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Position-Specific Isotope Analysis of Xanthines: A ^{13}C Nuclear Magnetic Resonance Method to Determine the ^{13}C Intramolecular Composition at Natural Abundance

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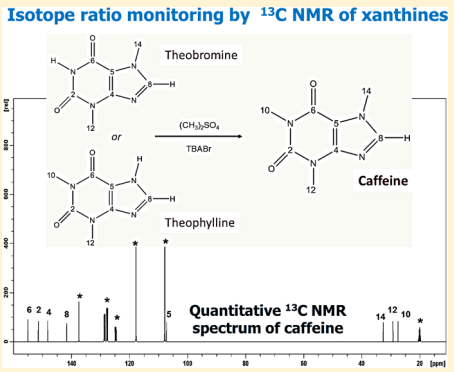
ABSTRACT: The natural xanthines caffeine, theobromine, and theophylline are of major commercial importance as flavor constituents in coffee, cocoa, tea, and a number of other beverages. However, their exploitation for authenticity, a requirement in these commodities that have a large origin-based price-range, by the standard method of isotope ratio monitoring by mass spectrometry (irm-MS) is limited. We have now developed a methodology that overcomes this deficit that exploits the power of isotopic quantitative ^{13}C nuclear magnetic resonance (NMR) spectrometry combined with chemical modification of the xanthines to enable the determination of positional intramolecular $^{13}C/^{12}C$ ratios ($\delta^{13}C_i$) with high precision. However, only caffeine is amenable to analysis: theobromine and theophylline present substantial difficulties due to their poor solubility. However, their *N*-methylation to caffeine makes spectral acquisition feasible. The method is confirmed as robust, with good repeatability of the $\delta^{13}C_i$ values in caffeine appropriate for isotope fractionation measurements at natural abundance. It is shown that there is negligible isotope fractionation during the chemical *N*-methylation procedure. Thus, the method preserves the original positional $\delta^{13}C_i$ values. The method has been applied to measure the position-specific variation of the $^{13}C/^{12}C$ distribution in caffeine. Not only is a clear difference between caffeine isolated from different sources observed, but theobromine from cocoa is found to show a ^{13}C pattern distinct from that of caffeine.



Since the first report of the existence of a nonstatistical isotope distribution in amino acids,[1] it has been recognized that isotope fractionation in plant metabolism can only be fully understood by the analysis of isotope redistributions at the submolecular level. However, until recently, relatively few position-specific $\delta^{13}C_i$ values (see Table 1 for definitions) in metabolites[2,3] and enzymatic isotope effects[4,5] have been determined. Mostly, only data for whole-molecule $\delta^{13}C$ values (global or bulk value, $\delta^{13}C_g$) have been obtained by isotope ratio monitoring by mass spectrometry (irm-MS)[6] or by high resolution absorption measurements in the near-infrared (NIR) spectral range based on cavity ring-down spectroscopy.[7] These techniques analyze the CO_2 obtained after a complete combustion of the molecule, destroying any position-specific fractionation. Limited positional $\delta^{13}C$ values ($\delta^{13}C_i$) can be accessed by irm-MS but only via complex and tedious (bio)chemical degradations. This approach was developed and exploited by the team of Schmidt during the 80s and 90s and applied to glucose of C_3 and C_4 origin,[8] glycerol,[9] and caffeine.[10] Even so, the chemical and biochemical degradations involved only allowed a direct measurement for some of the intramolecular $\delta^{13}C$ values: the others were deduced by difference. Although this work clearly showed with a good precision that the ^{13}C distribution in natural metabolites is nonuniform, the use of this technique is impractical for large sample sets. More recently, controlled degradation coupled with online pyrolysis and GC separation of fragments coupled with mass spectrometry has been developed[11] but at present this is limited to small molecules, such as acetic acid, lactic acid, or ethanol.[12−15]

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Table 1. Symbols Used in This Work

symbol	meaning
$\delta^{13}C$	carbon isotope composition, carbon isotope ratio of the molecule relative to the international standard (Vienna Pee Dee Belemnite V-PDB)
$\delta^{13}C_g$	^{13}C mean isotope composition of a whole molecule measured by irm-MS (found also in literature as bulk ^{13}C content)
$\delta^{13}C_i$	^{13}C isotope composition of the carbon position i measured by ^{13}C NMR
f_i	mole fraction for a carbon position i measured by ^{13}C NMR = area of the peak S_i corresponding to the carbon position i divided by the sum of the area of all the carbon positions of the molecule ($f_i = S_i/(\Sigma_n S_i)$)
F_i	statistical molar fraction for a carbon position i, molar fraction for the carbon position i in case of a homogeneous ^{13}C distribution within the molecule (F_i = 1/8 for caffeine)
f_i/F_i	reduced molar fraction for a carbon i, any shift from 1 indicates an isotope fractionation.

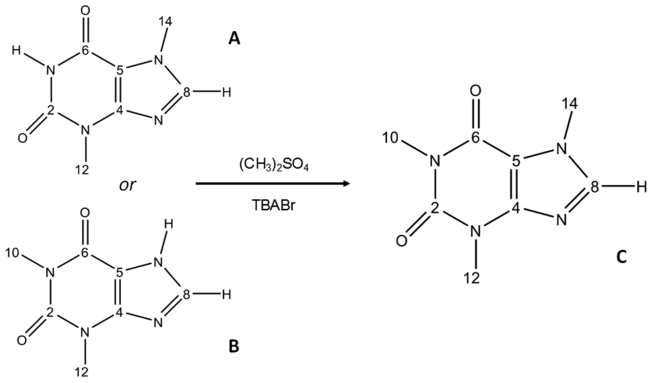

Figure 1. Schematic description of the *N*-methylation of theobromine (A) or theophylline (B) into caffeine (C), used as the molecular probe for irm-^{13}C NMR spectrometry. For clarity, the numbering of the methyl groups has been kept identical for the three methylxanthines. TBABr, tetrabutylammonium bromide.

Position-specific isotope analysis (PSIA) is conveniently performed using nuclear magnetic resonance (NMR) spectrometry (isotope ratio monitoring by ^{13}C NMR, irm-^{13}C NMR), that provides a general method for determining the intramolecular ^{13}C distribution of any low-molecular-weight soluble species. It analyzes directly the target molecule or a derivative thereof, but crucially, does so without need for prior chemical degradation. Position-specific natural isotopic fractionation studied by NMR has been carried out for δ^2H_i determinations since the 80s and now is routinely used for metabolic and climatic analyzes and as a tool in authentication.[16] However, the use of NMR for position-specific natural abundance ^{13}C presented more of a challenge because the natural range of isotopic variation in natural compounds is about 10-fold less for ^{13}C than for ^2H (about 50‰ and 500‰, respectively on the δ-scale). Hence, irm-^{13}C NMR requires 10-times higher precision. Once initial difficulties in isotopic ^{13}C NMR were overcome, appropriate long-term repeatability with sufficient precision was obtained.[17] Key features for reaching the target precision of 1‰ include (i) homogeneity and robustness of the ^1H decoupling of $^{13}C-^1H$ interactions by using appropriate adiabatic decoupling[18] and (ii) the reduction of the experimental time via the reduction of the longitudinal relaxation time by using relaxation reagents.[19] However, further fine-tuning and specific parameters (relaxation, line width, $^{13}C-^{13}C$ satellites, etc.) have to be established using elaborated NMR protocols, including an adiabatic INEPT pulse sequence[20] for each new molecular probe so as to exploit effectively the irm-^{13}C NMR tool. This has now been successfully applied to glycerol,[21] vanillin,[22] paracetamol and aspirin,[23] glucose,[24] fructose,[25] and the pharmaceutical traceability of ibuprofen and naproxen.[26] The reproducibility and the intercomparison of NMR spectrometers for irm-^{13}C NMR have recently been addressed. An interlaboratory collaborative irm-^{13}C NMR study was performed in which it was shown that the instrumental response (interspectrometer variability) can be monitored and adjusted when a systematic protocol is applied.[22] Then, conditions for performance qualification of the NMR spectrometer have been defined in order to express the $\delta^{13}C_i$ on the international δ-scale relative to established certified material references used in irm-MS.[27]

The purine alkaloids, notably caffeine (1,3,7-trimethylpurine-2,6-dione) and theobromine (3,7-dimethyl-1*H*-purine-2,6-dione) are of considerable commercial and biological interest (see Figure 1 for the structures and carbon numbering). Caffeine is found in relatively large amounts[28] in coffee (0.5−3%), tea (1−4%), mate (0.5−2%), guarana (2−5%), cola species (1−4%), and cocoa 0.1−0.4%), while theobromine is characteristic of cocoa and related *Theobroma* species. Because of their wide geographical cultivation and the considerable variation in quality, hence price, the is major economic interest in being able to determine the geographical origin of coffee, tea, or cocoa and to distinguish between sources of caffeine, including commercial (synthetic) ones.

Furthermore, although the biosynthesis and catabolism of methylxanthines is relatively well established,[29] there is some uncertainty as to the metabolic routes utilized preferentially in different plants. The carbon skeleton of purine alkaloids arises from three different sources, which can be summarized according to the numbering depicted in Figure 1: CO_2/HCO_3^- (C-6), C_1-activated-tetrahydrofolate (C-2 and C-8), and glycine (C-4 and C-5). The three methyl groups (C-10, C-12, and C-14) are derived from *S*-adenosylmethionine by sequential incorporation, leading to theophylline or theobromine as the ultimate step before caffeine. Both precursors have been implicated, depending on species.[29] Probing the intramolecular ^{13}C distribution in methylxanthines should help shed light on the substrate-product relationships and how these relate to species and/or geographical origins.

In the pioneering work of Schmidt and colleagues, it was shown that the $^{13}C/^{12}C$ ratio distribution in caffeine is nonstatistical.[10] Although this work proved that the range of $\delta^{13}C_i$ in natural caffeine is quite broad, with for example a low value (−40‰) for the mean of the methyl groups, this approach is both extremely tedious and incomplete, confirming the interest of performing PSIA by NMR. To provide direct, reliable and comprehensive measurements of the $^{13}C/^{12}C$ ratios of all individual carbon atom positions of methylxanthines, we have therefore developed an irm-^{13}C NMR method for these purine alkaloids. This objective is a priori very challenging, since both theobromine and theophylline (1,3-dimethyl-7*H*-purine-2,6-dione) are almost insoluble in organic solvents typically used for NMR. This was overcome by adopting a global strategy in which the transformation of theobromine or theophylline into caffeine via selective *N*-methylation under conditions that do not per se introduce isotopic fractionation makes it possible for caffeine to be exploited as a unique molecular probe for PSIA of methylxanthines. Despite the only moderate solubility of caffeine in some organic solvents, which limits the NMR sensitivity, a method for the determination of $\delta^{13}C_i$ in caffeine is described and examples given of its

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application to obtaining the intramolecular ^{13}C-patterns in caffeine, theobromine and theophylline.

MATERIALS AND METHODS

Chemicals and Plant Material. Ethanol and sulfuric acid (98%) were purchased from VWR, pyrrole and tris(2,4-pentadionato)chromium-III $Cr(Acac)_3$ (97%) from Acros Organics, caffeine from Aldrich, theobromine from Sigma, tetrabutylammonium bromide (99%) from EGA-Chimie, and Toluene-d_8 from Eurisotop. Theobromine was extracted from cocoa beans bought directly from the producers (harvested in 2009) or from chocolate bought from ethical and fair-trade shops (2012). Other sources of methylxanthines were obtained by purchase from local suppliers worldwide in 2011.

Extraction of Theobromine and Caffeine. Extraction of theobromine from cocoa beans (12 g): after separation from the shells, the cotyledons (10 g) were mechanically ground and extracted with cyclohexane (150 mL) at reflux during 2 h, to remove the lipids (cocoa butter). After the mixture was cooled and filtered, a residue of lean cocoa (~5 g) was recovered, to which was added an aqueous solution of 2 M sulfuric acid (150 mL). The whole was heated at reflux for 1 h to solubilize theobromine in its ionic form. The cooled solution was basified with aqueous ammonia (25%) and theobromine was recovered by extraction with 3 × 150 mL of chloroform/propan-2-ol (3/1, v/v). After solvent removal by rotary evaporation, pure theobromine was obtained from the residue by recrystallization from ethanol, with a yield ranging between 0.8 and 1.6% D/W of bean.

Extraction of caffeine from natural matrices: an appropriate mass of material (e.g., 200 g of ground coffee) was submitted to solid−liquid extraction with water (300 mL) at reflux in the presence of calcium carbonate. After filtration of the hot liquid, caffeine was recovered following cooling by extraction with dichloromethane (3 × 150 mL). After drying with anhydrous magnesium sulfate, solvent was removed by rotary evaporation, leading to a slightly greenish solid. From this, recrystallization from ethanol provided pure caffeine as a white powder (>99.5%), as probed by ^1H and ^{13}C NMR.

For the tea samples from Japan, crude tea (10 g dry weight per batch) was first crushed to a powder and then extracted with 200 mL of MeOH under sonication for 30 min. This procedure was repeated three times, and the combined MeOH extracts were evaporated to dryness. Residue was dissolved in 100 mL H_2O and the aqueous phase was extracted three times with 50 mL dichloromethane to extract caffeine. The dichloromethane layers were combined and evaporated to dryness. The residue was dissolved in 1.5 mL of MeOH/dichloromethane (1:1) and applied to preparative HPLC under the following conditions: column CAPCELLPAK ODS 20 × 250 mm (Shiseido, Japan); flow rate, 10 mL min^{-1}; mobile phase, A: H_2O (0.05% formic acid), B: MeOH; gradient, 5−40% (v/v) B/(A + B) over 30 min; column temperature 40 °C. The purified caffeine fraction (23.2−27.3 min) was concentrated to dryness on a rotary evaporator.

Chemical Conversion of Theobromine and Theophylline into Caffeine. The protocol was adapted from the work of Hedayatullah.[30] In a double-neck round-bottomed flask (250 mL), about 0.65 g of theobromine or theophylline were dissolved in 20 mL of aqueous sodium hydroxide (10 mM). Then, 30 mL of a solution of 1,2-dichloroethane and 0.23 g of tetrabutylammonium bromide, which acts as catalyst, were added. The mixture was heated to 90 °C and an excess of dimethyl sulfate (3 mL) was added. After it was refluxed for 1 h and then cooled, caffeine was extracted with chloroform (3 × 150 mL). After the combined organic layers were dried with anhydrous magnesium sulfate and filtered, solvent was removed on a rotary evaporator. From the residue, recrystallization from ethanol provided pure caffeine as a white powder (>99.5%) and with a yield of 75% or 80% from theobromine or theophylline, respectively (initial mass >0.5 g of caffeine). To determine the effect of lower yields on the isotope fractionation, the refluxing time was reduced.

NMR Spectrometry. *Spectral Acquisition Conditions.* Quantitative ^{13}C NMR spectra were recorded on a Bruker 500 Avance III spectrometer fitted with a 5 mm-i.d. $^{13}C/^1H$ dual cryoprobe carefully tuned to the recording frequency of 125.76 MHz. The temperature of the probe was set at 303 K, with no spinning of the tube. The experimental parameters for spectral acquisition were the following: pulse width 11.3 μs (90°), sampling period 1 s. The offsets for both ^{13}C and ^1H were set at the middle of the frequency range for caffeine. 32 scans were acquired using a repetition delay of 35 s leading to a signal-to-noise ratio (SNR) ≈ 700. Inverse-gated decoupling was applied in order to avoid nuclear Overhauser effect. The decoupling sequence for removing $^1H-^{13}C$ scalar coupling interactions employed a cosine adiabatic pulse with appropriate phase cycles, as described previously.[31] Each measurement consisted of the average of five independently recorded NMR spectra. In addition, a quantitative ^{13}C NMR reference spectrum was recorded on a Bruker 400 Avance III spectrometer fitted with a BBFO probe carefully tuned to the recording frequency of 100.62 MHz; this was used to calibrate the 500 MHz spectrometer.[27]

Sample Preparation. A mass of 160 mg of caffeine and 1.75 mg of relaxant $Cr(Acac)_3$ were separately weighed into two small vials. The caffeine was dissolved in a mixture of 360 μL of pyrrole and 240 μL of toluene-d_8. The relaxant was dissolved in100 μL of toluene-d_8. The solutions were mixed and filtered into a 5 mm o.d. NMR tube.

NMR Data Processing. Free induction decay was submitted to an exponential multiplication inducing a line broadening of 2 Hz. After baseline correction, a curve fitting was carried out in accordance with a Lorentzian mathematical model using Perch Software (Perch NMR Software, University of Kuopio, Finland).

Isotopic Data. Isotope $^{13}C/^{12}C$ ratios were calculated from processed spectra with the method of Silvestre et al.[23] Briefly, the positional isotopic distribution in a molecule was obtained from the ^{13}C mole fraction f_i (where i stands for the C atom position considered) as follows: $f_i = S_i/S_{tot}$, where S_i is the ^{13}C-signal (i.e., the area under the peak associated with the C atom in position i) and S_{tot} is the sum of all ^{13}C-signals of the molecule. Each S_i had to be corrected to compensate for the slight loss of intensity caused by satellites ($^{13}C-^{13}C$ interactions) by multiplying by $(1 + n \times 0.011)$, where n is the number of carbon atoms directly attached to the C atom position i and 1.1% (= 0.011) is the average natural ^{13}C-abundance (see Tenailleau et al.[32] and Silvestre et al.[23] for a detailed explanation). If F_i denotes the statistical mole fraction (homogeneous ^{13}C-distribution), then the position-specific relative deviation in the ^{13}C-abundance at any C atom position i is $d_i = f_i/F_i - 1$. The d_i values were converted to $\delta^{13}C$ values using the isotope composition of the whole molecule obtained by irm-EA/MS.

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Table 2. Mean Isotopic Composition $\delta^{13}C_i$ (‰) and Standard Deviation (SD, ‰) Determined from 10 Independent Measurements (5 Spectra Each) of a Commercial Sample of Caffeine Spread over One Year[a]

	C-6	C-2	C-4	C-8	C-5	C-14	C-12	C-10
$\delta^{13}C_i$ mean (‰)	−31.9	−33.7	−20.0	−36.4	−29.4	−44.1	−40.0	−40.9
SD (‰)	0.6	0.5	0.8	0.8	1.5	0.6	0.5	0.7

[a]The carbon numbering follows IUPAC and the order of the carbon positions, as for the other tables, follows the order of the ^{13}C NMR signal on the spectrum from the highest to lowest chemical shift.

Table 3. Mean Isotopic Composition $\delta^{13}C_i$ (‰) and Standard Deviation (SD, ‰) Determined for Caffeine Obtained by the N-Methylation of Commercial Theobromine with Three Yields and with Duplicates at Optimum Yield (75%)

$\delta^{13}C_i$ (‰)	C-6	C-2	C-4	C-8	C-5	C-14	C-12	C-10
75%	−29.9	−37.2	−24.5	−30.6	−53.5	−47.7	−45.8	−83.0
75%	−30.7	−37.3	−26.8	−32.3	−52.0	−46.3	−45.3	−82.1
50%	−28.6	−36.5	−24.8	−30.1	−57.7	−46.1	−44.2	−80.9
mean (‰)	**−29.7**	**−37.0**	**−25.4**	**−31.0**	**−54.4**	**−46.7**	**−45.1**	**−82.0**
SD (‰)	**1.1**	**0.5**	**1.3**	**1.1**	**2.9**	**0.9**	**0.8**	**1.0**
mean-75% (‰)	***−29.0***	***−36.7***	***−24.5***	***−30.8***	***−55.8***	***−46.9***	***−45.1***	***−81.8***
SD-75% (‰)	***0.6***	***0.1***	***1.6***	***1.1***	***1.1***	***1.0***	***0.4***	***0.6***

irm-EA/MS. The ^{13}C abundance of whole molecules, $\delta^{13}C_g$ (‰) was determined by elemental analysis (EA) coupled to irm-MS (irm-EA/MS) using a Flash HT-Delta V Advantage mass spectrometer (ThermoFinnigan). About 0.8 mg of sample was sealed in a tin capsule and the $\delta^{13}C$ determined by reference to a working standard of glutamic acid standardized against calibrated international reference material (NBS-22, IAEA-CH-6, IAEA-CH-7). Thus

$$\delta(‰) = \left(\frac{R}{R_{std}} - 1\right) \times 1000$$

where R is the isotope ratio of the sample and R_{std} is the isotope ratio of Vienna Pee Dee reference standard (V-PDB) (R_{std} = 0.0112372).

RESULTS AND DISCUSSION

NMR Conditions, Repeatability, and Reliability. irm-^{13}C NMR requires that quantitative conditions are established for each target molecule (see Materials and Methods). Once this is achieved, the analytical repeatability is primarily dependent on the signal-to-noise ratio (SNR): a standard deviation of 1‰ requires an SNR > 500. The number of moles of caffeine in the NMR tube is therefore of prime importance for good sensitivity. As purine alkaloids have a very poor solubility in common organic solvents, attaining sufficient solubility was a primary hurdle for this method. Caffeine shows its best solubility in halogenated solvents ($CHCl_3$ and CH_2Cl_2), but these are also too volatile for irm-NMR. We explored other mixtures which should provide both adequate solubility and avoid overlapping NMR signals. The best conditions we identified were to dissolve 160 mg of pure caffeine in 360 μL of pyrrole and 340 μL of deuteriated toluene (for comparison, 1 mL C^2HCl_3 dissolved 130 mg of caffeine). Using these conditions and a 400 MHz NMR spectrometer (one used routinely for irm-^{13}C NMR measurements and used during the ring test[22]) the duration of the analysis is more than 9 h for the acquisition of one spectrum. Since five spectra are usually recorded, the total time for analyzing one sample is prohibitive. Therefore, we opted to use a much more sensitive instrument: a 500 MHz spectrometer equipped with a cryoprobe, which enabled the acquisition of one spectrum within 20 min. This is, to the best of our knowledge, the first time that such a probe is used for $\delta^{13}C_i$ determination. As the $\delta^{13}C_i$ can show a dependency on the type of spectrometer,[27] a correction factor is applied on each $\delta^{13}C_i$. This is not a problem since the instrumental repeatability (precision of the measurement) is met. A correction factor for each position is calculated from measurement performed on a machine which is known to produce "true" values, i.e. it is calculated from the difference between the results of the 500 MHz and the 400 MHz spectrometers. The latter has been identified during a previous work as an instrument enable to give the true $\delta^{13}C_i$ values.[27] Accordingly, the $\delta^{13}C_i$ obtained (and presented in the present work) are expressed on the international δ-scale, allowing intercomparison.

To test repeatability, a commercial caffeine sample was analyzed 10 times in the same conditions (each measurement constituted of 5 consecutive spectra) over one year. The standard deviations are lower than 1‰, except for the C-5 atom position, for which SD = 1.5‰ (Table 2). The NMR peak of C-5 is very close to a peak of pyrrole, which makes integration of this signal problematic. It should be noticed that a better stability and a lower SD are observed during the more recent measurements, indicating that the ^{13}C NMR protocol becomes more robust with practice.

Selection of the N-Methylation Procedure. As the direct analysis of theobromine or theophylline is not possible because of low solubility, N-methylation to caffeine was carried out to increase solubility and render these two methylxanthines amenable to irm-^{13}C NMR analysis. The added advantage of transforming theobromine and theophylline into caffeine is that only one molecular probe, caffeine, is required. The first strategy was to use the classic methylation reagent, methyl iodide,[33] but we were unable to obtain caffeine in a yield >50%. In addition, reaction with methyl iodide gave caffeine and minor side-products, which could be O-methylation of a structure derived from theobromine. No better yield or purity was obtained with dimethyl carbonate or dimethyl sulfate. It appears that the difficulty of substitution at position 1 of theobromine can be attributed to the electronic deactivation of the nitrogen in position 1 and the steric effect of adjacent carbonyl groups. To overcome these difficulties, we relied on the method of alkylation phase transfer catalysis,[30] in which N-

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Table 4. Mean Isotopic Composition $\delta^{13}C_i$ (‰) and Standard Deviation (SD, ‰) Determined for Caffeine Obtained by the N-Methylation of Commercial Theophylline with Three Yields and with Duplicates at Optimum Yield (80%)

$\delta^{13}C_i$ (‰)	C-6	C-2	C-4	C-8	C-5	C-14	C-12	C-10
80%	−6.3	−37.0	−25.9	−28.6	−18.6	−79.2	−30.1	−33.0
80%	−8.0	−39.1	−26.5	−28.3	−19.8	−76.7	−30.7	−30.3
50%	−10.8	−45.1	−34.2	−36.9	−32.2	−101.4	−45.6	−46.7
mean (‰)	**−8.4**	**−40.4**	**−28.9**	**−31.2**	**−23.6**	**−85.8**	**−35.4**	**−36.7**
SD (‰)	**2.3**	**4.2**	**4.6**	**4.9**	**7.6**	**13.6**	**8.8**	**8.8**
mean-80% (‰)	***−7.2***	***−38.0***	***−26.2***	***−28.4***	***−19.2***	***−77.9***	***−34.0***	***−31.7***
SD-80% (‰)	***1.2***	***1.4***	***0.4***	***0.2***	***0.9***	***1.8***	***0.4***	***1.9***

Table 5. Mean Isotopic Composition $\delta^{13}C_i$ (‰) and Standard Deviation (SD, ‰) Determined for Caffeine Obtained by the N-Methylation of Natural Theobromine Extracted in Duplicate from Cocoa Beans from the Same Batch from the Ivory Coast

$\delta^{13}C_i$ (‰)	C-6	C-2	C-4	C-8	C-5	C-14	C-12	C-10
1	−17.0	−24.6	−25.3	−17.4	−26.6	−50.1	−35.3	−92.7
2	−18.3	−24.4	−26.5	−18.8	−24.7	−49.9	−35.2	−91.6
mean (‰)	−17.6	−24.5	−25.9	−18.1	−25.6	−50.0	−35.2	−92.2
SD (‰)	0.9	0.2	0.8	1.0	1.3	0.2	0.1	0.8

Table 6. Position Specific Isotopic Composition $\delta^{13}C_i$ (‰) Determined for Caffeine from Natural Origins Extracted from Several Plants and from Commercial Origins[a]

plant	origin	C-6	C-2	C-4	C-8	C-5	C-14	C-12	C-10[b]
cocoa	Ivory Coast (Gagnoa)	−18.2	−27.2	−26.8	−22.1	−15.3	−53.8	−34.6	
cocoa	Ivory Coast (Oumé)	−13.4	−24.9	−25.4	−20.4	−27.5	−45.2	−32.9	
cocoa	Ivory Coast (Sinfra)	−11.7	−23.1	−24.6	−18.1	−29.6	−56.2	−36.2	
cocoa	equator	−15.4	−21.5	−22.2	−16.0	−21.9	−53.5	−33.4	
cocoa	Venezuela	−16.2	−21.6	−24.1	−16.1	−25.0	−56.0	−35.5	
coffee	Ivory Coast (Gagnoa)	−13.8	−5.2	−12.5	3.2	−26.9	−61.3	−49.0	−49.7
coffee	Ivory Coast (Sinfra)	−12.1	−5.9	−13.6	1.1	−22.9	−62.0	−49.9	−49.9
coffee	Brazil	−10.3	−12.5	−12.9	−7.5	−20.1	−54.4	−48.2	−46.4
coffee	Colombia	−16.2	−15.4	−13.4	−8.7	−15.6	−52.5	−48.5	−45.4
guarana	commercial	−17.8	−12.9	−19.0	−3.9	−32.0	−47.2	−42.0	−52.4
cola nut	Ivory Coast	−3.8	−12.1	−27.8	−5.6	−31.2	−49.3	−33.2	−54.6
maté	Argentina	−10.0	−3.2	−15.7	6.7	−30.9	−57.2	−55.1	−47.1
tea	China	−3.5	−21.0	−16.9	−15.7	−25.0	−48.7	−40.6	−53.7
tea	Japan (Kyoto)	−14.4	−32.7	−27.2	−29.5	−34.3	−67.5	−53.2	−73.7
commercial caffeine	Aldrich (Europe)	−31.8	−32.7	−19.7	−36.5	−32.0	−44.8	−41.3	−41.8
commercial caffeine	Fluka (Japan)	−19.4	−27.2	−21.9	−39.0	−30.5	−92.8	−37.4	−38.9
commercial theobromine	Sigma (Europe)	−29.7	−37.0	−25.4	−31.0	−54.4	−46.7	−45.1	

[a]For cocoa extract and commercial theobromine, caffeine was obtained by the *N*-methylation of theobromine. [b]For theobromine, this $\delta^{13}C$ value reflects the methylation reagent used: it is not shown since it cannot be used for the purpose of the discrimination of origin.

methylation with dimethyl sulfate is enhanced by catalysis with tetrabutylammonium bromide (Figure 1). Optimization of this method gave caffeine with yields of 75 and 80% from theobromine and theophylline, respectively. In addition to increased yield, this method gives an almost pure product, which, following recrystallization from ethanol, is of sufficient purity for NMR, i.e. with no impurity or if any it is at very low level and with no interaction with the ^{13}C NMR spectrum. This protocol is repeatable on pure commercial theobromine (Table 3) and theophylline (Table 4) with SD values of the same order as for the NMR repeatability. A similar precision was obtained from theobromine extracted from cocoa beans (Table 5).

Is There a $^{12}C/^{13}C$ Isotope Fractionation Associated with N-Methylation? Since the yield of the chemical N-methylation of theobromine or theophylline is well below quantitative, any isotope fractionation associated with the methylation reaction could influence the measurement of the $^{13}C_i$ abundance. As depicted in Figure 1, the reaction consists in a substitution of H by CH_3 on a nitrogen atom without change in hybridization status (position 1 for theobromine and position 7 for theophylline). It can, therefore, be anticipated that any secondary isotope effect leading to ^{13}C fractionation on the neighboring carbon positions (C-2 and C-6 in theobromine and C-8 in theophylline) should be negligible, even if the reaction does not go to completion. It was confirmed that no position-specific isotope value was perturbed by purposefully lowering the isolation yield of the obtained caffeine. As can be seen, the intramolecular composition is very similar at 50% yield for caffeine derived from theobromine (Table 3) or theophylline (Table 4). We can therefore conclude that any variation in the $\delta^{13}C_i$ because of N-methylation-associated isotope effects is insignificant compared with that due to natural $\delta^{13}C_i$ variation. It is worth noting that the N-methylation, consisting of the addition of only one carbon, is optimal in term of sensitivity because the molecular weight is increased by only 14 Da (<8%).

Intramolecular $\delta^{13}C$ in Caffeine and Theobromine from Different Sources. The intramolecular ^{13}C distribution

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in caffeine and theobromine purified from several plant species and from a number of commercial sources are shown in Table 6. Several noteworthy features can be identified.

First, the range of the $\delta^{13}C_i$ values within the molecule (theobromine or caffeine) is very broad with the extreme values of over 60‰. This is large, but not unreasonable for natural products, particularly those containing methyl groups which are known generally to be impoverished.[34] In the methylxanthines, the highest $\delta^{13}C$ values are found for the C-8 of some natural samples, for which the $\delta^{13}C$ is positive relative to the V-PDB reference. That is, this position is significantly enriched compared with the ^{13}C content in atmospheric CO_2 (−8‰ relative to V-PDB). To our knowledge, such a high enrichment has not been observed previously in natural products and will be discussed elsewhere. Second, by observing the $\delta^{13}C_i$ of the 8 carbons of caffeine and the 7 carbons in theobromine, it is clear that the methylxanthines display intramolecular ^{13}C profiles characteristic of each plant. Third, the natural ^{13}C profiles obtained are different from those of commercial synthetic caffeine and theobromine.

The $\delta^{13}C_i$ values obtained for natural caffeine agree well with those obtained by chemical degradation and irm-MS measurement on some fragments.[10] However, it offers much higher resolution than this earlier study, in which, for example, the $\delta^{13}C$ values of the three methyl groups in caffeine were averaged. These authors reasonably assumed that the ^{13}C content of the methyl at position N-7 (C-14) should be identical to the other two methyl groups C-10 and C-12. The present work clearly shows that it is not the case: C-14 is significantly and consistently impoverished compared to C-12 in caffeine. Similarly, the C-10 displays a profile distinct from the C-12 and C-14. In fact, it appears that the $\delta^{13}C$ of each methyl group is specific, which could reflect their sequential incorporation in the order C-14, C-12 then C-10 during biosynthesis. Interestingly, C-2 and C-8 do not have the same $\delta^{13}C$ yet they originated from the same source, C_1-THF, with the C-8 systematically enriched whatever the plant species. This may reflect their not coming from the same pool of the equilibrated precursor: the C-8 from 5,10-methenyl-THF and the C-2 from 10-formyl-THF. Disequilibrium between these pools could explain the observed difference. Similarly, disequilibrium because of the extensive use of 5,10-methenyl-THF for the synthesis of the methyl groups at C-10, C-12, and C-14 via methionine and SAM can also be advanced as a reason for the extreme enrichment at C-8 of the samples of coffee variety Robusta, a high caffeine accumulator. Such interpretations of the PSIA in relation to biosynthesis will be developed elsewhere.

CONCLUSIONS

Our results clearly show that it is now possible to access the position-specific isotope fractionation in caffeine and theobromine obtained from plants by exploiting irm-^{13}C NMR to determine the intramolecular $\delta^{13}C_i$ values. A new methodology was necessary to ensure repeatable data in an acceptable analysis time. It is evident that considerable interspecies variation is present, which might be linked to variable metabolic processes. The present analytical method provides a tool that could be applied in origin authentication, once more data from samples from different plant species and from further geographical locations is obtained. The high sensitivity of the method could allow work on as little as 80 mg of caffeine, while still within an acceptable duration of analysis. Thus, it can be envisaged to extract caffeine in cocoa or theophylline from tea, which will make possible a detailed picture of the C1 pool biosynthesis.

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Author Contributions

G.S.R., N.H., and N.W. planned and managed the project. D.G.D, E.M., A.G., P.N., K.Y., A.M., and I.T. carried out the research. D.G.D, E.M., G.S.R., and A.G. processed the data. G.S.R., R.J.R., and A.G. wrote the paper.

Notes

The authors declare no competing financial interest.

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