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Benchtop (60 MHz) proton NMR spectroscopy for quantification of 16-O-methylcafestol in lipophilic extracts of ground roast coffee ☆

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ABSTRACT

We present a method for analysing the lipophilic fraction extracted from ground coffee beans using 60 MHz proton (^{1}H) NMR spectroscopy. In addition to the triglycerides from coffee oil, spectral features are seen from a range of secondary metabolites, such as various diterpenes. We demonstrate quantitation of a peak attributed to one such compound, 16-O-methylcafestol (16-OMC), which is of interest as a coffee species marker. It is present in low concentrations (<<50 mg/kg) in *Coffea arabica* L. ('Arabica') beans, but in orders of magnitude greater concentrations in other coffees, in particular the other commercially grown species *C. canephora* Pierre ex A. Froehner (commonly known as 'robusta'). A series of coffee extracts spiked with 16-OMC analytical standard are used to establish a calibration, and to estimate 16-OMC concentrations in a range of different coffees (Arabicas and blends with robustas). To validate the method, values obtained are compared with an analogous quantitation method that uses high field (600 MHz) NMR spectroscopy.

- Quantitation of 16-O-methylcafestol in ground roast coffee extracts using benchtop (60 MHz) NMR spectroscopy
- Validated by comparison with quantitative high field (600 Mz) NMR spectroscopy
- Detection limit is sufficient for discovering adulteration of Arabica coffee with non-Arabica species

Specifications Table

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More specific subject area:	Food chemistry
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Method details

Background

NMR spectroscopy is widely used in the analysis of biological materials, for untargeted metabolomics and measuring individual metabolites. To achieve the strong magnetic fields required, high-resolution NMR spectrometers are based upon cryogen-cooled superconducting electromagnets. Due to cost and technical complexity, such facilities are mostly found in research laboratories and large companies. However, the emergence of ‘compact’ or ‘benchtop’ instruments over the past decade is bringing NMR spectroscopy to a much wider range of settings. Equipped with a permanent magnet and operating at lower field strengths (<100 MHz), these are versatile analytical tools that are gaining traction for applications such as quality assurance and integrity.

Some of the earliest benchtop NMR studies of natural products were of plant-derived oils. These are comprised largely of mixed triglyceride esters, a compound class amenable to analysis at low field strengths. Oils require little or no preparation before spectral acquisition, and by using a suitable extraction procedure, a variety of other sample matrices can also be examined such as seeds, spices and dried herbs. Of the standard NMR solvents, chloroform is the most effective for accessing the triglycerides along with other lipophilic compounds.

We present a method for analysing the lipophilic fraction extracted from ground coffee beans using 60 MHz proton (^{1}H) NMR spectroscopy. In addition to the triglycerides from coffee oil, spectral features are seen from a range of secondary metabolites, such as diterpenes. We demonstrate quantitation of a peak attributed to one such compound, 16-O-methylcafestol (16-OMC), which is of interest as a marker for authenticating coffee species. Cases of coffee fraud usually involve the substitution of *Coffea arabica* L. (‘Arabica’) with the other commercially important species, *C. canephora* Pierre ex A. Froehner (commonly known as ‘robusta’); the former typically trades at twice the price of the latter. 16-OMC is present in low concentrations (<<50 mg/kg) in Arabica beans but orders of magnitude more in robusta, in which its concentration can range between 1000-2000 mg/kg [1]. Detecting 16-OMC at above a certain level is therefore strongly suggestive of the presence of robusta.

In this work, a series of coffee extracts spiked with 16-OMC analytical standard are used to establish a calibration, and to estimate 16-OMC concentrations in a range of different coffees (Arabicas and blends with robustas). The calibration range covers amounts of 16-OMC commensurate with robusta contents of 0 ~ 10%w/w, as the original focus of the work was detection of low-level adulteration of Arabica coffees. The technical error associated with the measurement is estimated from duplicate analyses. To validate the method, values obtained are compared with an analogous quantitation method carried out using high field (600 MHz) NMR.

Materials and equipment

Solvents and standards

- Chloroform (Fisher Scientific UK Limited)
- Deuterated chloroform (chloroform-d), 99.8 atom % D (Merck Life Sciences UK Limited)
- 16-O-methylcafestol (Merck Life Sciences UK Limited)
- NMR sealed standard sample of TMS (tetramethylsilane) in chloroform-d (Oxford Instruments, UK)

Consumables, glassware and general laboratory equipment

- Filter paper (Whatman No. 1)
- Cotton wool
- Glassware: Sovirel tubes (or Pyrex glass SVL culture tubes with screwcap and PTFE-lined rubber disc inserts to ensure compatibility with chloroform), Pyrex glass beakers, glass measuring cylinder (100 ml)
- Pipettes (glass, displacement)
- 5mm NMR tubes (Merck Life Sciences UK Limited)
- Magnetic stirrer
- Centrifugal (or vortex) evaporator
- Coffee grinder (for samples of whole coffee beans)
- Analytical microbalance, resolution 0.1 mg (Sartorius AG, Germany)

NMR spectrometer

- Benchtop 60 MHz ‘Pulsar’ NMR spectrometer (Oxford Instruments, Tubney Woods, Abingdon, Oxford, UK) installed with SpinFlow instrument driver and data acquisition software (v1, Oxford Instruments).

Software

- Mnova (Mestrelab Research, Santiago de Compostela, Spain)
- Matlab (The Mathworks, Cambridge, UK)

Method steps

Extraction of lipophilic fraction from ground roasted coffee

- Ground roast coffee is required for the preparation procedure.
  - Whole coffee beans should be ground in a coffee grinder using a medium setting. This requires a minimum of around 100 beans with a mass of 12 – 15 g.
  - Commercially ground roast coffee can be used directly, irrespective of grind (French press, filter, espresso, etc.).
  - The particle sizes of ground roast coffee are typically in the range 0.3 – 1 mm, assessed by gradation tests.
- Using an analytical microbalance with 0.1 mg resolution, 10g of the ground roast coffee is weighed into a glass beaker using a spatula. Ideally, this is a sub-sample taken from a larger quantity of ground coffee (e.g. 100 g) which should be shaken to maximize homogeneity and give a representative sample.
- 30ml of chloroform is measured into a glass measuring cylinder and poured into the beaker containing the coffee. The mixture is stirred with a magnetic stirrer (600 rpm) at room temperature for 5 minutes.
- The extract is filtered through filter paper into another clean glass beaker and the filtrate transferred to Sovirel tubes.
  - The expected volume of extract recovered at this stage is 8 ml +/- 0.5 ml.
- The filtrate is dried in a centrifugal evaporator for approximately 3 hours.
- The dried extract is reconstituted with 0.8 ml of chloroform-d (2×0.4ml using a displacement pipette).
- Using a glass pipette, the reconstituted extract is filtered through a small amount of cotton wool into an NMR tube.

NMR acquisition and processing

- ^{1}H NMR measurements are performed using a (nominal) 60 MHz benchtop NMR spectrometer (precise operating frequency = 59.7 MHz).
- Before analysis of any samples, a linewidth measurement is made on an NMR sealed standard sample of TMS (tetramethylsilane) in chloroform-d, to check that the chloroform FWHM (full width half maximum) does not exceed a benchmark value for the instrument (e.g. 0.6 Hz). Shimming is undertaken as necessary until the FWHM value is within specification.
  - This check is performed at least daily, and as required should a decrease in spectral resolution occur. This helps achieve consistent peak widths, which is important for reliable peak integration and accurate quantitation.
- For each coffee extract, 256 free induction decays (FIDs) are collected using a filter width of 5000 Hz, acquisition time of 6.55 s and a recycle delay of 2 s.
- The 90° pulse length is 13.28 μs as determined by the machine's internal calibration cycle.
- The temperature of the spectrometer magnet is 37 °C, which gives a temperature inside the probe of ~ 35 °C during acquisition.
  - These parameters result in a total acquisition time of approximately 35mins per extract. The relatively large number of co-added scans is required to give sufficient signal-to-noise in the spectrum for quantifying the minor peak from 16-OMC at the required limit of detection. The relatively short recycle delay gives an acceptable total acquisition time as well as accurate quantitation of the peak of interest. For analysis of other chemical species, a longer delay may be required to ensure relaxation equilibrium is achieved between scans.
- Processing of the FIDs is carried out using SpinFlow and MNova (Mestrelab Research, Santiago de Compostela, Spain) software packages, and post-processing, visualization and statistical analysis using Matlab (The Mathworks, Cambridge, UK).
  - FIDs were zero-filled, Fourier-transformed and co-added to obtain a single frequency-domain spectrum of 65,536 data points at a spacing of 0.001277 ppm on the chemical shift scale from each coffee extract.
  - Phase correction was conducted using an automated template.
  - All spectra were smoothed using a second order Savitsky-Golay filter with a window width of 11 data points.

Referencing the chemical shift scale

- Mixed triglycerides are the main component of the lipophilic fraction extracted from coffee, and present in all samples are prominent resonances from the glycerol backbone (-CH_2OCOR, see Fig. 1). The chemical shift scale can be referenced by setting the central maximum of this group of peaks to 4.28 ppm as indicated. This is preferable to using the residual d-chloroform peak at 7.26 ppm, as at 60 MHz field strength it is overlapped in some samples by resonances from minor constituents of coffee. Further, by referencing to a consistent spectral feature from the sample itself, some of the movement in peak positions due to variable extract efficiency is mitigated.

Quantifying 16-O-methylcafestol

- A notable singlet in the robusta coffee spectrum occurs at 3.16 ppm. This arises from the diterpene 16-O-methylcafestol (16-OMC), which is recognised as a 'marker' compound for robusta and other non-Arabica coffee species [4,5]. The integral of this peak has been reported for quantification of 16-OMC in coffees by high field NMR spectroscopy [2].
  - At 60 MHz field strength, although peaks are generally broader and more overlapped, the 3.16 ppm resonance remains sufficiently isolated from its neighbours to be amenable to integration.

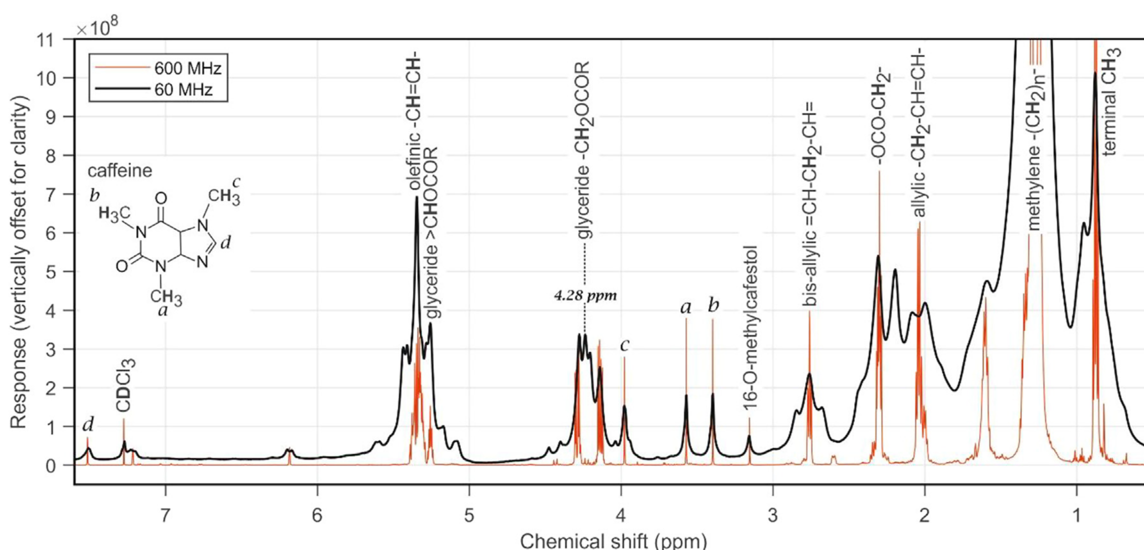

Fig. 1. The ^{1}H 60 MHz spectrum of a sample of ground roast robusta coffee, compared with the 600 MHz spectrum of the same extract. Both spectra are dominated by features from triglycerides. In the benchtop spectrum there is considerable band overlap, much more so than at the high field strength. However, some of the spectral features can be attributed to specific functional groups, as indicated. Although peaks are wider at the lower field strength, note that the central peak positions of multiplets as well as singlets are field-independent, so when making assignments it is helpful to draw on relevant high-field studies in the literature for example Schievano, Finotello, De Angelis, Mammi, and Navarini [2] and Monakhova et al. [3].

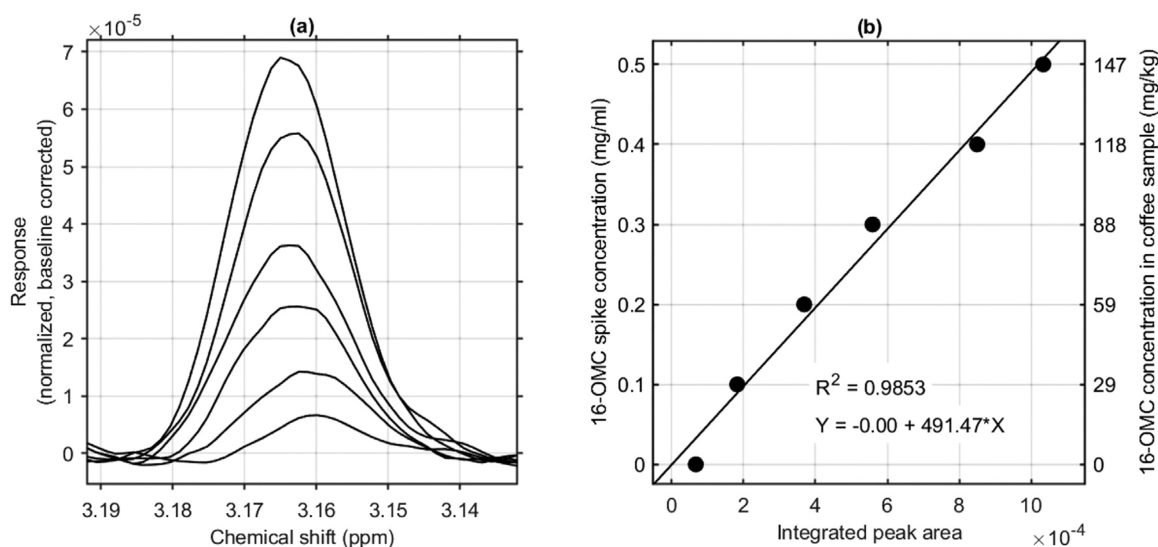

Fig. 2. (a) The 3.16 ppm peak in a series of extracts from an Arabica coffee spiked with increasing amounts of 16-OMC. The data are shown after normalization to the area of the glyceride region (4.02 - 4.58 ppm) and local baseline correction of the region of interest. Peak integrals are calculated using region limits as shown (3.13 – 3.19 ppm) and the values obtained are plotted in (b) versus the 16-OMC spike concentration.

  - A calibration can be established using serially spiked samples to link peak integral values to the concentration of 16-OMC present in the sample.
- Dried extracts of an Arabica coffee are prepared using the method described above, and serially spiked with different concentrations of 16-OMC (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml in chloroform-d).
- Fig. 2(a) shows the region around the 3.16 ppm peak in the 60 MHz spectra of this series.
  - The spectra have been normalized by ratioing to the integral of the glyceride resonances between 4.02 and 4.58 ppm. This is a useful method of scaling data to mitigate variations in overall extract concentration due to solvent evaporation [6].

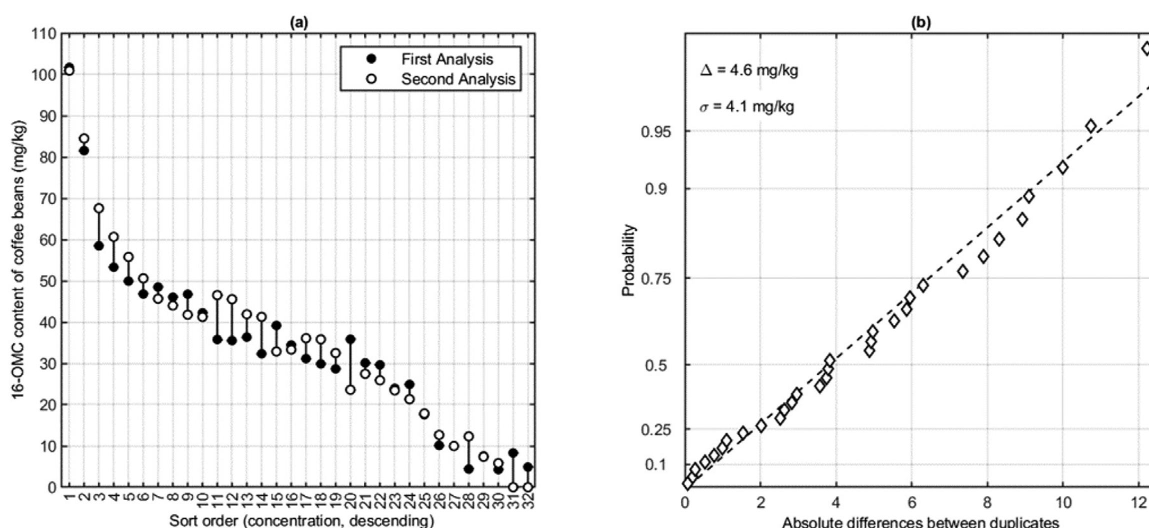

Fig. 3. (a) 16-OMC concentration predictions by low-field NMR for duplicate analyses of 32 coffees, from a range of either pure Arabica coffees or blends with <10% Robusta. (b) Probability plot between the half-normal distribution and the absolute differences of the outcomes of the pairwise duplicate analyses.

  - The region of interest (3.13 – 3.19 ppm) is shown after Whitaker baseline correction [7], which is provided in MNova as well as other NMR and signal processing software.
- The region of interest is integrated between the limits as shown. Linear regression of the spike concentration values onto the integrals yields the calibration line shown in Fig. 2(b).
  - The spectrum of the 0 mg/ml sample also has a small peak at 3.16 ppm and a non-zero peak integral, consistent with the presence of 16-OMC at a low level. It is known that Arabica coffee beans can contain small but non-negligible quantities of this compound [8–12].
  - The 16-OMC analytical standard contains the freeform compound, whereas the native form in coffee is mostly esterified. High-field studies show a small difference (<0.01 ppm) in the nominal 3.16 ppm peak associated with each form [10,2]. However, at benchtop field strengths the peaks are much broader and this difference cannot be resolved, making it of no consequence for the quantitative analysis.
  - In high field NMR spectra, a singlet attributed to another diterpene present in some coffees, 16-O-methylkahweol (16-OMK), can also occur in this region, at almost the same position as the 3.16 ppm 16-OMC peak. However, concentrations of 16-OMK are typically two orders of magnitude lower than 16-OMC [13]. The lower sensitivity at benchtop field strengths means this is undetectable and does not affect the quantitation of 16-OMC.
- The gradient of regression line in Fig. 2(b) is used to estimate the total concentration of 16-OMC in an NMR tube:

$$\text{16OMC } concentration \left(\frac{mg}{ml}\right) = gradient \ \times peak\ integral$$

- This can be expressed as mg/kg in the original coffee using the measured extraction efficiency (8 ml recovered out of 30 ml = 8/30) and the volume of sample in the NMR tube (0.8 ml) as follows:

$$\frac{NMR\ tube\ volume\ (ml)\ \times\ \text{16OMC}\ concentration\ (mg/ml)}{extraction\ efficiency\ \times mass\ of\ coffee\ used\ (kg)}$$

- The corresponding mg/kg values are marked on the right-hand axis in Fig. 2(b).
  - Note that a value of <50 mg/kg has become accepted within the coffee sector as an upper limit for authentic Arabicas. This is well above the limit of detection achievable using benchtop NMR.

Method validation

Estimation error in the 16-OMC content

- The measurement precision of the method can be estimated by repeating the complete analysis procedure (sampling, extraction, spectral acquisition) on multiple coffee samples.
- Fig. 3(a) shows the outcomes for 32 different coffee samples analysed in this way. The samples comprise a variety of different Arabicas and blends with low (< 10 %w/w) amounts of robusta.

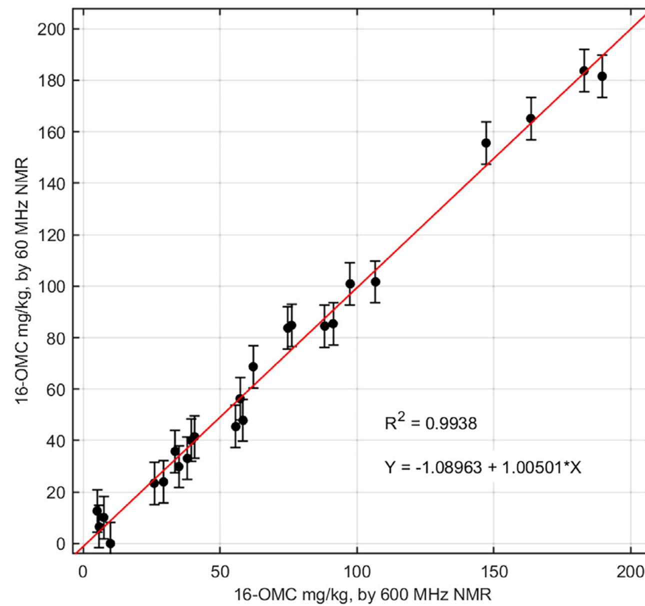

Fig. 4. Predictions of 16-OMC concentrations by low-field NMR versus those from the reference method (high-field NMR) for a collection of 25 coffees (pure Arabicas and blends with robusta). The expected error with low-field NMR is indicated with error bars. The regression line for the low upon high field outcomes, its equation and R^2 value are also shown.

- The calculated 16-OMC contents are all within the calibration range of the spike series. The data in Fig. 3(a) are presented in descending order of pairwise mean calculated 16-OMC concentration. There is no association between the absolute differences between duplicates and the mean concentration, nor with the analyses' chronological order.
- The absolute differences between duplicates are half-normally distributed with a mean value of 4.6 mg/kg (see the probability plot in Fig. 3(b)). From this it is calculated that the standard deviation associated with individual measurements is approximately 4.1 mg/kg.
- This benchmarking exercise was conducted at regular intervals over the period 2017 – 2021. Sample preparation was carried out by multiple researchers.
- Each pair of analysis duplicates took place at intervals of typically a few days, and never more than one month.

- The sources of variation that contribute to imprecision include:
  - Biological and sampling variability.
  - Sample preparation variability (weighing, pipetting, extraction efficiency).
  - Spectral noise, which affects the baseline correction and the precision of the peak integral.
  - Spectrometer shimming, which may drift during acquisition and compromise the spectral linewidth.

Comparison with outcomes from a high field NMR quantitation

- The accuracy of the present method was estimated through comparing predictions to those obtained by using a well-established method for 16-OMC quantification, high-field (600 MHz) NMR spectroscopy [2]. A calibration, analogous to that above, was constructed from a spiked extract series analysed using 600 MHz NMR spectroscopy. This is reported in detail in Gunning et al. [10].
- A collection of 25 different coffees (Arabicas and blends with robustas) were analysed by the calibrations from both field strengths. The 16-OMC concentrations calculated by each technique are plotted against one another in Fig. 4.
  - The accuracy of the low-field predictions is demonstrated by the excellent correspondence between the two methods through the whole of the concentration range spanned by these coffee samples.
  - Error bars are marked for the 60 MHz values to reflect the estimated measurement precision (+/- 2 standard deviations). Predictions for three of the coffees somewhat exceed the range spanned by samples used in the calibration, but nevertheless show good agreement between the two techniques.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Y. Gunning: Investigation, Methodology, Writing – review & editing. **M. Defernez:** Methodology, Data curation, Writing – review & editing. **E.K. Kemsley:** Supervision, Data curation, Software, Writing – original draft.

Data availability

The data that has been used is confidential.

Ethics statements

N/A to this work.

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Supplementary material *and/or* additional information [OPTIONAL]

N/A

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