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Single-step transesterification with simultaneous concentration and stable isotope analysis of fatty acid methyl esters by gas chromatography-combustion-isotope ratio mass spectrometry

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Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is increasingly applied to food and metabolic studies for stable isotope analysis ($\delta^{13}C$), with the quantification of analyte concentration often obtained via a second alternative method. We describe a rapid direct transesterification of triacylglycerides (TAGs) for fatty acid methyl ester (FAME) analysis by GC-C-IRMS demonstrating robust simultaneous quantification of amount of analyte (mean $r^2 = 0.99$, accuracy ±2% for 37 FAMEs) and $\delta^{13}C$ (±0.13‰) in a single analytical run. The maximum FAME yield and optimal $\delta^{13}C$ values are obtained by derivatizing with 10% (v/v) acetyl chloride in methanol for 1 h, while lower levels of acetyl chloride and shorter reaction times skewed the $\delta^{13}C$ values by as much as 0.80‰. A Bland-Altman evaluation of the GC-C-IRMS measurements resulted in excellent agreement for pure oils (±0.08‰) and oils extracted from French fries (±0.49‰), demonstrating reliable simultaneous quantification of FAME concentration and $\delta^{13}C$ values. Thus, we conclude that for studies requiring both the quantification of analyte and $\delta^{13}C$ data, such as authentication or metabolic flux studies, GC-C-IRMS can be used as the sole analytical method.

Gas chromatography-combustion–isotope ratio mass spectrometry (GC-C-IRMS) is a highly sensitive technique that allows the precise and accurate determination of stable isotope ratios down to 10^{-5},[1] allowing natural and low-level enrichment of stable isotope ratios to be precisely quantified. The first applications and development of the technique sprang from the geological sciences and rapidly led to significant discoveries in that field.[2,3] Others quickly recognized the potential value of the sensitivity imparted by GC-C-IRMS, leading to its application in forensics,[4,5] ingredient authentication,[6–9] archaeology,[10,11] nutrition,[12–14] metabolic flux measurements,[15–17] and performance-enhancing drug testing,[18] as well as recent recognition of its potential in metabolomics.[19] This technique has a potential use in any science that is based on measuring chemical fluxes through systems, as evidenced by its rapidly increasing application.[20,21]

Unlike other forms of mass spectrometry in which a mass analyzer often scans ranges of mass-to-charge ratios (*m/z*) (e.g., ion traps which may give spectra between *m/z* 50 and 650), the isotope ratio mass spectrometer is a magnetic sector with multiple single-channel Faraday cup detectors each of which is tuned to a specific *m/z* value. This design leads to high sensitivity and precision in measuring stable isotope ratios but limits the analysis to simple compounds that yield ions with *m/z* values below 50 (e.g., N_2, CO_2).[20,21] Therefore, organic molecules must be quantitatively converted into CO_2 for stable carbon isotope ratio ($\delta^{13}C$) measurement. During the 1980s and 1990s the first instrumental designs allowing the on-line conversion of molecules separated by GC were introduced.[2] In these methods, the GC effluent is directed to a narrow quartz or ceramic tube with a CuO and/or NiO wire catalyst heated to 850°C, and the resulting CO_2 isotopologues are analyzed by IRMS to give precise stable isotope ratio determinations.[1,20,21]

GC-C-IRMS is useful in the study of fatty acid methyl esters (FAMEs) and has been exploited in the food sciences to determine the adulteration of fine vegetable oils by natural abundance $\delta^{13}C$ measurements,[6,7,9,22,23] and, in combination with other stable isotopes, the geographic origin of oils.[23] GC-C-IRMS has also garnered attention for its ability to quantify very low-level enrichment in ^{13}C-labelling studies to understand *in vivo* fatty acid metabolism in animal models, and it is more precise and accurate than other MS-based methods for this purpose.[15,17]

Stable isotope values are often coupled with quantitative data (e.g., fatty acid composition profiles) thus providing a very powerful assessment of partitioning in mixed systems (e.g., [8,9]). Such studies typically obtain the analyte concentration data independent of the $\delta^{13}C$ data via GC-flame ionization detection (FID). This requires researchers to analyze samples twice in order to obtain matched analyte concentration and stable isotopic data. In addition to doubling the analysis time, such an approach may not be desirable for samples of limited quantity or those that must be processed rapidly (such as forensic samples). From the inception of GC-C-IRMS, it has been recognized that robust and accurate stable isotope values require quantitative consistency through all steps of the analysis (extraction,

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purification, derivatization, and conversion of analyte into CO_2).[2,20,21] Therefore, it follows that both analyte concentration data and stable isotope values could be measured simultaneously within a single GC-C-IRMS run. Such a principle was recently suggested;[19] however, the focus of that work was to demonstrate optimal stable isotope analysis, and the quantitative results are not presented in detail.

Here we demonstrate the simultaneous measurement of fatty acid distribution and $\delta^{13}C$ profiles through a single GC-C-IRMS run. An evaluation of the quantitative response of GC-C-IRMS and an investigation of the impact of sample preparation on FAME concentration and $\delta^{13}C$ values are presented. The analysis is applied to vegetable oils as well as oils isolated from restaurant French fries, representative of a typical food product.

EXPERIMENTAL

Materials and reagents

All glassware was washed at 550°C for 6 h to eliminate organic contaminants. Acetyl chloride (≥99.0%, Fluka®, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and HPLC-grade hexane, dichloromethane and methanol (Fisher Scientific, Pittsburgh, PA, USA) were used without further purification. Deionized water (18.2 MΩ) and ACS-grade sodium bicarbonate (Fisher Scientific) were extracted with dichloromethane three times and stored in ashed glass bottles with Teflon-lined caps until use. Pentadecanoic acid (C15:0) (≥99%), nonadecanoic acid (C19:0) (≥99%), and naphthalene (Np) (≥99%) (Sigma–Aldrich, St. Louis, MO, USA) were used as internal isotope and recovery standards. A stock solution of 25 mg/mL of each compound was prepared in dichloromethane and stored at −40°C until use as the multiple component internal standard (IS). Compound identification and stable isotope calibrations were achieved using Supelco 37 (Supelco Inc., Bellafonte, PA, USA) and F8 fatty acid ester mixture (ethyl and methyl derivatives of C14:0, C16:0, C18:0 and C20:0, 100 ppm each; A. Schimmelmann, Indiana University, Bloomington, IN, USA), respectively. Vegetable oils used for method development and testing were purchased from a local grocery store, and mixtures were prepared quantitatively by mass. Commercial French fries were purchased from restaurants on the island of Oahu, Hawaii, USA,[26] and stored at −40°C until extraction with dichloromethane.

Transesterification reagent and reaction

Methanol (30 mL) was transferred to an ashed glass tube, capped and placed in a freezer at −20°C for at least 30 min. Acetyl chloride (AcCl) was then added to the cooled methanol and shaken to ensure even dissolution. Previous authors have noted the exothermic reaction between AcCl and methanol and recommended keeping the reaction mixture cold over dry ice while slowly adding AcCl.[24] However, we found that pre-cooling the methanol was a more convenient approach that rendered the reaction much less violent. The transesterification reagent (TR) was stable at higher concentrations (10% AcCl) for at least 2 weeks if sealed and kept at −40°C.

Approximately 1.2 mg of a total lipid sample was placed in an ashed 2 mL GC vial with a Teflon-lined screw cap (Supelco Inc.), followed by 4 µL IS, 500 µL hexane and 500 µL TR. The mixture was immediately vortexed and placed on a heating block held at 95°C. The reaction was quenched with 50–100 µL of a saturated $NaHCO_3$ solution, briefly vortexing and transferring the upper hexane layer to an ashed 2 mL GC vial. The solution was then ready for GC-C-IRMS analysis. Varying concentrations of AcCl (2, 5 and 10% v/v) and reaction times (10–60 min) were tested for free fatty acids (FFAs) and triacylglycerides (TAGs).

Instrumentation

An Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Isoprime isotope ratio mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK) via a combustion interface, controlled by MassLynx 4.0 software (Isoprime Ltd), was used. The gas chromatograph was equipped with a DB-WAX column (30 m × 0.250 mm × 0.25 µm; J&W Scientific Inc., Folsom, CA, USA) preceded by a deactivated fused-silica guard column (2 m × 0.250 mm; Fisher Scientific). Samples were injected manually with a 10 µL syringe (SGE Analytical Science Pty. Ltd., Ringwood, Victoria, Australia). The temperature program was as follows: 80°C with a 2 min hold, then a 25°C/min ramp to 160°C, followed by a 10°C/min ramp to 240°C and held for 10 min. The carrier gas was He, set to a constant flow rate of 1.2 mL/min. IRMS systems cannot handle the volume of solvent typically injected during a GC analysis.[2] Therefore, a 'heart-split' valve was used to control the direction of flow, either to the IRMS instrument, or to waste. For $\delta^{13}C$ measurement, the GC effluent was diverted to the combustion interface via a transfer line held at 350°C into a silica tube (50 cm × 6 mm) containing a CuO/NiO wire catalyst heated to 850°C in a He atmosphere (a pressure of 2.5 psi, at a flow rate of 68 mL/min). A Nafion membrane with a cross-flow of He (a pressure of 2.5 psi, at a flow rate of 25 mL/min) removed water from the stream exiting the combustion interface effluent, and the CO_2 isotopologues were directed to the mass spectrometer inlet. The flow from the combustion interface was joined with a second He flow at the mass spectrometer inlet that delivered a pulsed stream of isotopically characterized monitoring CO_2 gas for the calculation of relative isotope abundance (see Eqn. (1) and related discussion below). The sample peaks were integrated automatically with background subtraction carried out by the software and data was exported as an excel file for further processing. Bulk stable isotope measurements of the IS were conducted on a Eurovector elemental analyzer (Eurovector Inc., Milan, Italy) coupled to the above IRMS system (EA-IRMS), as described by Richter *et al.*[23]

Stable carbon isotope ratio ($\delta^{13}C$) determination

Isotope ratio mass spectrometers measure isotope values to a very high precision; however, accuracy can only be achieved by comparing the measured sample isotope ratio with the isotope ratio of a known standard.[20,21,26,27] Therefore, several calculations and correction procedures must be applied to raw GC-C-IRMS data in order to obtain accurate

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stable isotope values. Historically, the delta notation has been used, following the equation:

$$\delta^{13}C_m = \frac{\left(^{13}C/_{12}C\right)_m - \left(^{13}C/_{12}C\right)_s}{\left(^{13}C/_{12}C\right)_s} \times 1000 \quad (1)$$

where ^{13}C and ^{12}C are the integrated peak areas of the two isotopes (measured as $^{13}CO_2$ and $^{12}CO_2$) of the measured sample (m) and standard (s), respectively. In this study, the 'standard' was the monitoring CO_2 gas with a known $\delta^{13}C$ value. Since this reference gas did not pass through the chromatographic system, internal isotope standards were co-injected with the analyte to account for potential biases introduced during the analysis.[20,21] The International Atomic Energy Agency (IAEA, Vienna, Austria) has calibrated a number of compounds to act as stable isotope standards; however, at this time, there are none for $\delta^{13}C$ that are directly amenable to GC. Therefore, it is necessary to identify and calibrate secondary standards suitable for each analysis. In this case the standards selected were pentadecanoic acid ($\delta^{13}C = -34.09 \pm 0.02$‰ as FFA, $\delta^{13}C = -34.39 \pm 0.05$‰ as the FAME) and nonadecanoic acid ($\delta^{13}C = -29.99 \pm 0.02$‰ FFA, $\delta^{13}C = -30.63 \pm 0.01$‰ as the FAME) as representative FAMEs and naphthalene ($\delta^{13}C = -25.90 \pm 0.02$‰), which was not affected by the derivatization reaction but provided a $\delta^{13}C$-elevated anchor for the calibration. The $\delta^{13}C$ values of the IS compounds have been calibrated to international standards NBS19 and L-SVEC (IAEA) according to Coplen *et al.*[26] The internal standards were subjected to all phases of the sample preparation to account for any potential sources of isotope fractionation.

$\delta^{13}C$ calibration and correction

Further data processing which is applied to the $\delta^{13}C_m$ value includes correction factors for signal intensity dependence (referred to as the 'linearity effect'), instrumental drift, and derivatization.[20,21] The linearity effect (LE) can sometimes affect IRMS results, and previous authors have noted strong effects with GC-C-IRMS.[17] Calibrations to account for the LE were carried out through multiple injections of increasing mass of the IS mix and F8 solutions, and plotting the $\delta^{13}C$ offset (measured – actual) vs. $\log_{10}$(peak area).[17] A three-point linear calibration with the IS (raw $\delta^{13}C$ vs. actual $\delta^{13}C$) was used to correct for instrumental drift.[26] Each run was corrected individually. Since isotope values are ratios and not absolute magnitudes, the accuracy and precision are reported in the conventional units (‰), and not as relative standard deviations (s.d.).

An additional consideration for FAME analysis is that the $\delta^{13}C$ value measured by the instrument is actually the weighted sum of the $\delta^{13}C$ value of the original fatty acid carbon and the $\delta^{13}C$ value of the added methanol carbon from the derivatization. To illustrate, a fatty acid with the formula $C_nH_{2n}O_2$ and $\delta^{13}C_{FA}$ is derivatized with methanol having $\delta^{13}C_{CH3OH}$, and the resulting FAME is $C_{n+1}H_{2(n+1)}O_2$, with $\delta^{13}C_m$ measured by GC-C-IRMS. Thus, the $\delta^{13}C_{FA}$ was calculated from the mass balance equation:

$$\delta^{13}C_{FA} = \frac{\left[\delta^{13}C_m \times (1+n) - \delta^{13}C_{CH_3OH}\right]}{n} \quad (2)$$

The $\delta^{13}C_{CH3OH}$ was determined by measuring C15:0 and C19:0, as well as their methyl derivatives, by EA-IRMS, and using a similar mass balance equation.

Quantification

To demonstrate the quantitative nature of GC-C-IRMS, external calibration with Supelco 37 FAME mix was conducted. Individual solutions were prepared and absolute response factors (peak area:mass injected) were derived from the linear regression of the calibration. The chosen FAME internal standards were either present (C15:0-Me) or overlapped with other components (C19:0-Me) in Supelco 37. Therefore, relative response factors were not calculated for this treatment. Variability was expressed as the relative standard deviation. Limits of detection were calculated from the linear regression for each compound[28] and expressed in terms of moles of analyte.

Reconstructed bulk $\delta^{13}C$

Morrison *et al.* demonstrated that accuracy and bias in the measurement of mixtures by GC-C-IRMS can be evaluated through a comparison of bulk $\delta^{13}C$ values measured by EA-IRMS ($\delta^{13}C_B$) with those calculated through mass balance obtained from quantitative and isotope data:[19]

$$\delta^{13}C_R = \frac{\sum\left[\delta^{13}C_i \times A_i\right]}{\sum A_i}; \quad (3)$$

where $\delta^{13}C_R$ is the recalculated bulk value, $\delta^{13}C_i$ and A_i are, respectively, the measured stable isotope value and concentration of a given compound i, and the product is summed for all measured compounds. The value of $\Delta^{13}C$ is the difference between $\delta^{13}C_R$ and $\delta^{13}C_B$. Bland-Altman plots ($\Delta^{13}C$ vs. $\delta^{13}C_B$) were constructed to visualize the comparability between $\delta^{13}C_R$ and $\delta^{13}C_B$ for pure vegetable oils and oils extracted from restaurant French fries to evaluate the method for bias.[19,29]

RESULTS AND DISCUSSION

Optimization of GC

The main instrument components that could affect the determination of $\delta^{13}C$ by GC-C-IRMS (i.e., injection port and combustion column) have been reviewed.[20,21] Here, we found that the addition of a 2 m deactivated silica guard column affected neither the quantitative response factors nor the $\delta^{13}C$ values, although the retention times did shift by ~2 s. Split injection led to improved resolution and more reproducible results with other detection methods (e.g., FID, MS); however, this results in a potential fractionation of the ^{13}C composition.[20,21] Therefore, all analyses were carried out under splitless injection at a temperature of 250°C. Supelco 37 (a 37-component FAME mixture) was used to optimize the temperature profile and heart-split valve switching (Fig. 1(a)). The method was set up so that C10:0-Me through C23:0-Me were measured by IRMS. All FAMEs above C10:0-Me were sufficiently resolved for the precise and accurate determination of $\delta^{13}C$ by GC-C-IRMS,[20,21] with the exceptions already noted by the manufacturer of this column

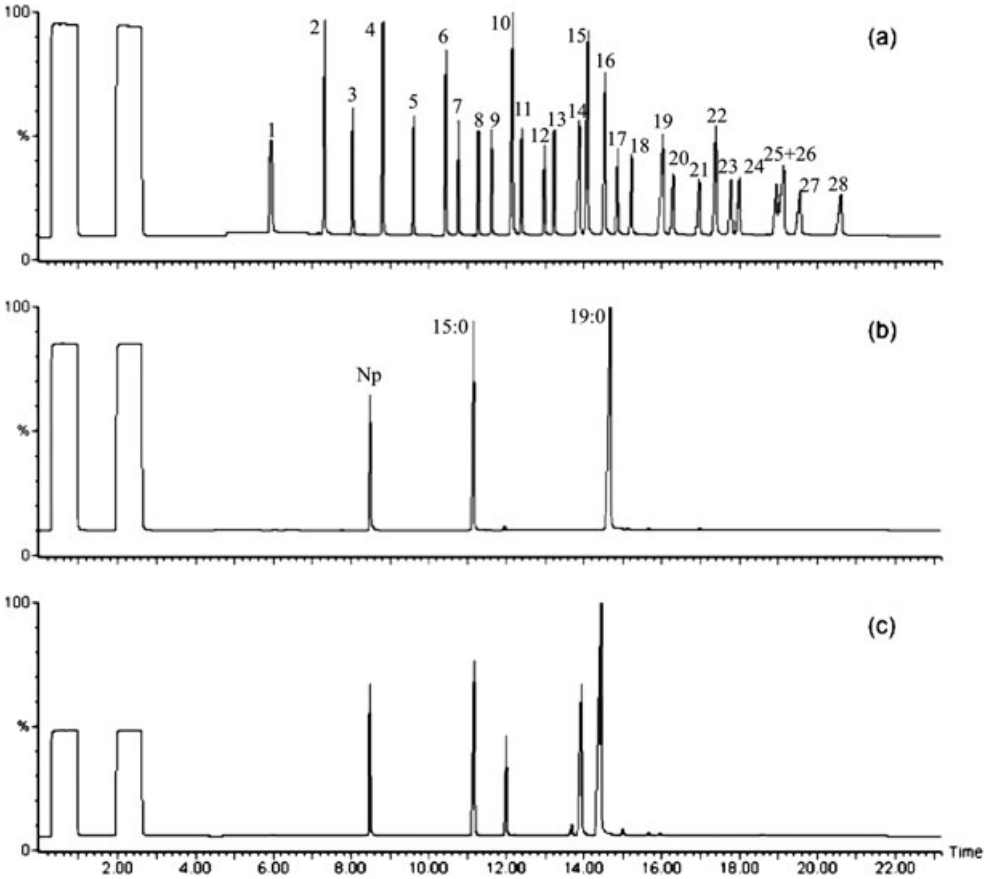

Figure 1. (a) Total ion count (TIC) chromatogram (*m/z* 44 + 45) of Supelco 37 FAME mix. See Table 1 for peak designations. (b) TIC chromatogram of internal standards (naphthalene (Np), C15:0-Me and C19:0-Me). (c) TIC chromatogram of a corn oil with internal standards.

(Supelco bulletin 907): *cis/trans* isomers are not resolved; C20:3n6-Me and C21:0-Me co-elute; and significant overlap of C20:5n3-Me with C22:0-Me occurs.

Selection of internal standards

To reduce significant inaccuracies in GC-C-IRMS analyses it is imperative to properly test for and select IS compounds that will be suitable for a given sample set. C15:0 and C19:0 fatty acids are appropriate internal standards for the target samples in our laboratory, but may fail for environmental or bacterial FAME analyses. C23:0 is another possible candidate and it is often used as the quantitative IS for FAME analysis.[30] It was recently shown that the most accurate and consistent $\delta^{13}C$ results are obtained with at least two internal standards for mass calibration.[27] Naphthalene was chosen as an additional standard because it is readily amenable to GC, elutes in a region where no FAMEs are expected in our samples, and provides a high $\delta^{13}C$ value that anchors the mass calibration (Table 1). In addition, because it is not affected by the derivatization reaction, it can be used as a quality control check for extraction efficiency and potential troubleshooting. Within a given run, the internal standards are highly reproducible, with a typical variability of ±0.10‰, and within 0.09‰ of their calibrated $\delta^{13}C$ values (Table 1).

$\delta^{13}C$ measurement of FAME standards

Reference mixture 'F8', constituted by eight ethyl and methyl fatty acid esters, whose $\delta^{13}C$ values have been independently determined, was used to test the GC-C-IRMS system for accuracy and precision (Table 1). The slope of the relationship between the measured raw and reported $\delta^{13}C$ values for repeated 3 μL injections was 1.03 ($r^2 = 0.99$, $p < 0.05$), with an average accuracy of ±0.11‰ from the reported values and precision ±0.10‰ (n = 5). Injections of increasing amount on-column of the F8 compounds (50–500 ng) showed no systematic size-based fractionation, maintaining the same order of accuracy (±0.13‰); however, the standard deviation did increase to an average of ±0.20‰. The source of this decrease in precision due to non-systematic error is uncertain; however, an appropriate internal mass calibration has been shown to reduce the error.[1] Using the compounds with extreme $\delta^{13}C$ values as internal reference standards (16:0-Et and 18:0-Me, $\delta^{13}C$ = −30.92 and −23.24‰, respectively) and applying a mass calibration did reduce the average uncertainty to ±0.13‰. This highlights the necessity of using internal standards to correct the raw $\delta^{13}C$ values for more robust GC-C-IRMS analysis, especially when analyte concentrations can vary. Overall, these results indicated no systematic fractionation in the GC-C-IRMS system.

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Table 1. Accuracy and precision of GC-C-IRMS for eleven $\delta^{13}C$ standard compounds

Compound	Solution	Reported $\delta^{13}C$ (‰)*	s.d.	Measured $\delta^{13}C$ (‰)#	s.d.
C14:0-Me	F8	−29.98	0.02	−29.99	0.02
C14:0-Et	F8	−29.13	0.03	−29.23	0.09
C16:0-Me	F8	−29.90	0.03	−29.86	0.18
C16:0-Et	F8	−30.92	0.02	−30.88	0.00
C18:0-Me	F8	−23.24	0.01	−23.46	0.18
C18:0-Et	F8	−28.22	0.01	−28.17	0.02
C20:0-Me	F8	−30.68	0.02	−30.50	0.15
C20:0-Et	F8	−26.10	0.03	−26.11	0.17
Naphthalene	IS	−25.90	0.02	−25.66	0.12
C15:0-Me	IS	−34.39	0.05	−34.41	0.10
C19:0-Me	IS	−30.63	0.01	−30.59	0.15

*F8, $n = 3$, determined by off-line IRMS analysis by A. Schimmelmann. IS $n = 3$ determined in-house by EA-IRMS.
#F8 determined by 3 µL injections by GC-C-IRMS analysis (n = 5). Naphthalene, C15:0-Me and C19:0-Me are determined by 1 µL injections (n = 10).

Table 2. Quantitative and $\delta^{13}C$ response of Supelco 37 FAME mix*

Peak #	Parent fatty acid	$\delta^{13}C$ (‰)	± s.d.	RF	LOD (nmol)
1	C10:0	−32.60	0.11	3.78	2.44
2	C11:0	−28.96	0.13	3.63	1.11
3	C12:0	−30.54	0.13	3.62	1.90
4	C13:0	−31.48	0.25	3.62	0.86
5	C14:0	−30.15	0.21	3.78	1.58
6	C14:1	−19.16	0.19	3.78	0.77
7	C15:0	−27.64	0.21	3.92	0.72
8	C15:1	−20.78	0.16	3.97	0.65
9	C16:0	−31.38	0.13	4.17	1.92
10	C16:1	−28.92	0.24	4.07	0.64
11	C17:0	−31.58	0.23	4.07	0.55
12	C17:1	−29.01	0.14	4.34	0.56
13	C18:0	−30.22	0.18	4.52	1.00
14	C18:1n9c + t	−30.78	0.14	5.10	1.10
15	C18:2n6c + t	−31.21	0.07	4.54	0.97
16	C18:3n3	−31.35	0.19	4.25	0.49
17	C18:3n6	−31.42	0.22	4.33	0.50
18	C20:0	−30.58	0.28	5.04	0.71
19	C20:1n9	−29.14	0.26	4.95	0.36
20	C20:2	−32.18	0.22	4.88	0.37
21	C20:3n3	−15.55	0.28	4.30	0.39
22	C20:3n6 + C21:0	−30.52	0.12	–	–
23	C20:4n6	−32.80	0.38	4.50	0.37
24	C20:5n3#	–	–	4.71	0.31
25	C22:0#	–	–	4.96	0.58
26	C22:1n9	−30.62	0.23	5.13	0.29
27	C22:2	−32.99	0.15	5.04	0.26
28	C23:0	−29.34	0.23	5.14	0.31

*Response factor (RF) is the slope of a calibration expressed as µg/nA × s, from 3 µL injections of serially diluted stock solution (6 calibration levels, 10–1320 ng individual FAME, $r^2 = 0.99$ for all compounds). Co-eluting peaks are considered additive.
#C20:5n3 and C22:0 were significantly overlapping; thus their $\delta^{13}C$ values are omitted.
The reported limit of detection (LOD) is the amount of analyte injected on-column.

Unlike those of the F8 mixture and IS compounds, the $\delta^{13}C$ values of the Supelco 37 FAMEs have not been determined. They thus serve to evaluate the precision of the $\delta^{13}C$ measurement of FAMEs expected in biological samples and food lipids (Table 2). On injecting increasing concentrations of Supelco 37, we did not find a systematic dependence of $\delta^{13}C$ value with peak area. Therefore, we conclude that a correction for linearity effect was not necessary for these analyses. However, the mean standard deviation of the measurement is ±0.21‰, similar in magnitude to the variability introduced with increasing mass from the F8 solution. As noted above, internal standards were not included in this analysis, thus precluding the linear mass calibration usually applied to samples. However, as the variability was comparable with that obtained for the F8 solution, this indicates that the $\delta^{13}C$ measurements were robust for all the FAMEs expected in the analysis of biological and food lipids.

Quantitative response of IRMS

The Supelco 37 FAME mix was used to assess the quantitative response and precision of the GC-C-IRMS system (Table 2). Calibration curves for each compound were calculated through a least-squares regression analysis of the measured total ion count (TIC, *m/z* 44 + 45) peak area as a function of concentration for triplicate injections. Co-eluting *cis/trans* isomers were considered to be additive. The quantitative response of each compound was linear with a mean $r^2 = 0.99$ (for 28 peaks) over a range from 50 to 1320 ppm, which is comparable with results from GC-FID or GC-MS systems used to quantify FAMEs.[30] The response factor increased regularly with chain length, reflecting the mechanism of detection whereby the analyte is quantitatively converted into CO_2 (Table 2). Whereas other techniques, such as GC-MS or GC-FID, are characterized by limits of detection (LODs) in the sub-picomole range injected on-column,[30] the LODs here are in the low- to sub-nanomole range injected on-column, which is typical for IRMS instrumentation.[20] The sensitivity of the IRMS system can be increased to decrease the LOD.

It is often assumed in the analysis of FAMEs that as the response factors are fairly similar, the relative proportion of a compound in a mixture can be calculated by taking the ratio of one FAME area to the sum of the areas of all the FAMEs. Dodds *et al.* clearly demonstrated the error in this for several detection methods including FID,[30] and the same holds true for GC-C-IRMS for similar mechanistic reasons: the detector is sensitive to CO_2, not to the parent FAME, and therefore the response factor must increase with chain length. Re-calculating the actual percentage composition of each FAME component in Supelco 37 to reflect those that are detected (C10:0-Me to C23:0-Me), we compared the two methods for the injection of 1 μL of a 1:1 diluted solution. The average precision of the integrated area for each peak was ±1.3% (n = 4). Using only the total areas to calculate the relative composition of each FAME in the mix resulted in a mean relative accuracy of ±5.8%, while using the calibration regression obtained for each component to calculate the mass of the components gave a mean accuracy of ±1.6%. The results obtained from the full calculation of FAME concentrations in the sample, therefore, provide the more accurate approach for quantitative GC-C-IRMS analysis.

Transesterification

With the aim of increasing sample throughput as well using a milder reagent, the transesterification reaction was tested for catalyst concentration and reaction time using corn oil, comparing corn oil-derived triacylglycerides (TAGs) and free fatty acids (FFAs), C15:0 and C19:0 (Fig. 2). The reaction was quantitative for FFAs tested over a range from ~20 to 1000 ng (40–2000 ppm final solution) ($r^2 = 0.99$, six levels) with highly reproducible and accurate $\delta^{13}C$ values (−34.17 ± 0.04‰ and −30.23 ± 0.08‰, n = 6 for 15:0 and 19:0, respectively) at all [AcCl] and time levels tested. Thus, FAMEs can be prepared under the mildest conditions tested from FFAs. Lower levels and faster reaction times were not evaluated.

FAMEs derived from TAGs, however, did show increasing yield as a function of both reaction time and [AcCl] (Fig. 2(A)). The maximum yield of FAMEs from TAGs (mean of 97.2 ± 1.4% for 16:0, 18:1 and 18:2) came from the use of 10 % AcCl for 60 min. The fatty acid compositional profile (% of each fatty acid) remained invariant regardless of [AcCl] or reaction time (Table 3). Unlike what was found for the FFAs, the $\delta^{13}C$ values of the FAMEs derived from the TAGs did show fractionation with regard to reaction conditions (Fig. 2(B)). The $\delta^{13}C$ values increased with reaction yield, by an average of 0.73 ± 0.04‰, from the mildest (2% AcCl for 10 min)

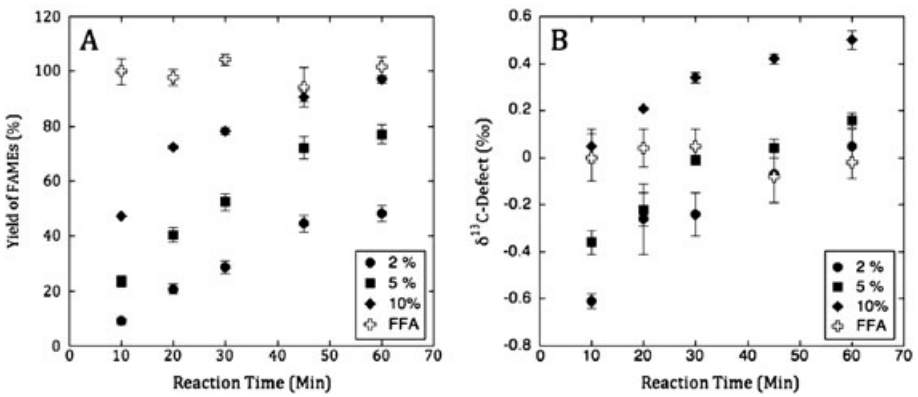

Figure 2. (A) Yield and (B) $\delta^{13}C$ defect of TAG (dark) and FFA (clear) at differing catalyst levels as a function of time. Yield is based on mass of TAG originally in the sample. Percent values refer to acetyl chloride concentration.

Table 3. Relative quantitative and isotope values of corn oil derived FAME subject to variable reaction conditions*

Compound	% (± s.d.)	Range (%)	$\Delta\delta^{13}C$ (‰) ± s.d.#	Range
16:0	13.6 ± 0.6	13.1–15.1	0.88 ± 0.16	0.46–1.15
18:1	23.1 ± 0.2	22.6–23.4	0.12 ± 0.09	−0.08–1.09
18:2	63.4 ± 0.4	62.4–63.7	−0.23 ± 0.04	−0.03 to −0.17

*Mean from 15 experiments in total, covering 3 concentration levels and 5 time periods.
#Relative to the reconstructed bulk $\delta^{13}C$ value.

to the strongest (10% AcCl for 60 min) conditions for the three FAMEs measured. As with the compositional profile data, the relative $\delta^{13}C$ value of each fatty acid remained consistent (Table 3), with the order (from heaviest to lightest) being 18:2 > 18:1 > 16:0, as reported for some maize oils.[8,9] These results indicated that the optimal reaction conditions for FAME sample preparation prior to GC-C-IRMS analysis depends largely on the goals of the experiment; i.e., whether the study requires accuracy for an 'absolute' $\delta^{13}C$ value (e.g., authentication) or if the aim is to obtain a relative enrichment factor (e.g., tracer flux studies). In the case where accuracy is desired, an assessment of reaction yield must be conducted during method development because less than complete derivatization will systematically skew the $\delta^{13}C$ value, potentially resulting in erroneous interpretation. The FFAs did not demonstrate any such consistent differences and the mildest conditions will give quantitative and isotopic results that are both accurate and reproducible.

Evaluation of transesterification bias

One of the more common applications of GC-C-IRMS in the food sciences is in the analysis of vegetable oils which, when coupled with quantitative data, provides a powerful tool for their authentication.[6–9] The above method was applied to a suite of seven vegetable oils (canola, palm, soy, peanut, corn and olive), and their mixtures, as well as oils extracted from 30 French fry samples. The pure oils and oils extracted from French fries had previously been measured for their $\delta^{13}C$ values by EA-IRMS.[25] Recalculating the bulk $\delta^{13}C$ value with Eqn. (3) is a useful approach to determine bias if the GC-C-IRMS system measures the components that make up the majority composition of a sample. Depending on purity, fatty acids comprise over 90% of the total mass of oils so this approach should work well. The specific fatty acid profile and $\delta^{13}C$ values of the pure oils matched well with their literature values,[31] while the recalculated isotope values ($\delta^{13}C_R$) using Eqn. (3) compared excellently with their independently measured bulk values ($\delta^{13}C_B$) (Table 4). The difference between values ($\Delta^{13}C$) did not exceed the standard deviation of the calculation. A Bland-Altman analysis showed no systematic bias in $\Delta^{13}C$ with increasing $\delta^{13}C_B$ and the mean offset is 0.08‰ (Fig. 3(A)). Oils that were extracted from fries resulted in a higher confidence limit interval and increased bias (Fig. 3(B)), but again did not show a systematic trend with changing $\delta^{13}C$ value. The offset of ~0.5‰ from the extracted oils can be explained since the EA-IRMS analysis will encompass additional impurities that are outside the GC-C-IRMS window of analysis for this study. Therefore, one should be cautious if using a Bland-Altman plot to evaluate the accuracy of GC-C-IRMS data compared with bulk since components that fall outside the analytical window of this technique could skew the interpretation. However, the generally good agreement between the bulk and GC-C-IRMS data indicates that the simultaneous quantitative and isotope approach works very well and it should be exploited when the two values are desired.

Table 4. Quantitative and $\delta^{13}C$ profiles of a selection of vegetable oils analyzed by GC-C-IRMS with recalculated bulk $\delta^{13}C$ values. Only major constituents present in appreciable amounts (>1%) in most oils are shown*

	Canola		Palm		Soy		Peanut		Corn		Olive	
Fatty acid	%	$\delta^{13}C$	%	$\delta^{13}C$	%	$\delta^{13}C$	%	$\delta^{13}C$	%	$\delta^{13}C$	%	$\delta^{13}C$
16:0	5.4	−30.36	47.3	−29.72	12.5	−31.65	11.7	−29.63	13.2	−16.45	16.7	−30.01
18:0	n.d.	n.d.	5.0	−30.39	5.1	−32.79	2.8	−30.69	1.8	−16.00	2.9	−31.45
18:1	62.6	−29.89	39.1	−29.20	23.5	−31.02	51.5	−28.79	23.1	−16.01	65.8	−29.52
18:2	23.0	−30.56	7.6	−29.92	58.9	−31.50	28.2	−29.47	63.4	−16.10	13.0	−31.30
20:0	8.9	−33.92	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$\delta^{13}C_R$ (‰)†	−30.4		−29.6		−31.5		−29.3		−16.1		−29.9	
s.d.†	1.6		1.2		1.3		0.9		1.0		1.2	
$\Delta^{13}C$ (‰)#	0.4		1.2		0.3		<0.1		0.7		0.4	

*From triplicate analyses of each oil. Other fatty acids detected: palm oil 14:0 (0.9%, −31.49‰), peanut oil 22:0 (1.3%, −32.31‰), 22:1 (1.6%, −29.40‰), 24:0 (3.0%, −31.21‰), olive oil 16:1 (1.7%, −30.28‰). n.d. = not detected.
†Based on Eqn. (3). Standard deviation is based on the propagated error and is reported in ‰.
#Difference between $\delta^{13}C_R$ and the measured bulk value.

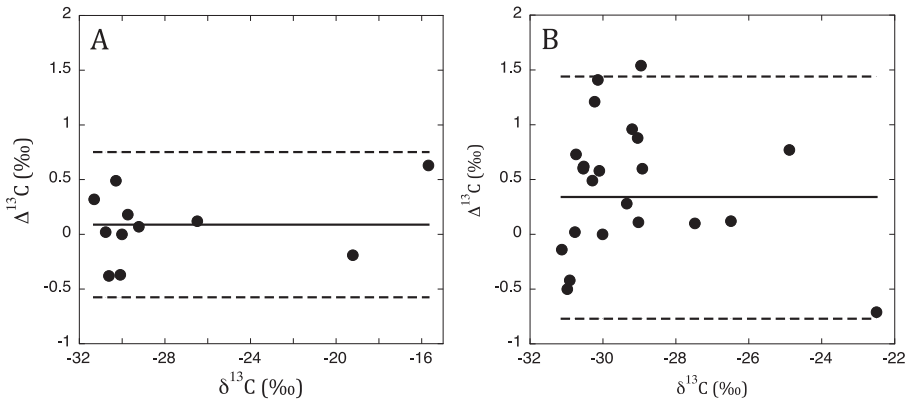

Figure 3. Bland-Altman plots comparing bulk $\delta^{13}C$ and $\delta^{13}C_R$ for (A) pure commercial vegetable oils and (B) oils extracted from restaurant French fries. Dashed lines are the 95% confidence interval limits and the solid line is the average bias within each data set.

CONCLUSIONS

In this work, we demonstrated that GC-C-IRMS can be used as the method of choice where both $\delta^{13}C$ data and analyte concentration data are desired, thus eliminating the need for additional analyses such as GC-FID. We also further highlighted that internal standards must be included for more precise $\delta^{13}C$ analysis. In addition, the single-step preparative method for FAME analysis is a safe and rapid approach that results in accurate, precise and reproducible analyte concentration data in addition to $\delta^{13}C$ values. Therefore, the approach reported here is ideal for applications such as ingredient authentication or in metabolic flux studies.

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