al. [6] reported on the lipid class composition of Arabica green coffee oils as determined by analytical silica gel TLC. The experimental results were in excellent agreement with those previously reported as far as triacylglycerols ($75.5 \pm 1.7\%$ w/w), diterpene esters ($15.1 \pm 1.2\%$ w/w) and sterol esters ($1.2 \pm 0.1\%$ w/w) are concerned, and new lipid classes were quantified (free sterols: $1.5 \pm 0.2\%$ w/w; free fatty acids: $1.2 \pm 0.2\%$ w/w; partial acylglycerols: $5.5 \pm 0.3\%$ w/w and polar lipids: $0.6 \pm 0.2\%$ w/w) thus reducing the unidentified components to $1.0 \pm 0.1\%$ w/w. The relatively high quantity of partial acylglycerols was interpreted by the authors as the result of both formation during storage and natural occurrence in green coffee beans [6]. The same research group [7] showed that the lipid class composition may vary according to coffee ripening and to post-harvest coffee treatments (sorting and drying). Specifically, TAG and DTE can be in the range 72.1–75.2 and 13.1–14.8% w/w, respectively. The high percentage of partial acylglycerols was confirmed, with DAG ranging from 2.7% to 5.4%. In all cases, the samples were obtained from dry-processed green coffee beans.

In Table 1, the lipid class composition determined by ^{13}C NMR is reported for the first time, as far as we know. The percentage of TAG is slightly higher and that of DAG is lower than those previously reported. The results on the other lipid classes are in good agreement with previous data. Because of the relative molecular complexity and the low amount, sterol esters and polar lipids cannot be quantified by NMR under the present chosen experimental conditions. As shown in Table 1, the Robusta sample is characterized by a higher content of free fatty acids and a lower content of DTE than Arabica samples, in agreement with the literature [3].

An important difference from earlier reports is that all the samples studied in the present paper were obtained from wet-processed green coffee. It is possible that the two different post-harvest processes may affect differently the relative proportions between TAG and partial acylglycerols. In fact, it is known that the two main coffee processing methods have a measurably different effect on the sugars and other flavor precursors present [66–68] and that the dry-process induces an active seed stress metabolism if compared with the wet-process [61,66]. It could be of remarkable interest to parallel TLC and NMR determinations on the same set of green coffee oil samples to evaluate the effect of the main post-harvest coffee processes on lipid composition.

The use of NMR as a possible technique to obtain fast quantification of a large number of species in green bean coffee oil was presented in this work. The potential for the development of methods that could replace several time-consuming analyses in a routine setting was demonstrated. From here, several avenues can be envisioned. Among others, the NMR spectrum of coffee oil could be the basis for a metabolomic analysis that could be used to determine geographic origin. Alternatively, a quantitative analytical determination of 16-O-methylcafestol could be conceived to detect the presence of Robusta in a commercial blend. A third possibility is the fast detection of caffeine in green coffee oil. Although the solubility of caffeine in coffee oil is not high, leading to large errors, its presence can be easily detected using ^1H NMR. The official method for caffeine determination (ISO 20481/2008) would be much more time consuming, since an extraction in aqueous solution is necessary with the obvious problems of complete recovery and consequently of reduced reliability. A fast and reliable method could be applied in the cosmetic industry where green coffee oil is used.

To develop any applicable analytical method, a substantial amount of work is needed, and a suitable strategy has to be devised to adjust the procedure we used to the desired end. For example, the field at which the spectra are collected should be chosen carefully. At 600 MHz, we obtained well separated signals with good signal to noise ratio. This suggests that good results could be achieved even at lower fields, much more appropriate for routine applications. Also, a much higher number of samples need to be analyzed to validate any protocol. The purpose of the work presented here was to break a ground still untouched in the literature and to determine whether NMR can indeed be used in this field.

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