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Journal of Chromatography A, 752 (1996) 233–241

JOURNAL OF CHROMATOGRAPHY A

Influence of gas chromatographic parameters on measurement of $^{13}C/^{12}C$ isotope ratios by gas–liquid chromatography–combustion isotope ratio mass spectrometry. I

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Received 10 April 1996; revised 13 May 1996; accepted 23 May 1996

Abstract

A potential influence of gas chromatographic parameters on measured $^{13}C/^{12}C$-isotope ratios of saturated fatty acid methyl esters in gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) has been studied. A dependence of measured isotope ratios on temperature gradients was observed and differences in measured $\delta^{13}C$-values for individual fatty acid methyl esters varied by 0.5 to 3‰. Possible reasons for this isotopic fractionation and its implications for ^{13}C natural abundance work are discussed.

Keywords: Combustion isotope ratio mass spectrometry; Isotope effects; Fatty acid methyl esters; Carbon 13

1. Introduction

Since the commercial availability of gas isotope ratio mass spectrometers (IRMS) directly coupled to a gas chromatograph (GC) via an on-line combustion interface (C), based on the design by Matthews and Hayes [1], GC–C–IRMS has become a powerful tool in all areas of applied analytical chemistry [2].

One inherent problem of this technique, however, has attracted little interest so far. The analysis of medium to high boiling point organic compounds by gas–liquid chromatography (GLC) is associated with two potential sources for isotopic fractionation. Kinetic isotope effects are associated with all derivatisation reactions that proceed by the cleavage or the formation of a carbon-containing bond. This effect has been noted for example in trifluoroacetylation of amino acids [3] and an in-depth examination of this effect in general has been reported recently [4].

Apart from that kinetic isotope effect there also exists a thermodynamic isotope effect that is associated with GLC of isotopically substituted compounds [5]. This effect is commonly known as the "inverse" chromatographic isotope effect [6] because the heavier isotopomer elutes earlier than the unlabelled substance. For ^{14}C-labelled fatty acids of higher molecular weight this effect can be observed by gas chromatography mass spectrometry [7]. To the best of our knowledge, its consequences for GC–C–IRMS measurements of organic compounds of natu-

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PII S0021-9673(96)00498-0

ral abundance in ^{13}C have not been investigated so far.

In this paper, we report on isotopic fractionation of saturated fatty acid methyl esters, naturally abundant in ^{13}C, observed during $\delta^{13}C$ measurements at varying gas chromatographic conditions. A tentative explanation for the surprisingly high magnitude of the isotope effect and conclusions as to practical consequences are presented.

2. Experimental

2.1. Chemicals

Individual fatty acid methyl esters (FAME) and National Health Institute (NHI) FAME reference mixtures were obtained from Sigma (St. Louis, MO, USA). Solvents were obtained from Fluka (Buchs, Switzerland) and were of >99.7% purity.

Sample 1 was prepared by mixing 2 μl of each individual FAME (C6, C10, C11, C12 and C14) and diluting this mixture in 1 ml of *n*-heptane. Sample 2 was prepared by adding 2 μl of each C6 and C11 FAME to 10 μl of FAME mixture NHI-C, containing FAMEs C8 through to C20 in increasing weight%, and diluting the resulting mixture in 1 ml of *n*-heptane.

2.2. Gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS)

Studies were carried out on an ORCHID GC–C–IRMS system (Europa Scientific, Crewe, UK) unless stated otherwise. The combustion interface, comprising a combustion furnace operated at 820°C (CuO/Pt wires), a Nafion tube acting as water separator, and a reduction furnace operated at 600°C (Cu wires) transformed sample peaks eluted from the GC into dry CO_2 and N_2. In ^{13}C-studies, measured isotope ratios are automatically corrected for contributions from ^{17}O [8] and expressed as δ-values in per mille [‰] units:

$$\delta^{13}C = (R_{sample} - R_{standard})/R_{standard} \times 1000 \quad (1)$$

where R_{sample} is the $^{13}C/^{12}C$ ratio of the sample and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of the working standard. Since this study was only concerned with changes rather than absolute values, in all experiments methylcaproate (C6) was initially chosen as internal standard and its $\delta^{13}C$-value was arbitrarily set to zero.

2.3. Gas chromatography (GC)

The gas chromatograph was an HP 5890, Series II, fitted with an electronic pressure control (EPC) thus permitting the carrier gas management to be set either to constant pressure or to constant flow mode.

Helium of 5.0 purity was used as carrier gas and was passed through a high capacity gas purifier (Supelco, Poole, UK) before entering the GC. Column head pressure was controlled by an EPC. In either mode, carrier gas was set to one of three linear-velocities at a column temperature of 70°C. These velocities were 22.1 cm/s, 28.4 cm/s and 35.4 cm/s, respectively.

Separation of saturated FAMEs was achieved on columns coated with stationary phases of different polarity: a CP-Sil 8 CB capillary column (Chrompack, Middelburg, Netherlands), dimensions 25 m × 0.25 mm, 0.25 μm film thickness and a CP-Sil 19 CB, dimensions 25 m × 0.25 mm, 1.2 μm film thickness. The columns were connected to a retention gap, 1 m × 0.25 mm, that was cyanopropyl/phenyl/methyl deactivated [9].

Column temperature was held at 70°C for 6 min and subsequently programmed to 270°C at one of three temperature gradients. These gradients were 4°C/min, 6°C/min and 8°C/min, respectively. Under these conditions methylcaproate (C6) always eluted at the end of the isothermal step (t_R = 387 s) on the CP-Sil 8 CB column and thus was not subject to any change in GC conditions. For this reason methylcaproate (C6) was initially referred to as internal standard.

The injector temperature was set to 230°C, split flow to 30 ml/min, and identical aliquots of samples 1 and 2 were injected in split and splitless mode, respectively. In case of the latter, split was kept closed for initial 6 s and kept open afterwards. Sample volumes injected were 0.5 μl and 0.2 μl for split and splitless mode, respectively. Thus, in splitless mode 1.74 nmol of methylundecanoate (C11) was injected on the column. With a split ratio of 1:40 at the open split in front of the mass spectrometer ion

source 522 pmol of CO_2 entered the ion source giving rise to a m/z 44 signal area of 12.9 nA s.

2.4. Data analysis

Data were analysed using the manufacturer provided software (Europa Scientific ORCHID POST-PROCESSOR). Peaks are detected by comparing calculated ascending and descending gradients with user-defined threshold slopes for peak start and stop. In this case, slope thresholds of +3 pA/s and −3 pA/s were chosen for start and stop, respectively. Once peak start and stop have been defined for channel 1, i.e. beam m/z 44 in the case of CO_2, the corresponding beams simultaneously measured on channel 2 and 3 are automatically associated with channel 1 at any point in time. Peaks are automatically baseline corrected.

Once a chromatographic peak has been identified, the isotope ratio for that compound is determined by a linear regression fit. The software plots e.g. beam 1 (x axis) against beam 2 (y axis) to produce a profile of the ratio as we move from the bottom of the peak to the top of the peak. A least squared fit (LR^2) is applied to the scatter plot, the result of which is a slope of the line which corresponds to the isotope ratio. The LR^2 fit has the shape of an ellipse which is a function of the isotope shift. The 2/1 and 3/1 ratios of the analytes and the reference compound are determined in exactly the same way.

Data collected on the Delta S (Finnigan, Bremen, Germany) was analysed using the Finnigan software ISODAT. Peaks are detected by calculating start and stop slope for one data channel (beam 1 = m/z 44) and comparing the result to user-defined threshold slopes. Slope thresholds were set to 0.35 mV/s. Peaks are automatically baseline corrected. Once the peak start and stop have been defined for the single channel, the values are extrapolated to the other two channels. Peak maxima are determined for all channels and a time shift is used to correct for chromatographic separation of isotopes [10–13].

3. Results and discussion

3.1. Influence of temperature gradient

Setting the carrier gas to a linear velocity of 28.4 cm/s at the initial temperature of 70°C, sample 1 was analysed at three different temperature gradients and two different carrier gas modes for each gradient. Furthermore, in order to investigate any potential influence of the design of the open split in front of the mass spectrometer on the outcome of the measurement, this experiment was repeated substituting an open split according to Hayes [14] for the open split-cum-isolation valve design used in the ORCHID.

The first experiments with sample 1 clearly showed the presence of an isotope effect (Table 1).

Table 1
Dependence of relative $\delta^{13}C$-values of split injected saturated fatty acid methyl esters on temperature gradient

	C10 Const. pressure[a]	C11 Const. pressure[a]	C12 Const. pressure[a]	C14 Const. pressure[a]
Orchid open split				
4°C/min	−8.41±0.04	−8.09±0.15	−6.32±0.18	−6.89±0.06
6°C/min	−6.09±0.11	−5.84±0.15	−3.76±0.17	−5.22±0.12
8°C/min	−5.10±0.11	−4.88±0.09	−3.36±0.21	−4.73±0.18
$\Delta\delta$ (4°–8°)	+3.31±0.12	+3.21±0.17	+2.96±0.28	+2.16±0.19
J. Hayes open split				
4°C/min	−8.00±0.08	−7.87±0.15	−5.63±0.19	−6.54±0.26
6°C/min	−6.12±0.03	−5.49±0.16	−3.85±0.20	−5.21±0.14
8°C/min	−5.50±0.21	−4.73±0.11	−3.37±0.09	−4.47±0.10
$\Delta\delta$ (4°–8°)	+2.50±0.22	+3.14±0.22	+2.26±0.21	+1.83±0.28

All relatives $\delta^{13}C$-values in [‰] are given as mean±standard deviation of 5 repetitions. Identical aliquots of sample 1 were injected in split mode. Split flow was set to 30 ml/min.

[a] Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C. Column used was a CP-Sil 8 CB.

The shift towards higher ^{13}C-enrichment was independent of the design of the open split in front of the mass spectrometer ion source.

To rule out the influence of isotopic fractionation during injection (see below) and to study the extent of this effect on higher boiling FAMEs further experiments with sample 2 were carried out using splitless injection.

The results of these experiments are presented in Fig. 1A. Data were evaluated and tested for significant difference on the basis of 95% confidence limits as well as the observed precision ($\pm 2\sigma$). Choosing C6 as internal reference peak, a significant shift from lower to higher δ-values could be detected when the results obtained for 4°C/min were compared with those obtained for 6°C/min and 8°C/min. On changing from 4°C/min to 8°C/min ramp rate, the difference in δ-values ranged from +2.58 to +1.02‰ with differences between 4 and 8°C/min being 2 to 3 times higher than corresponding differences between 6 and 8°C/min. The $\delta^{13}C$-values for FAMEs C14 to C20 measured at 6 and 8°C/min showed no significant differences within 2σ.

The way samples were injected showed, not surprisingly, a noticeable effect. The δ-values for FAME C11 should have shown no variation for a given set of GC parameters when compared to results obtained for sample 1, yet they were shifted to higher δ-values by 1.43‰ on average when samples were injected splitless for 6 s. In the absence of any other variables this effect was attributed to isotopic fractionation in the injector of the relatively low boiling FAME C6 that was used as internal standard.

In order to rule out influences beyond the control of a standardised protocol, we re-evaluated our data from the experiments with sample 2 setting either C11 or C18 as the active internal reference peak (Fig. 1B and C). With C11 as reference peak, $\delta^{13}C$-values for FAMEs C14 to C20 were no longer significantly different within 2δ. When C18 was chosen as reference peak all but two $\delta^{13}C$-values became virtually identical ($\pm 2\sigma$).

These results prompted us to repeat these experiments on a column coated with a stationary phase of medium polarity. Polymethylsiloxane (PMS) phases that have a 7% phenyl, 7% cyanopropyl substitution (e.g. CP-Sil 19 CB) generally show a higher selectivity for FAMEs than apolar phases such as CP-Sil 5 CB (100% PMS) or PMS with a 5% phenyl substitution (e.g. CP-Sil 8 CB).

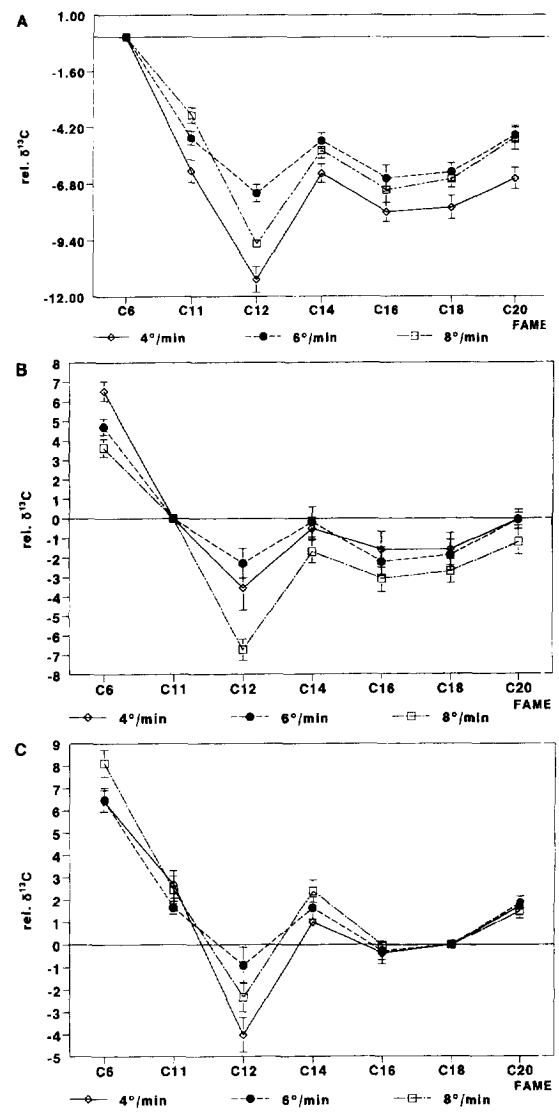

Fig. 1. Effect of GC parameters and choice of reference peak on relative $\delta^{13}C$-values without time shift correction. Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 8 CB column. Linear carrier gas velocity was set to 28.4 cm/s at a column temperature of 70°C. Values are the mean of 5 repetitions. Error bars are $\pm 2\sigma$. (A) $\delta^{13}C$-values calculated vs. $\delta^{13}C(C6) = 0.000‰$; (B) $\delta^{13}C$-values calculated vs. $\delta^{13}C(C11) = 0.000‰$; (C) $\delta^{13}C$-values calculated vs. $\delta^{13}C(C18) = 0.000‰$.

Interestingly enough, with this stationary phase δ^{13}-values for all FAMEs were identical within $\pm 2\sigma$

when calculated using C6 as internal reference peak (Fig. 2A). When calculated vs. C11 as internal reference peak δ^{13}C-values for C16, C18 and C20 obtained at 4°C/min were significantly higher by 2‰ on average compared to their corresponding δ^{13}C-values obtained at 6 and 8°C/min (Fig. 2B). When C18 was set as internal reference peak all but three δ^{13}C-values became virtually identical within $\pm 2\sigma$.

Since all experiments were carried out under standardised conditions with respect to sample, injected sample volume, carrier gas velocity, temperature gradients, data evaluation and instrumental set-up, the different outcome of the experiments depicted in Figs. 1 and 2 could only be attributed to the differences in stationary phase properties. Furthermore, these findings clearly demonstrate that the calculated isotopic abundance depends on the choice of reference peak, i.e. on the overall chromatographic conditions (GC plus combustion interface) at the point in time represented by this peak. Hence, when no time shift correction is employed to compensate for chromatographic isotope effects the choice of internal reference peak has a significant impact on observed δ^{13}C-values.

The same set of experiments with sample 2 was carried through on another GC–C–IRMS instrument (Delta S). Since Finnigan's software ISODAT employed a time shift correction to compensate for chromatographic separation of isotopomers [10–13] we anticipated not being able to observe this effect here. This, indeed, was the case as only a slight trend towards lower ^{13}C-values was detected when changing the temperature gradient from 8°C/min to 4°C/min (Fig. 3). However, the measured changes in δ^{13}C-values ranging from 0.13 to 0.65‰ lay well within the observed precision ($\pm 2\sigma$).

When we compared the results from both instruments obtained at 6°C/min on an apolar stationary phase with C6 as internal reference peak, time shift corrected δ^{13}C-values were on average 1.31‰ higher.

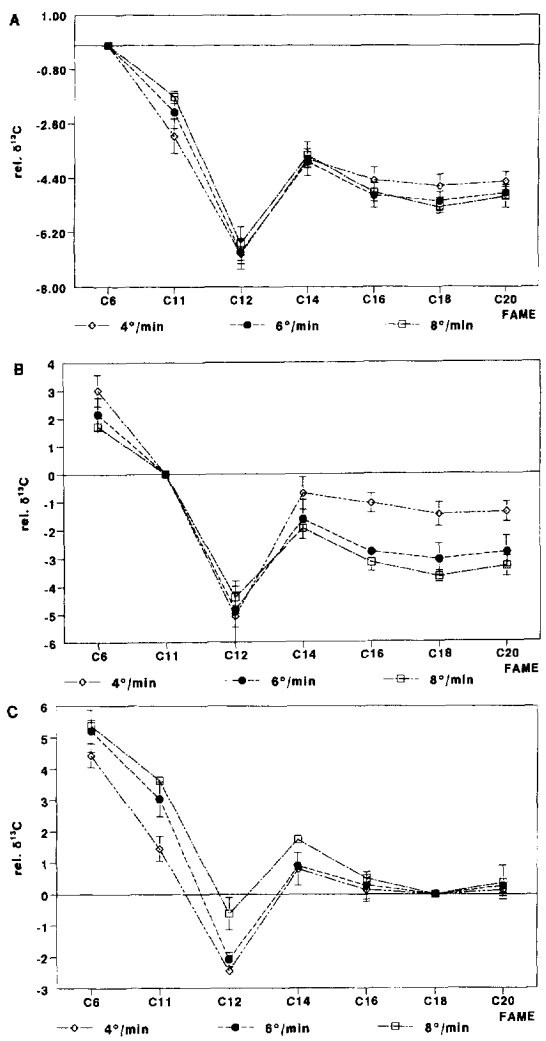

Fig. 2. Effect of GC parameters and choice of reference peak on relative δ^{13}C-values without time shift correction. Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 19 CB column. Linear carrier gas velocity was set to 28.4 cm/s at a column temperature of 70°C. Values are the mean of 3 repetitions. Error bars are $\pm 2\sigma$. (A) δ^{13}C-values calculated vs. δ^{13}C(C6)=0.000‰; (B) δ^{13}C-values calculated vs. δ^{13}C(C11)=0.000‰; (C) δ^{13}C-values calculated vs. δ^{13}C(C18)=0.000‰.

3.2. Influence of carrier gas flow

The studies on the influence of temperature gradient on ^{13}C/^{12}C isotope ratios were carried out by setting the carrier gas flow to its optimum linear velocity of 28.4 cm/s [15] corresponding to a flow-rate of 1.12 ml/min. To monitor a potential influence of actual carrier gas flow, these studies were run under both constant pressure and constant flow conditions. When a constant column head pressure is applied the actual carrier gas flow decreases as the temperature increases because of the gas viscosity-temperature dependency. To compensate for that, in

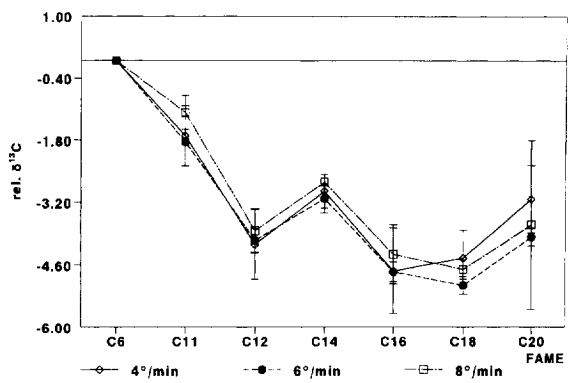

Fig. 3. Effect of GC parameters and choice of reference peak on relative δ^{13}C-values when time shift correction is employed (Delta S). Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 5 CB column. Linear carrier gas velocity was set to 36 cm/s at a column temperature of 70°C. Values are the mean of 3 repetitions; error bars are $\pm 2\sigma$; δ^{13}C-values were calculated vs. δ^{13}C(C6) = 0.000‰.

constant flow mode the column head pressure is increased electronically over the temperature gradient.

Although the observed differences in δ^{13}C-values were not significantly different, at temperature gradients of 6 and 8°C/min a trend towards slightly lower δ^{13}C-values going from constant pressure to constant flow mode could be noticed and the results for 6°C/min are presented in Table 2.

Analyses run at higher column head pressure or higher flow rate yielded slightly lower δ^{13}C-values as compared with δ^{13}C-values observed at lower pressure or lower flow rate (Table 2), an effect that appeared to decrease with increasing chain length. Comparing results between a constant pressure setting and its corresponding constant flow setting also did not show significant differences. Once again a trend could be detected showing a slight ^{13}C-depletion for FAMEs run at constant flow compared with δ^{13}C-values obtained from the FAMEs when run at corresponding constant pressure mode.

3.3. Discussion

Although the observation of an isotope effect in GC-combustion came as no surprise, however, the magnitude of this effect was unexpected. The influence of GC parameters on the separation of stable isotope labelled compounds from their unlabelled analogues is well known [6,16–19]. Employing e.g. ^{2}H-labelled compounds, this effect is exploited in stable isotope dilution analysis as well as for internal standardisation and quantification [20] because introduction of deuterium atoms into a molecule results in a significantly decreased retention time [21]. Matucha et al. [7] investigated this phenomenon on deuterated *n*-alkanes and ^{14}C-labelled methyl esters of palmitic and oleic acid. Uniformally ^{14}C-labelled FAMEs eluted 1.8 s earlier than their unlabelled analogues. Matucha and coworkers clearly showed that this phenomenon was caused by lower molar volumes of the labelled, and thus heavier, compounds and resulting changes in chromatographic solute–stationary phase interaction. Our own observations showed that for di-tBDMS derivatives of $^{2}H_3$-labelled L-leucine and unlabelled L-leucine separation increased by 0.9 s going from 8°C/min to 4°C/min at a constant pressure of 8 p.s.i. and increased by 0.48 s going from 8 p.s.i. to 16 p.s.i. at a temperature gradient of 6°C/min.

As to the exact nature of the isotope effect observed in this study we can but speculate. However, our observations are consistent with the assumption that the separation of isotopomers in the GC column, i.e. solute-stationary phase interaction dominated by Van der Waals dispersion forces leading to an earlier elution of the heavier isotopomer [7], is one key factor in the observed isotope effect.

To test this hypothesis we analysed sample 2 again using a temperature gradient of 4°C/min but having set the column head pressure to 18.2 p.s.i. Under these conditions the heavier isotopomers should elute even earlier and, hence, should result in even lower δ^{13}C-values compared with the values obtained for 4°C/min at 14.4 p.s.i. This experiment yielded δ^{13}C-values on average 1‰ lower than those observed for 4°C/min and 14.4 p.s.i. which substantiated our findings.

Using different temperature gradient under constant column head pressure had a markedly effect on retention time and the separation of the FAMEs. Peak shapes however, were not significantly effected (Table 3). Despite the considerable changes in separation of the individual FAMEs we were not able to determine the difference in retention times for the isotopomers. Time difference between mass

Table 2
Dependence of relative $\delta^{13}C$-values of splitless injected saturated fatty acid methyl esters on carrier gas parameters

	11.1 p.s.i. Const. pressure[a]	0.8 ml/min Const. flow[a]	14.4 p.s.i. Const. pressure[b]	1.12 ml/min Const. flow[b]	18.2 p.s.i. Const. pressure[c]	1.54 ml/min Const. flow[c]	$\Delta\delta$ (11.1–18.2 p.s.i.)	$\Delta\delta$ (0.8–1.54 ml/min)
J. Hayes open split								
C10	−1.37±0.89	−1.64±1.05	−2.11±0.81	−3.34±0.06	−2.23±1.00	n.d.	−0.86±1.30	n.d.
C11	−4.35±0.19	−4.89±0.05	−5.05±0.04	−5.34±0.03	−5.12±0.31	−5.56±0.12	−0.77±0.36	−0.67±0.13
C12	−3.48±0.22	−3.57±0.17	−3.72±0.52	−3.67±0.07	−0.386±0.67	−3.38±0.09	−0.38±0.71	+0.19±0.10
C13	−3.92±0.18	−3.86±0.16	−3.97±0.22	−4.32±0.08	−3.91±0.11	−4.39±0.03	+0.01±0.21	−0.53±0.16
C16	−6.59±0.15	−6.69±0.13	−6.97±0.43	−6.76±0.08	−6.65±0.18	−6.89±0.20	−0.06±0.23	−0.20±0.24
C18	−6.36±0.10	−6.80±0.14	−6.79±0.31	−6.85±0.12	−6.67±0.20	−6.90±0.11	−0.31±0.22	−0.10±0.18
C20	−4.76±0.22	−5.08±0.15	−5.01±0.25	−5.18±0.02	−5.01±0.13	−5.05±0.10	−0.25±0.26	+0.03±0.18

All relative $\delta^{13}C$-values in [‰] are given as mean±standard deviation of 5 repetitions. Identical aliquots of sample 2 were injected in splitless mode. Split was kept closed for 6 s and opened thereafter for the rest of the run. Split flow was set to 30 ml/min. Column temperature was held at 70°C for 6 min and then programmed to 270°C at 6°C/min. Column used was a CP-Sil 8 CB.

[a] Column head pressure of 11.1 p.s.i. corresponding to a flow-rate of 0.80 ml/min and a linear velocity of 22.1 cm/s at a column temperature of 70°C.

[b] Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C.

[c] Column head pressure of 18.2 p.s.i. corresponding to a flow-rate of 1.54 ml/min and a linear velocity of 35.4 cm/s at a column temperature of 70°C.

Table 3
Retention times t_R and peak width t_W of saturated fatty acid methyl esters for various temperature gradients

		C6 Const. pressure[a]	C11 Const. pressure[a]	C12 Const. pressure[a]	C14 Const. pressure[a]	C16 Const. pressure[a]	C18 Const. pressure[a]	C20 Const. pressure[a]
4°C/min	t_R	387±0.5	1613±0.5	1812±0.5	2177±0.5	2507±0.5	2807±0.5	3084±0.5
	t_W	30±0.5	32±0.5	24±0.5	27±0.5	28±0.5	28±0.5	29±0.5
6°C/min	t_R	387±0.5	1329±0.5	1466±0.5	1714±0.5	1938±0.5	2142±0.5	2329±0.5
	t_W	30±0.5	31±0.5	24±0.5	27±0.5	28±0.5	28±0.5	29±0.5
8°C/min	t_R	387±0.5	1168±0.5	1274±0.5	1463±0.5	1634±0.5	1789±0.5	1934±0.5
	t_W	29±0.5	30±0.5	22±0.5	24±0.5	25±0.5	25±0.5	27±0.5
Δt_R (8–4°C)		0	445	538	714	873	1018	1150
Δt_W (8–4°C)		1	445	538	3	3	3	2

Retention times t_R and peak width t_W (s) are given as mean±standard deviation of 5 repetitions. Identical aliquots of sample 2 were injected in splitless mode. Split was kept closed for 6 s and opened thereafter for the rest of the run. Split flow was set to 30 ml/min.

[a] Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C. Column used was a CP-Sil 8 CB.

traces m/z 44 and m/z 45 was of the order of 100 ms apex to apex, but, changes from one temperature gradient to another were too small to be determined with sufficient precision.

However, the surprisingly high magnitude of this isotope effect for samples of natural abundance in ^{13}C in conjunction with one internal reference peak could be due to an amplification of this effect caused by the combustion interface. Since the compounds eluting from the column are combusted into CO_2 (and H_2O), a molecule of low molecular mass and as such much more susceptible to isotopic fractionation, that is subsequently transferred into the mass spectrometer passing on its way numerous connections and changes in capillary diameter, it can be speculated that the passage through the combustion interface amplifies the isotope effect in GLC. The distortion of peak shapes by the combustion interface and its resulting influence on precision has been discussed by Goodman and Brenna [13].

4. Conclusion

The isotope effect in GC–C–IRMS and the various influences of GC parameters on this effect have been confirmed for compounds of natural abundance in ^{13}C. It is small but reliably measurable with modern isotope ratio mass spectrometers thus adding studies relating to the theory of gas chromatography to the list of GC–C–IRMS applications, provided the data evaluation software allows to calculate the isotope ratios without time shift correction. On the other hand, the results of this study show that time shift correction is one powerful tool to compensate for any isotope effect occurring during GC-combustion.

Especially in typical GC–C–IRMS applications of natural abundance work such as food adulteration, aroma and perfume adulteration, forensics and environmental studies, the isotope effects occurring in GLC should be taken into account.

Practical consequences are (1) once established, to stick to one protocol for a certain application, (2) to inject samples splitless to avoid potential isotopic fractionation of lower boiling compounds, (3) not to compare, or only with caution, $\delta^{13}C$-values for a given compound obtained by different methods and different instruments, and (4) rather than comparing "absolute" values, to compare $\delta^{13}C$-values from an unknown sample with those from an authentic sample measured under identical conditions and/or to apply the "finger print" method [22].

Furthermore, these findings emphasise the necessity to use an internal standard of calibrated isotope ratio that is subject to the same physical conditions as the analytes of interest introduced into the GC–C–IRMS system [2]. The results reported in this paper show that adding one internal standard to the sample to be analysed may not be enough since the magnitude of the isotope effect varies over the course of the GC analysis and depends on the relative position of the reference peak in the chromatogram. Adding an internal standard mixture, however, will create unwanted problems caused by co-elution or at least overlap of analytes and individual internal standards. Investigations as to how this problem can be overcome are almost finished and will be reported at a later date [23,24].

Acknowledgments

This work was supported by the European Community, grant no. MAT1-CT-94031 as part of the third framework.

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