Stable Isotopes in Paediatric Nutritional and Metabolic Research

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High-Precision Measurement of $^{13}C/^{12}C$ Ratios by On-Line Combustion of GC Eluates and Isotope Ratio Mass Spectrometry

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Introduction

The stable isotopes of carbon (^{12}C,^{13}C) are widely used in natural sciences: in geology, by measuring isotope ratios; in organic compounds, or in human studies, by using artificially enriched compounds as tracers.

First, the natural variations of the $^{13}C/^{12}CO_2$ ratio are studied by means of isotope ratio mass spectrometry (IRMS). Various processes fractionate the carbon isotopes in nature. The observed isotope ratios provide information on these processes, for example on palaeotemperatures or on specific chemical or biological reactions; or they can be used, for instance, to trace the origin and/or genesis of a specific compound.

Secondly, the isotopic content of ^{13}C in samples artificially enriched with ^{13}C-labelled components is measured, usually with combined gas chromatography/mass spectrometry (GC/MS). This replaces ^{14}C labelling and thus avoids any radiation hazards. This method is used preferentially in biology, biochemistry or clinical and environmental research, where medical ethics discourage the use of radioactive tracers for investigative purposes.

In both application areas very small amounts of ^{13}C must be detected. On the one hand, significant natural variations in the approximately 1.1% ^{13}C abundance (in relation to ^{12}C) are as small as 0.0002 atom%. Hence, small natural variations in the amount of ^{13}C must be detected in the presence of 100 times more ^{12}C. This requires very high precision in measuring the $^{13}C/^{12}C$ ratio, as is normal with IRMS.

On the other hand, in labelling studies, normally only very small amounts of sample are available and the compounds are separated on line in the GC/MS. Hence, the capability for detecting small amounts of ^{13}C is mandatory. From an analytical point of view, this is totally equivalent to the requirement for high

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precision in the $^{13}C/^{12}C$ natural ratio determination in IRMS as only low amounts of ^{13}C-labelled material are present.

The type of compound (organic, inorganic, solid, liquid, gaseous) that must be handled by these mass spectrometric methods is very diverse. No mass spectrometer, IRMS or GC/MS is able to accept such a broad variety of sample types directly for an isotope ratio determination. In $^{13}C/^{12}C$ isotope ratio mass spectrometry each isolated compound of interest is combusted to CO_2 then measured. This greatly simplifies the mass spectrometer and offers two definite advantages towards the achievement of high precision and accuracy: the isotope ratio can always be measured by one and the same sample triple collector, adjusted for the simultaneous recording of masses 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$) and 46 ($^{12}C^{16}O^{18}O$) and one can always use the same inexpensive working standard gas (CO_2) to calibrate the mass spectrometer easily at short time-intervals.

This elegant methodological simplicity in IRMS is counterbalanced by the apparent need of a variety of sample preparation and isolation methods, which are more or less specially adapted to certain compound types. Such methods range from rather special ones, e.g. the reaction of carbonates with phosphoric acid to yield CO_2, to more universally applicable processes, such as the catalytic combustion of all kinds of organic compounds. If the isotope ratio of one or of all compounds in a mixture is requested, a separation process (e.g. a gas chromatographic separation) is required before the conversion to CO_2.

For all of these methods, there is one common requirement: they must separate and then convert the sample into CO_2 so that the isotope ratio of each sample is preserved in the CO_2 measured by IRMS. Apparently, this requirement is the critical step of the whole method.

Classical isotope ratio mass spectrometers use a dual gas-inlet system, where one sample container is used to store the sample CO_2 and the other one to store the working standard gas. Sample and standard, both pure CO_2, are repeatedly introduced intermittently at 4–8 seconds' time-intervals into the mass spectrometer and are directly compared to each other.

By this (batch-) type of sample to standard comparison method, the highest possible precision and accuracy is achieved. However, the method is totally incompatible to modern types of continuous-flow separation, such as gas chromatography or combustion devices, which normally use a carrier gas like helium and which make each sample available for measurement as a 'peak' for a few seconds only.

This incompatibility led to the (recent) development of the non-classical continuous-flow introduction method, which will be described in more detail below. We call this method in its combination with a gas chromatograph, 'GC combustion isotope ratio mass spectrometry (GC/C/IRMS)'.

Experimental set-up of GC/C/IRMS

Figure 14.1 shows the schematic layout of our complete system: a capillary gas chromatograph is coupled on-line to a triple collector (masses 44/45/46) isotope ratio mass spectrometer via a combustion interface. A second, switchable gas inlet

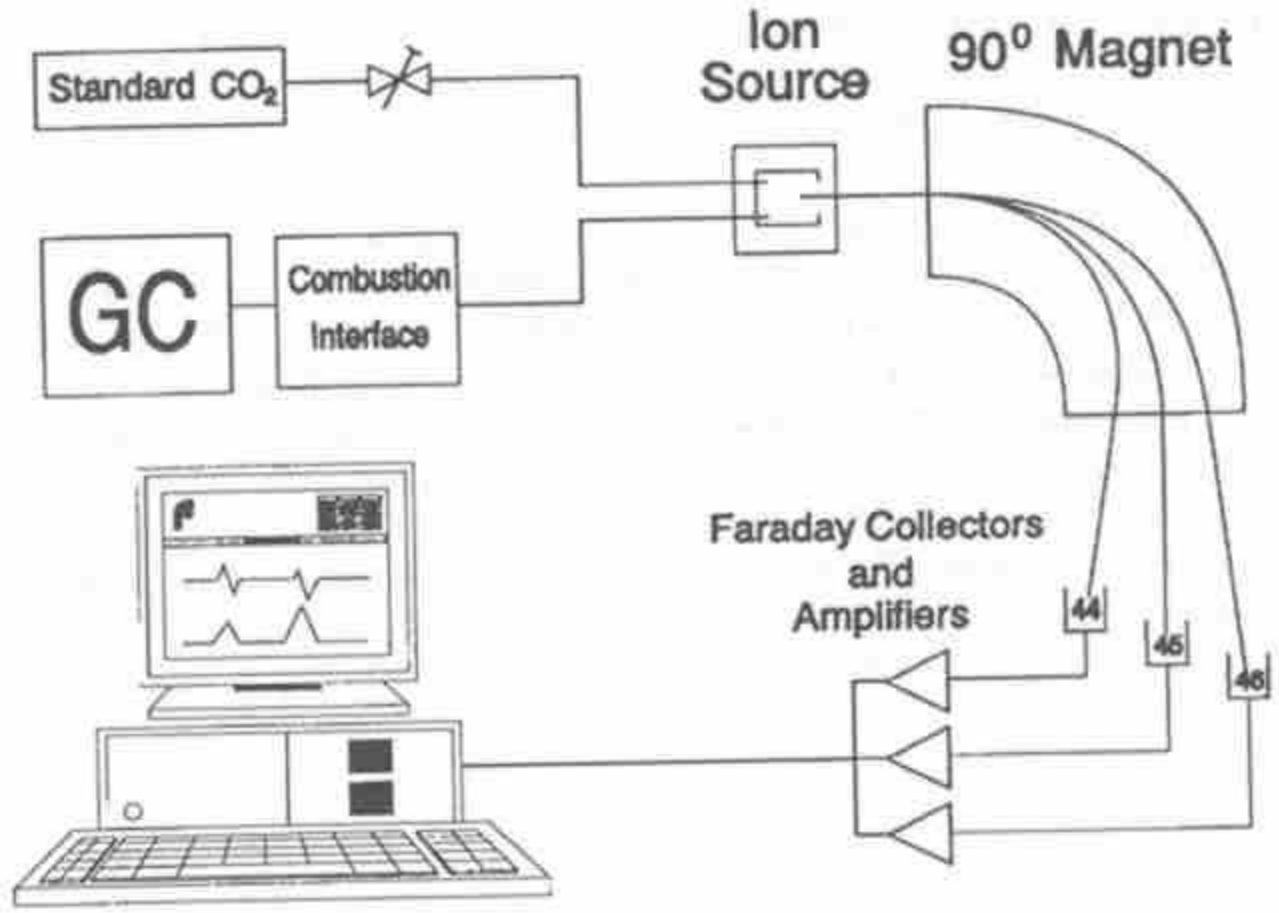


Figure 14.1 Schematic layout of the GC/C/IRMS (gas chromatograph/combustion/isotope ratio mass spectrometer) system.

line to the mass spectrometer is used to introduce a standard gas (CO_2). The mass spectrometric data are acquired and evaluated by a PC-based data system using a multitasking operating system with a graphic, menu-operated user interface.

In *Figure 14.2*, the combustion interface is shown in more detail. A capillary gas chromatographic column is connected to a high-efficiency microcombustion furnace via a post-column splitter. The second outlet of the splitter can be

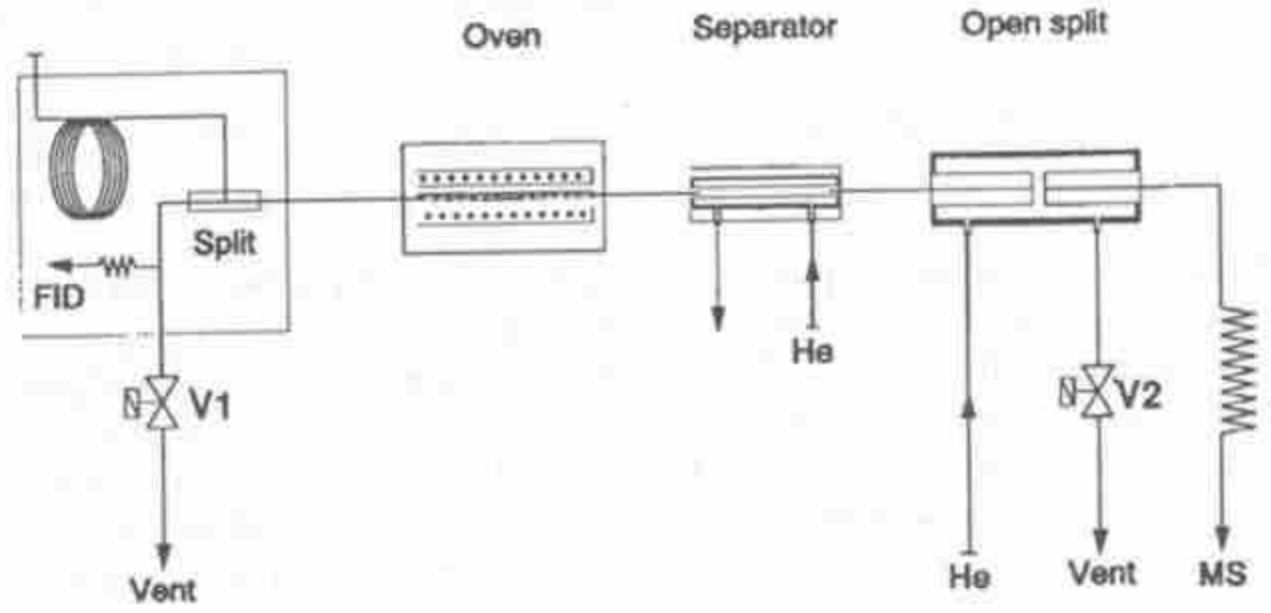


Figure 14.2 Combustion interface of the GC/C/IRMS system.

connected to any type of GC detector [flame ionization detector (FID), thermal conductivity detector (TCD) or ion trap detector/mass spectrometer (ITD/MS)] and is connected in parallel to a high-conductivity vent, which can be closed by valve V1.

The combustion furnace consists of a quartz capillary at 820°C, filled with platinum and copper oxide, serving as a combustion catalyst and as a source of oxygen. Behind the furnace, a capillary-shaped phase separator, made of selectively permeable membrane materials, is connected. This simple dead-space-free device removes all water from the mixture of combustion products. A conventional open split couples the combustion system to the mass spectrometer via a capillary, which limits the flow to 0.6 ml min^{-1}, practically independent of the flow conditions in the rest of the system. The open split is fed with a constant flow of make-up gas (He) which can leave the split device via valve V2.

The chromatographic resolution of the complete, virtually dead-space-free

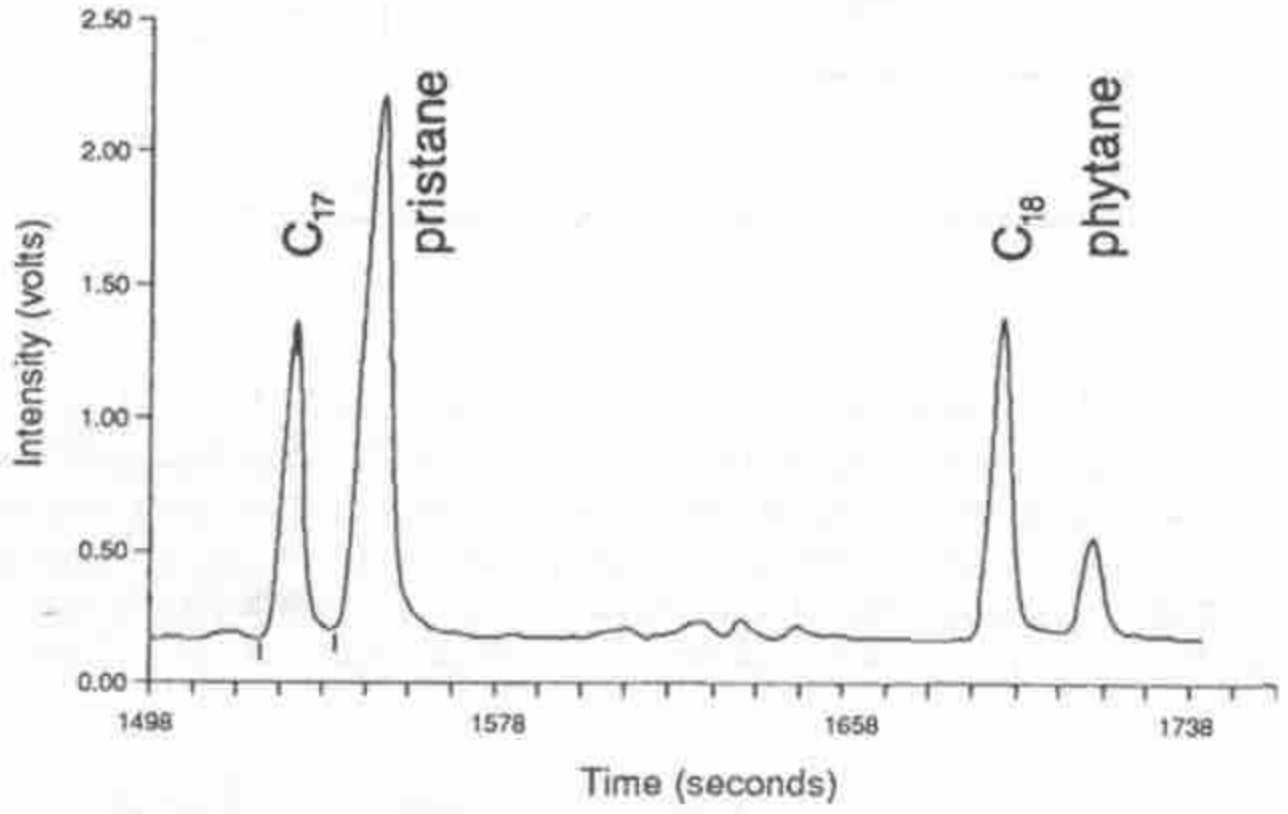


Figure 14.3 Detail of a combusted crude oil chromatogram: recording of the $^{12}C^{16}O_2^+$ ion current, m/z 44.

combustion system can be judged from *Figure 14.3*, which shows part of the mass 44 recording trace of a mixture of hydrocarbons.

Depending on the status of the two valves V1 and V2 (*Figure 14.2*), the combustion system operates in two different modes. First, during normal operation, when the GC eluates are to be combusted, V1 is closed and V2 is open. Thus, because of the closure of the high conductivity vent, the post-column split feeds the majority of the gas flow through the combustion system and only a small portion is flowing to the GC FID detector.

Secondly, in order to prevent the solvent, or any other undesirable eluate (which would possibly destroy the furnace's catalytic activity), from entering the combustion system, V1 is opened and V2 is closed. In this way, the flow through the system is reversed by the open split's make-up gas, and all GC eluates are now completely directed to vent via the post-column split. The flow to the GC detector is still maintained, as well as the pure helium flow to the mass spectrometer.

For illustrative simplicity, another feature of the combustion system is not shown in *Figure 14.2*: oxygen can be introduced into the furnace to regenerate the copper oxide that is slowly used by the combustion process.

In order to cope with the relatively high helium flow, the mass spectrometer itself is differentially pumped. For the same reason the gas conductance of the ion source is raised to approximately 1.6 l s^{-1} (air), which results in approximately half the ion-source sensitivity as compared to the normal operation of an isotope ratio mass spectrometer (without helium flow): for 1 nmol of CO_2 introduced into the ion source, one detects about 8×10^{10} ions at the mass 44 collector.

For the measurement of an isotope ratio of 1 : 100, the statistical limit allows a best relative precision of approximately 5×10^{-5} for one nmol of CO_2. In the Faraday cup recording system, the rise time of the DC amplifiers for the three ion collectors is carefully matched to an appropriate value [200 milliseconds (ms)], although they use working resistors which differ by more than a factor of 100 (because of the different abundances of the three ion currents). The ion current signals are integrated for selectable time-intervals (range 60–500 ms). Hence, at least 20 triple-collector readings can be taken for a 5 s GC peak.

Typical ion current (m/z 44) and isotopic ratio (m/z 45 : m/z 44) chromatograms are shown in *Figure 14.4*. The lower graph shows the intensity recording of the mass 44 ($^{12}CO_2$) trace, and is very similar to a direct FID trace of the gas chromatograph or a total ion current recording chromatogram of a conventional GC/MS system. The upper graph shows the plot of the ratios of the traces of masses 45 and 44 as a function of time. The scale is calibrated in δ-values, i.e. the relative difference of the measured ratio (in Pee Dee belemnite limestone) given in parts per thousand or per mil (‰). In addition, a total of three standard CO_2 gas injections are shown before and after peak number 4, benzaldehyde (*Figure 14.4*).

The isotope ratio traces of the gas chromatographic peaks exhibit a typical S-shape, whereas the standard gas injections, which are introduced directly into the ion source, show a nearly rectangular ratio response (*Figure 14.4*, upper trace). The reason for the S-shaped isotope ratio (*Figure 14.5*, lower trace) of the GC peak is that the 'heavier' isotopic species of a compound elutes more rapidly from the high-resolution capillary column that the light species (*Figure 14.5*, upper trace). This behaviour is the result of a vapour pressure isotope effect, and is an often observed phenomenon in GC/MS selected ion monitoring. The actual ratio is computed from the ratio of the area under the two isotopic peaks. Hence, care must be taken to integrate in currents across the full width of the chromatographic peaks.

During the run of a gas chromatogram, a real-time plot of the GC trace and of the $^{13}C/^{12}C$ ratio is shown on the screen of the computer. Data evaluation is performed fully automatically after the completion of the gas chromatogram. The user can manually change the integration program via a dialogue, if necessary, to advise the system of the proper choice of baseline ratio values.

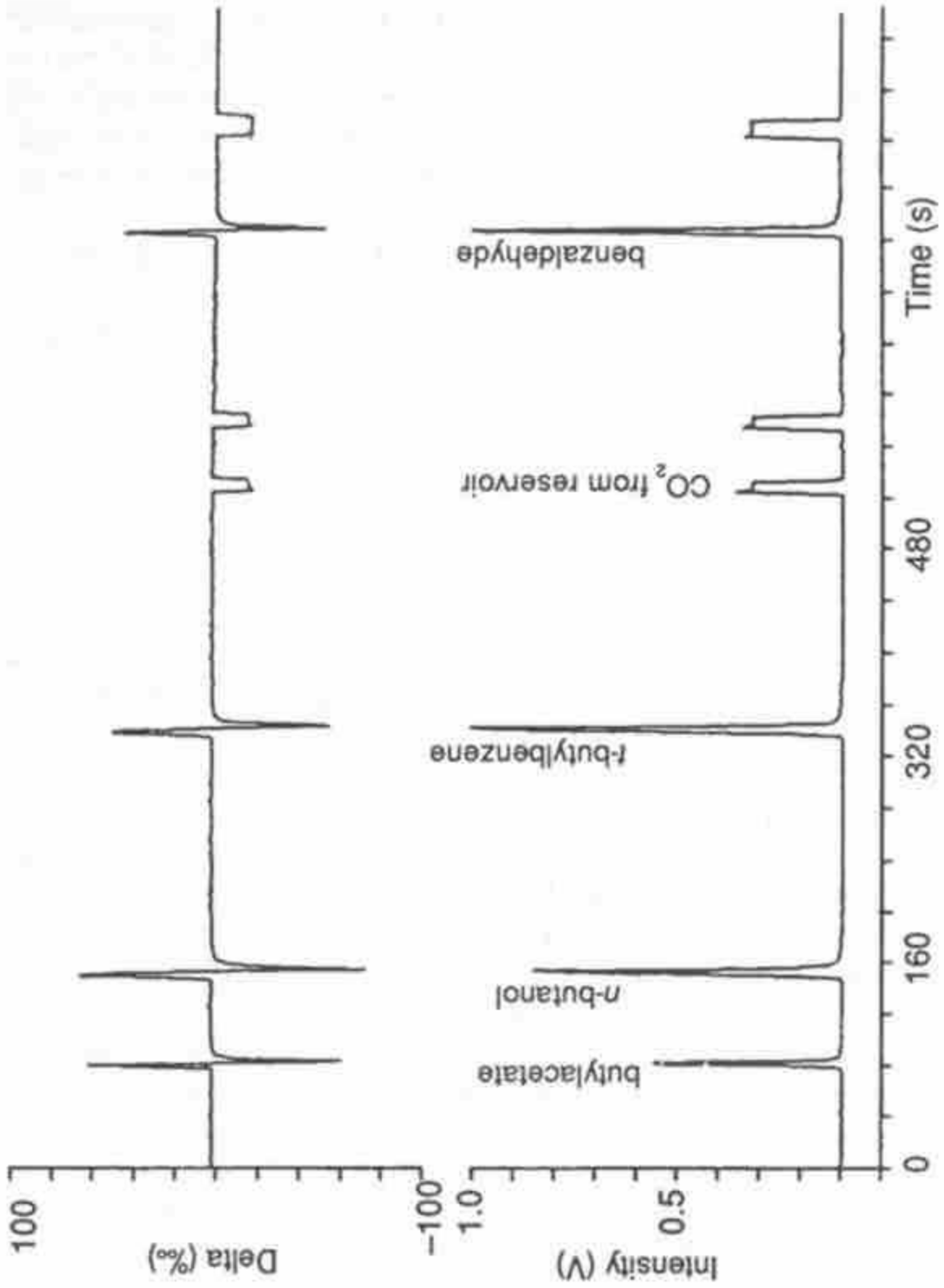
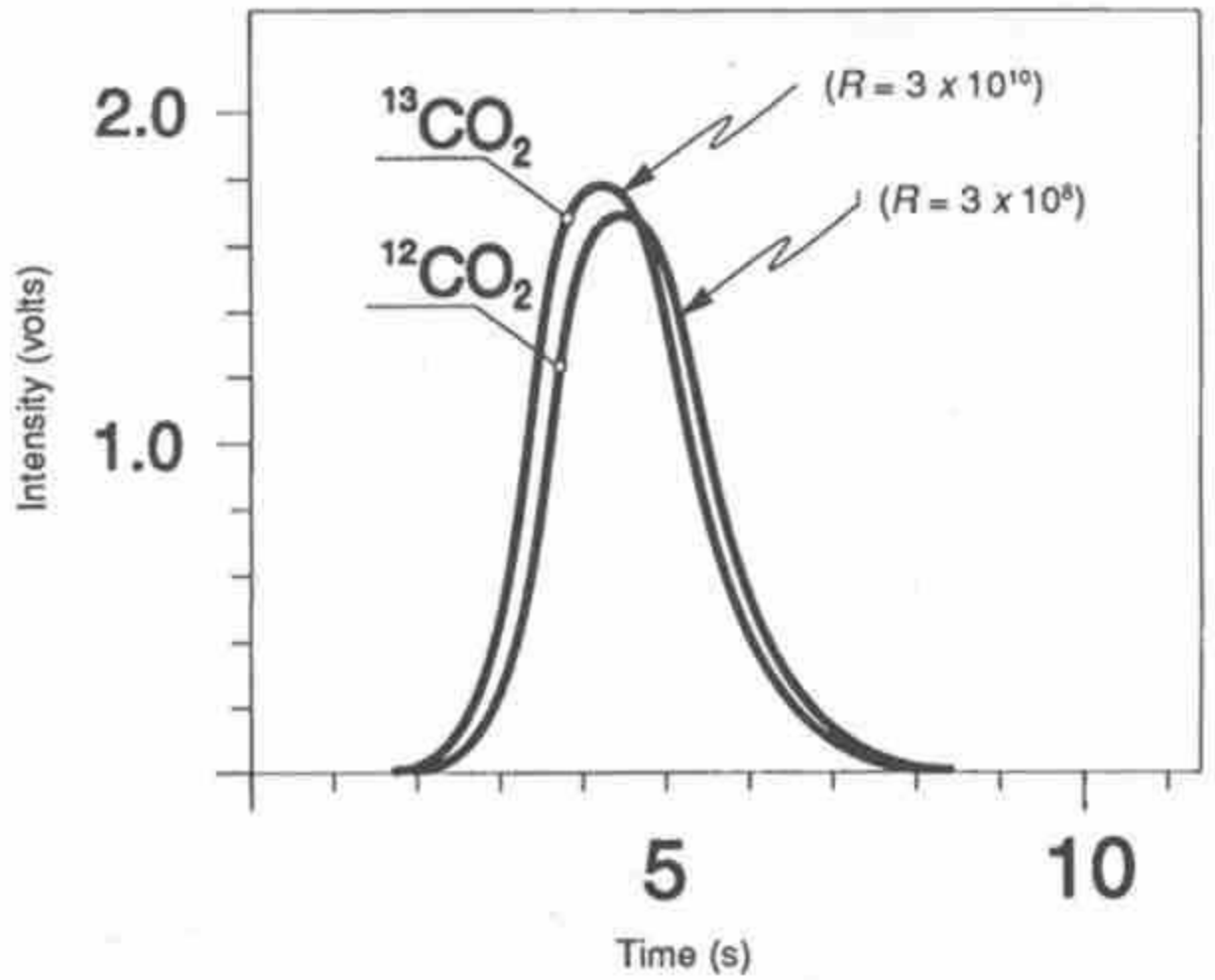


Figure 14.4 Chromatogram ($^{12}C^{16}O_2^+$ ion current, m/z 44) and isotopic ratio (in δ-notation) of a standard mixture, together with three CO_2 reference gas injections.



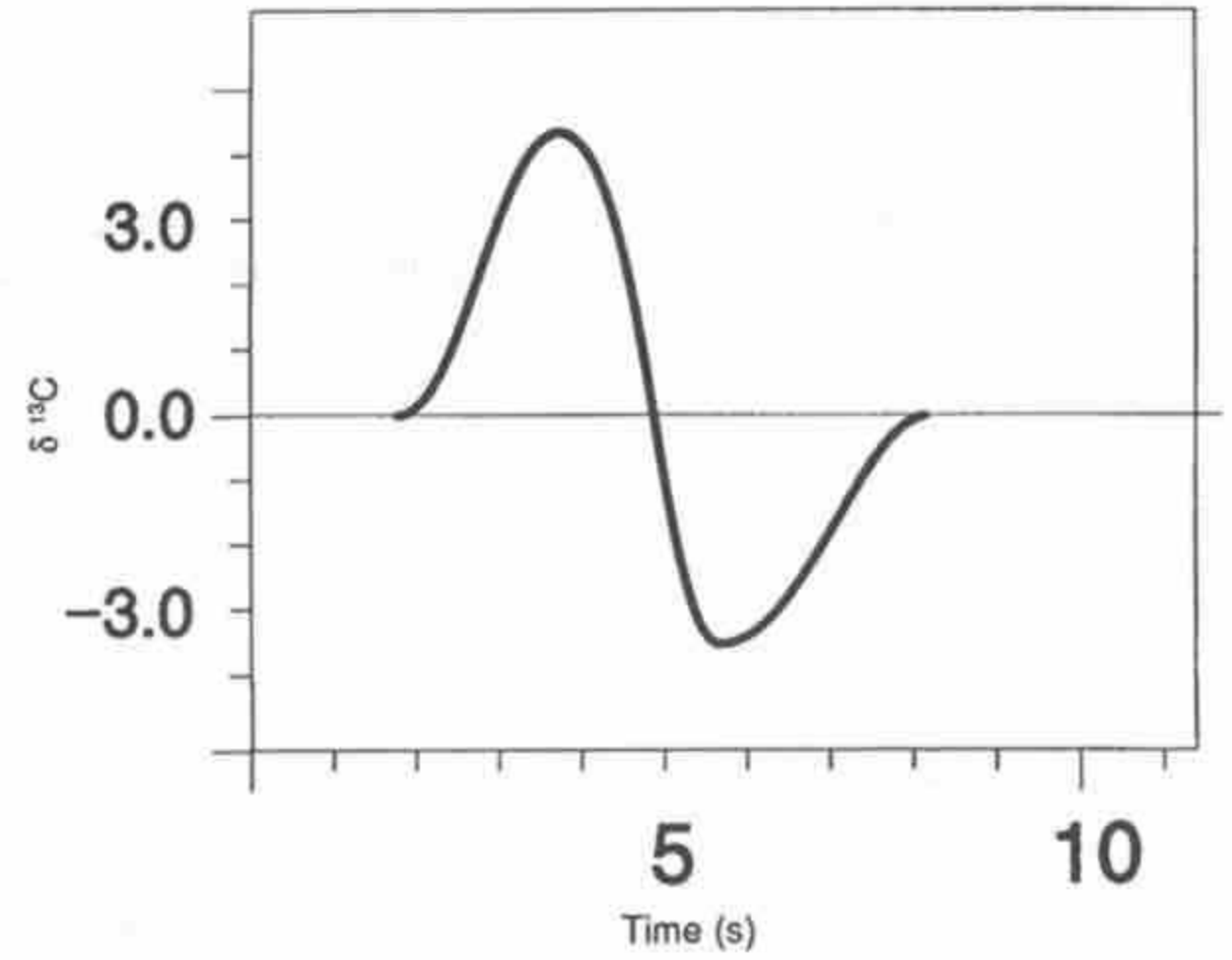


Figure 14.5 The origin of the S-shaped ratio output: the heavier isotopic species elutes more rapidly from the capillary column than the lighter (more abundant) ^{12}C species.

Nomenclature and methodology

The 'isotope ratio' (r) of a sample is the ratio of the number (n_i) of two isotopes ($i = 1,2$):

$$r = \frac{n_2}{n_1}$$

Therefore, the atom% value (a) of the (n_2) isotope is:

$$a = \frac{100\,r}{(r+1)} = \frac{(100\,n_2)}{n_1 + n_2}$$

The 'enrichment' of an isotope in a sample as compared to a standard value (a_s) is, therefore, given by atom% excess (APE):

$$\mathrm{APE} = a - a_s$$

An isotope ratio mass spectrometer measures the isotope ratio. The measured raw isotope ratio is, however, influenced by several mass discriminating effects which fluctuate with time and from instrument to instrument. In order to achieve the best precision and the best accuracy, each ratio measurement *must* be calibrated by the measurement of a standard with known isotopic composition. Otherwise, measurements made at different times on the same instrument and other instruments would be incomparable.

This is the basic reason why isotope ratios are always measured and reported relative to a standard value. However, classically with IRMS (and in contrast to the usual way to report the amount of isotopically labelled species in a sample, as with GC/MS) not the 'enrichment' versus a standard, i.e. the value APE (where r_s is the isotope ratio in the standard)

$$\mathrm{APE} = \frac{(r_s - r)\,100}{(r+1)\,(r_s+1)}$$

is reported, but the so-called δ-value:

$$\delta = \frac{r}{r_s - 1} \times 1000$$

This is the relative difference of a ratio r from a standard ratio r_s, given in parts per thousand, or per mil (‰).

The relationship between the enrichment and the δ-value is basically non-linear. For all details, how to 'convert' δ-values into atom% or atom% excess values and vice versa, *see Figure 14.6*.

If the measured isotope ratios are related to a standard near the natural level of ^{13}C (approximately 1.1%), a linear approximation can be used for δ-values up to approximately δ = 1000‰ (i.e. for $r \approx 2r_s$ or ≈ 1 atom% excess):

$$\mathrm{APE} = \frac{a_s \times \delta}{1000}$$

$$ape_u = a_s \frac{\delta_u \times (100 - a_s)}{a_s \times \delta_u + 10^5}$$

$$a_u = a_s \frac{\left(1 + \frac{\delta_u}{1000}\right) \times 10^5}{a_s \times \delta_u + 10^5}$$

$$\delta_u = \frac{ape_u \times 10^5}{100 - (1 + ape_u) \times a_s}$$

$$\delta_u = \frac{(a_u - a_s) \times 10^5}{(100 - a_u) \times a_s}$$

a_u , a_s , ape_u in %

δ in ‰

Figure 14. 6 The conversion of atom% values into δ-values and vice versa. a_u, atom% of sample; a_s, atom% of standard; ape_u, atom% excess in sample.

This relationship shows the 'high resolution' of the δ-scale as compared to the atom% scale: a δ-value of 1‰ corresponds to 0.001 atom% excess at natural level (1%).

Table 14.1 Reproducibility of the GC/C/IRMS system; the samples were measured over 6 weeks

Measurement	*n*-Butylacetate $\delta^{13}C$(‰)	*n*-Butanol $^{13}C/^{12}C$ ratio (%)	*t*-Butylbenzene $\delta^{13}C$(‰)	5-Nonanone $\delta^{13}C$(‰)	Benzaldehyde $\delta^{13}C$(‰)
1	–5.50	1.15626	–1.24		
2	–5.49	1.15636	–0.84		
3	–6.06	1.15625	–1.01		
4	–5.42	1.15486	–1.05		
5	–5.05	1.15546	–0.80	–4.25	
6	–5.32	1.15508	–0.58		
7	–5.33	1.15443	–0.35		
8	–5.36	1.15547	–0.94		
9	–5.51	1.15660	–0.84		
10	–5.36	1.15683	–0.97	–4.76	
11	–5.24	1.16439	–0.37		
12	–5.66	1.16277	–1.09		
13	–5.14	1.15995	–1.57		
14	–5.67	1.15955	–0.97		
15	–5.18	1.15900	-0.71		
16		1.15779	–1.07		
17	–5.18	1.15664			–2.82
18	–5.75	1.15485	–1.11		–2.89
19	–5.14	1.15492	–0.80		–2.46
20	–5.58	1.15494	–1.31		–2.75
21	–5.46	1.15720	–1.11		–2.63
22	–5.21	1.15993	–0.80		–2.39
23	–5.51	1.15987	–1.32	–4.59	–2.88
Mean	–5.41	1.15736	–0.95	–4.53	–2.69
SD	0.23	0.00261	0.29	0.21	0.19

n-Butanol was used as the internal standard.

For a complete GC run, basically two different ways of standardization are available. First, a dose of CO_2 of known isotopic composition is introduced directly into the ion source (or somewhere into the helium flow of the combustion interface) and the isotopic ratio of all GC peaks is related to this gas. Several standard injections can also be distributed across the whole run, if necessary.

Secondly, one or several compounds with known isotopic composition are used as internal standards in the sample mixture. Besides the proper use of a standard, the selection (or measurement) of the correct baseline values is important. It must be noted that the isotope ratio of the baseline may be different at different times during the GC run, due to a changing column bleed.

Table 14.1 summarizes the repeated measurements of the mixture of standards that was shown in *Figure 14.4*. *n*-Butanol was used as internal standard for measurement of the isotope ratios. The external standard deviation of these measurements is better than 0.3‰. This means that two samples whose isotopic composition differs by only 0.0003 atom% can be differentiated by this method.

In order to demonstrate the excellent linearity of the method over a wide range of (small) isotopic concentrations, we have measured several precise mixtures of [1-^{13}C]palmitate diluted with unlabelled palmitate as methyl ester derivatives. Our results are shown in *Figure 14.7*. The measured δ-values have been converted into atom% excess values, using the isotopic content of the unlabelled palmitate methyl ester as the reference. It should be noted that a range of approximately 7‰ in the δ-value covers almost two orders of magnitude in isotopic concentration of the sample.

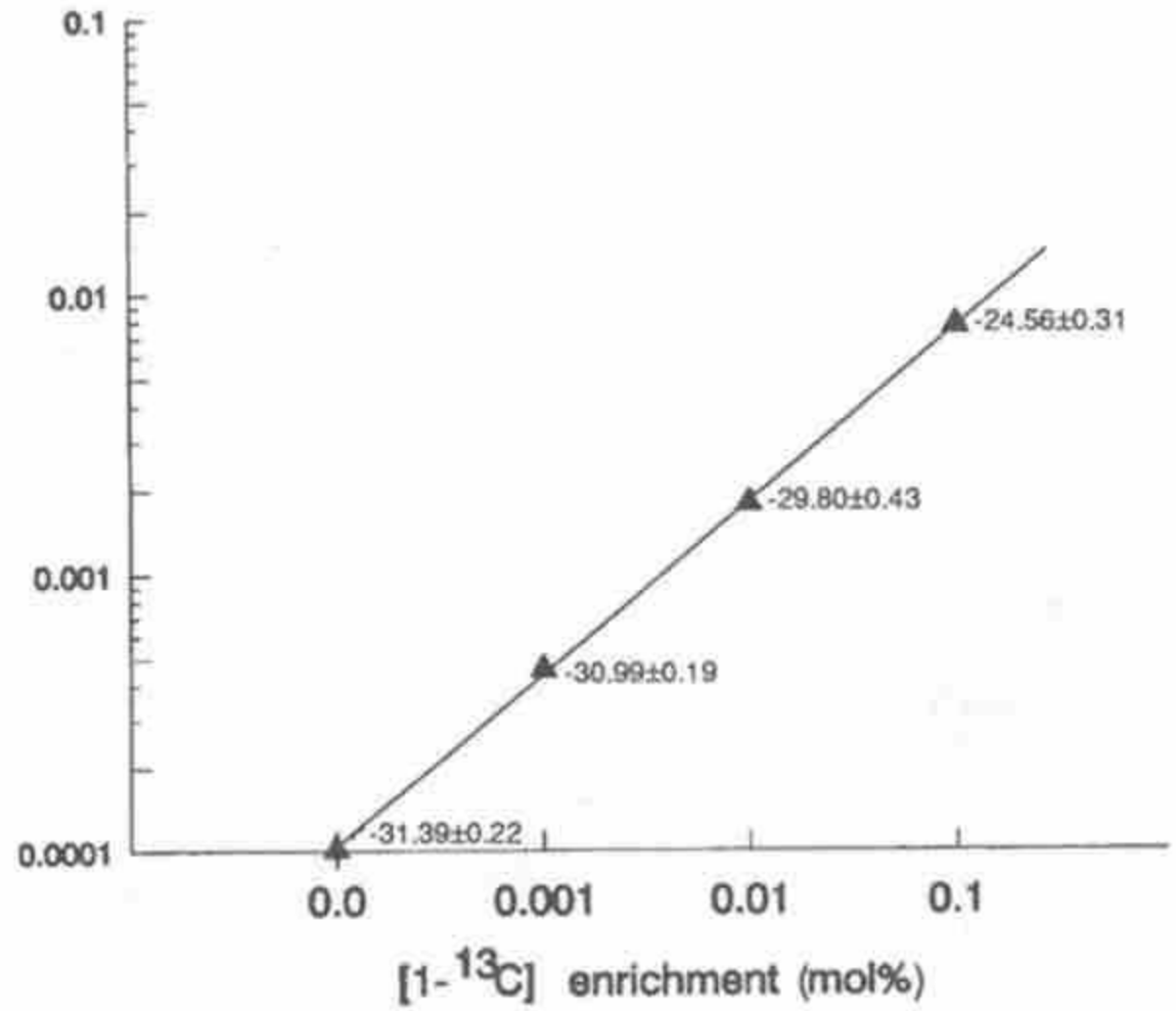


Figure 14.7 Calibration graph of a [1 -^{13}C]palmitate methyl ester dilution series. The measured δ-values (± SD), shown next to the data points, have been converted into atom % excess values. (Samples courtesy of T.E. Chapman, University Hospital Groningen, The Netherlands.)

In order to check not only the precision, but also the accuracy of our results (i.e. of our standardization procedure), the individual compounds of the test mixture (*Figure 14.4*) were analysed individually with the classical high-precision total combustion method (Dumas combustion). The mean isotope ratios (and their standard deviations) are shown in *Table 14.2*, together with the means from the GC combustion run of the mixture. The difference of the isotopic ratios obtained by both methods is smaller than 0.2‰ (δ-notation) in most cases and is, therefore, safely within the statistical uncertainties.

Table 14.2 Comparison of GC/C/IRMS and classical off-line combustion and IRMS isotope analysis of a standard mixture

Compound	GC/C/IRMS $\delta^{13}C_{PBD}$(‰) mean (±SD)	Dual inlet $\delta^{13}C_{PDB}$(‰) mean (±SD)	Difference in $\delta^{13}C$ (‰)
n-Butylacetate	–28.97 (0.23)	–28.78 (0.01)	0.19
n-Butanol	–23.56	–23.56 (0.06)	(0.00)
t-Butylbenzene	–24.51 (0.29)	–24.50 (0.02)	0.01
5-Nonanone	–28.06 (0.21)	–28.48 (0.02)	0.42
Benzaldehyde	–26.23 (0.19)	–26.15 (0.05)	0.08

n-Butanol was used as the internal standard in the GC/C/IRMS measurements, its $\delta^{13}C$ value was measured using off-line combustion and classical IRMS.
The difference in $\delta^{13}CO_2$(‰) values between the two techniques was GC/C/IRMS values minus the dual inlet values.
PDB, Pee Dee belemnite limestone.

Application examples

The carbon and hydrogen isotopic ratios of the light hydrocarbons in natural gas are useful indicators for gas and oil deposits. *Figure 14.8* shows a typical recording of the measurement of a natural gas sample, together with two reference CO_2 gas injections at the end of the gas chromatogram. The δ-values range from -32‰ to -25‰, with standard deviations in measurements ranging from 0.2‰ to 0.4‰ (n = 8) (*Figure 14.9*).

The *n*-alkanes in crude oil also show differences in isotopic ratios. An example for *n*-alkane up to C_{35} is shown in *Figure 14.10*.

Food flavours are normally mixtures of compounds. For economic reasons, very often such flavours are synthesized artificially instead of being extracted from plants. Synthesized flavour extracts show a different isotopic pattern from their natural counterparts, and this can be used to differentiate between them. One example (strawberry aroma) is given in *Figure 14.11*.

An interesting feature of the GC combustion isotope ratio method is shown in *Figure 14.12*. It shows a gas chromatogram of an extract from a fermentation experiment. At two locations in the chromatogram one can observe a big (non-S-shaped) isotope signal (upper trace) whereas virtually no corresponding peak can be found at m/z 44 (lower trace). This points to a highly ^{13}C-enriched compound (virtually no ^{12}C content) at a very low concentration.

In *Figure 14.13*, we therefore show the enlarged traces of masses 44 *and* 45: a $^{13}C^{16}O_2^+$ trace can be detected (10 mV), a $^{12}C^{16}O_2^+$ trace at natural level should be present with about the same intensity, but is, actually, much smaller. This is the

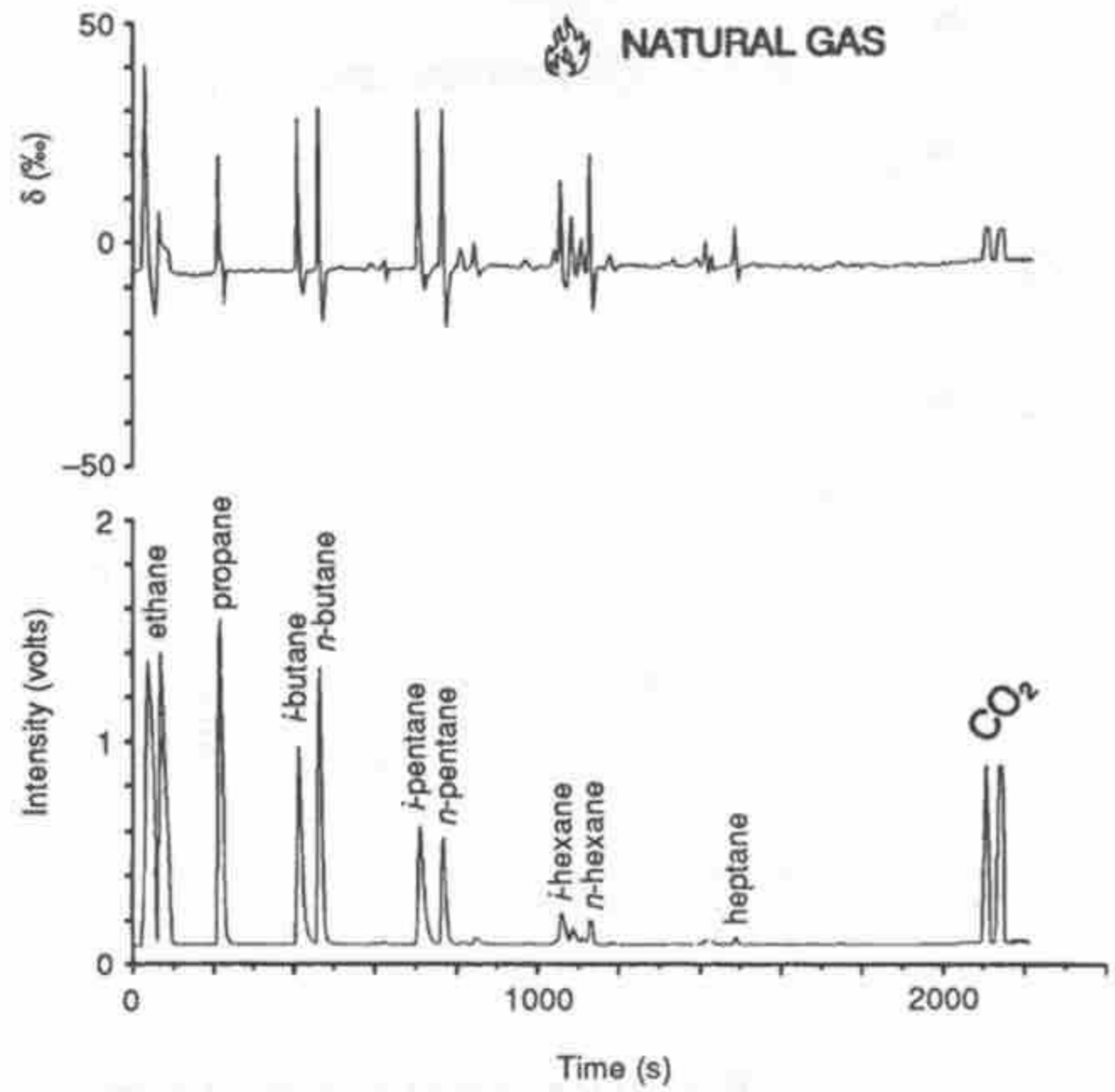


Figure 14.8 Raw data output from a natural gas sample: $^{12}C^{16}O_2^+$ ion current, m/z 44, and isotopic ratio (δ-notation).

explanation why the ratio signal does not show the typical S-shape. The isotopic content (corrected for baseline) is 4 atom% excess. The peak height at mass 45 corresponds to 2 pg of ^{13}C label, the signal-to-noise ratio of the ratio peak being better than 100 : 1 (*Figure 14.13*, upper trace). The 'detection limit' with a signal-to-noise ratio of 10 : 1 would, therefore, be estimated to be 200 fg.

With these few examples we have demonstrated the great potential of this new analytical method. We believe it will find many applications in the future.

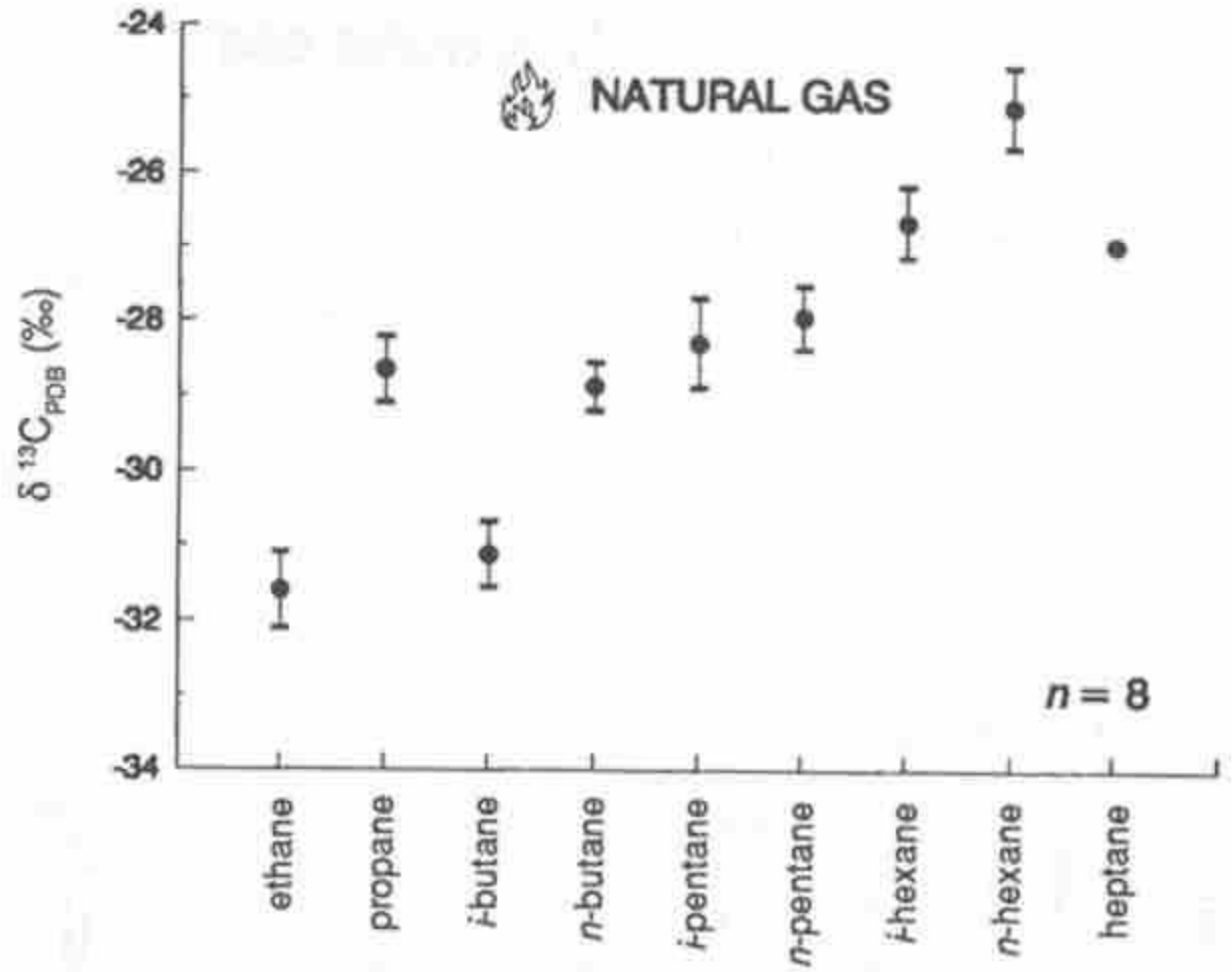


Figure 14.9 The isotope ratios of a natural gas sample, relative to Pee Dee belemnite limestone (PDB), the international standard.

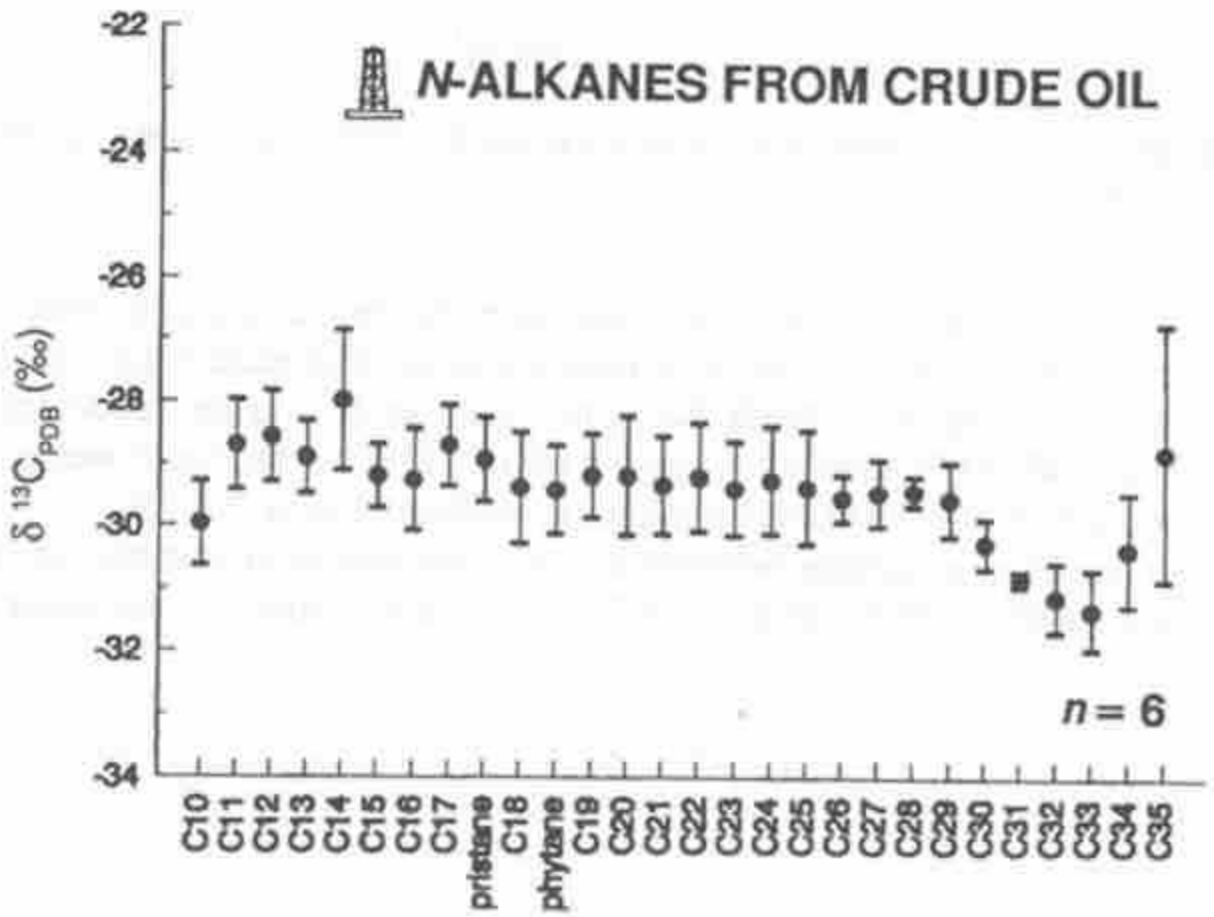


Figure 14.10 Isotopic pattern of the *n*-alkanes of a crude oil sample in the range C10–C35.

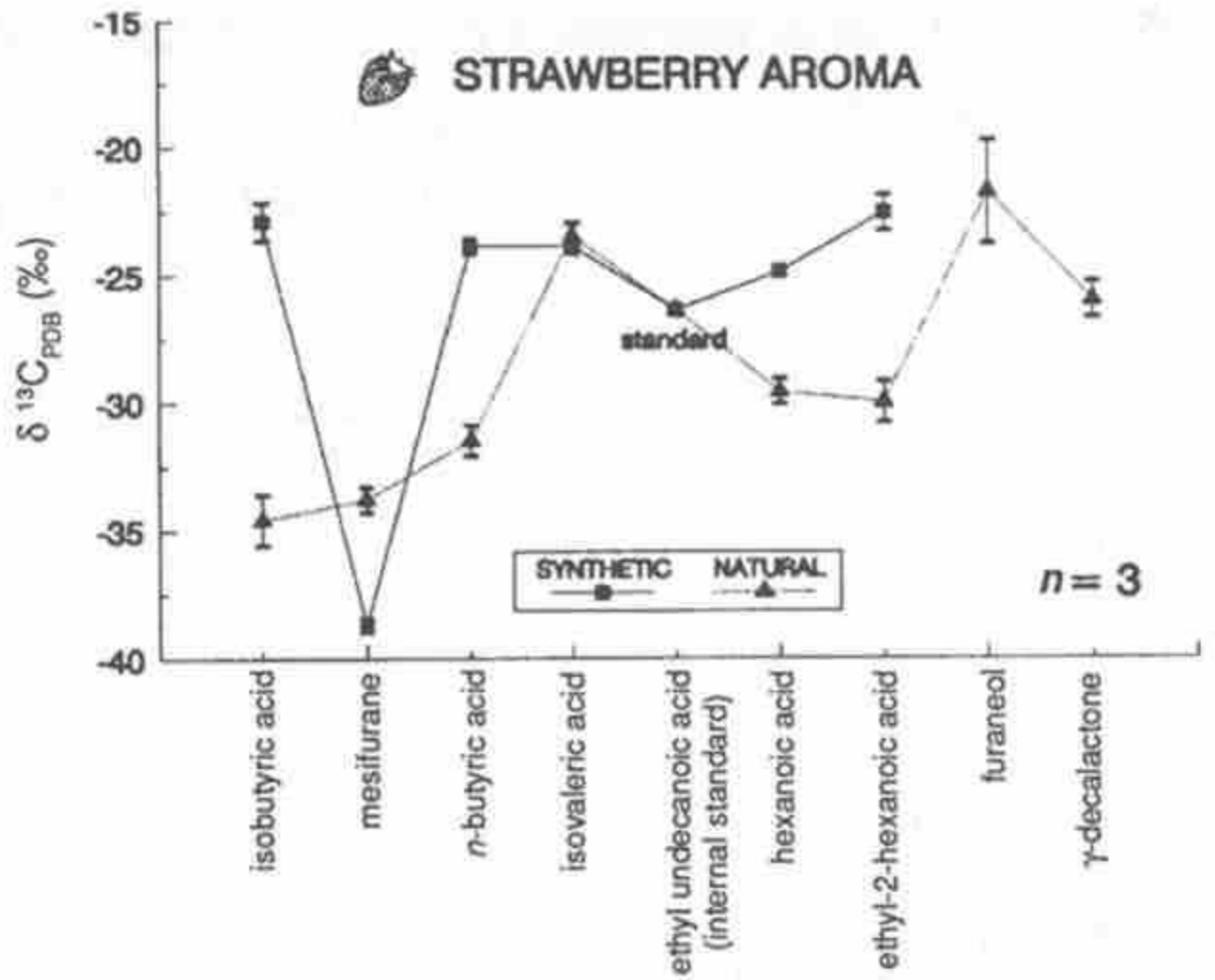


Figure 14.11 Isotopic pattern of compounds in a natural and synthetic strawberry aroma (courtesy of J. Koziet, Centre de Recherche Pernod Ricard, Créteil, France).

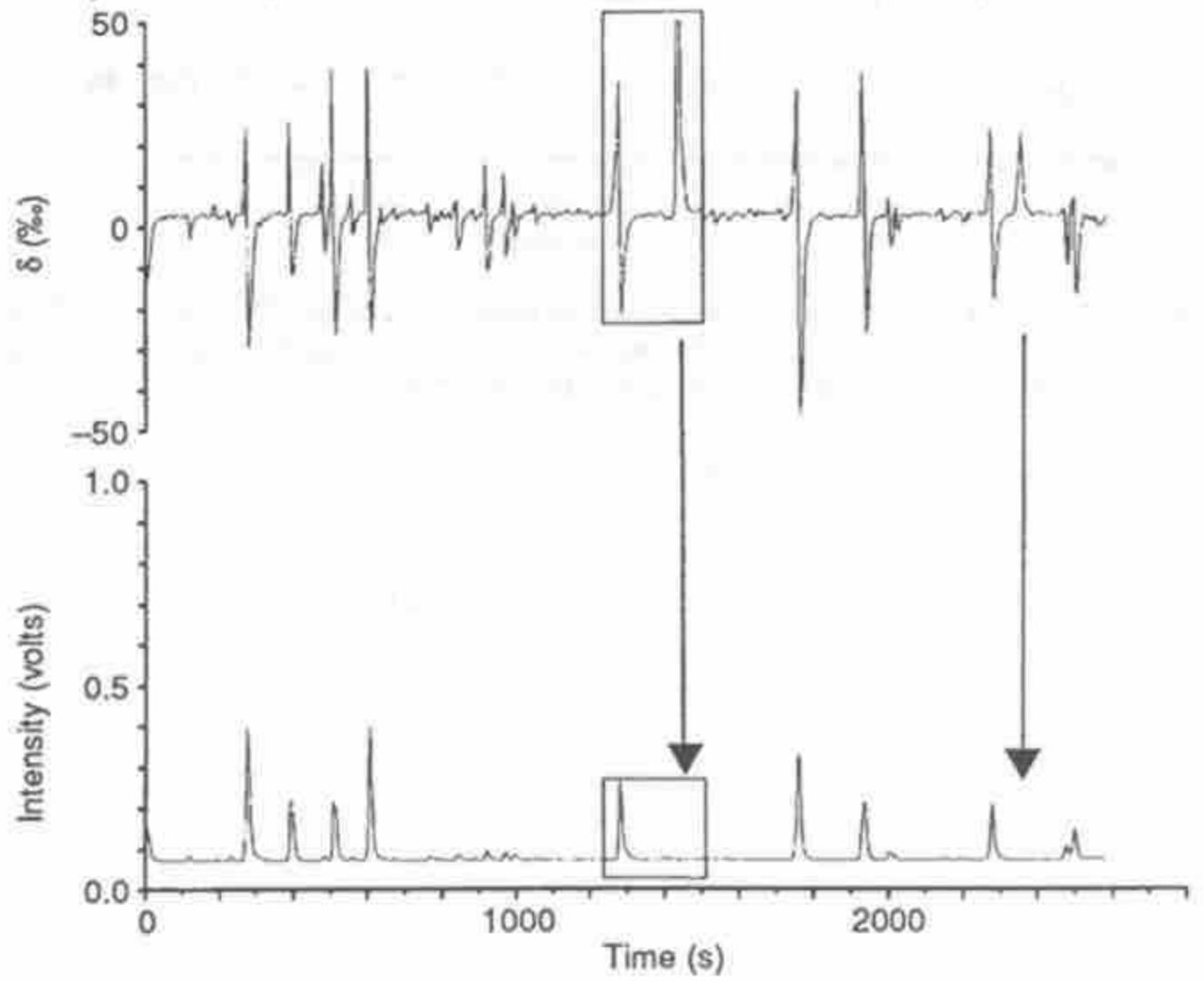
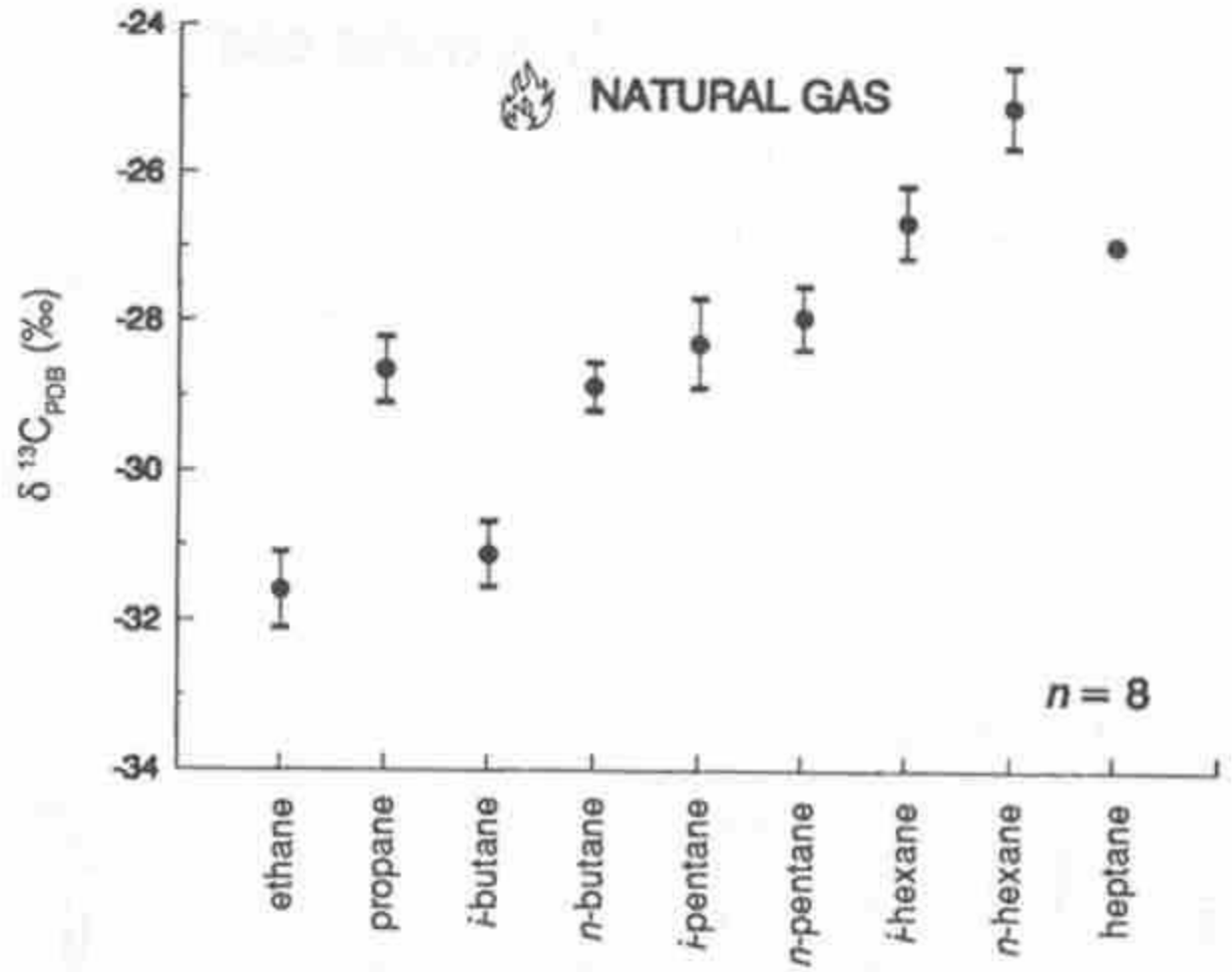


Figure 14.12 Raw data output from a mixture of metabolites. Boxes show area of chromatogram displayed in detail in *Figure 14.13*.



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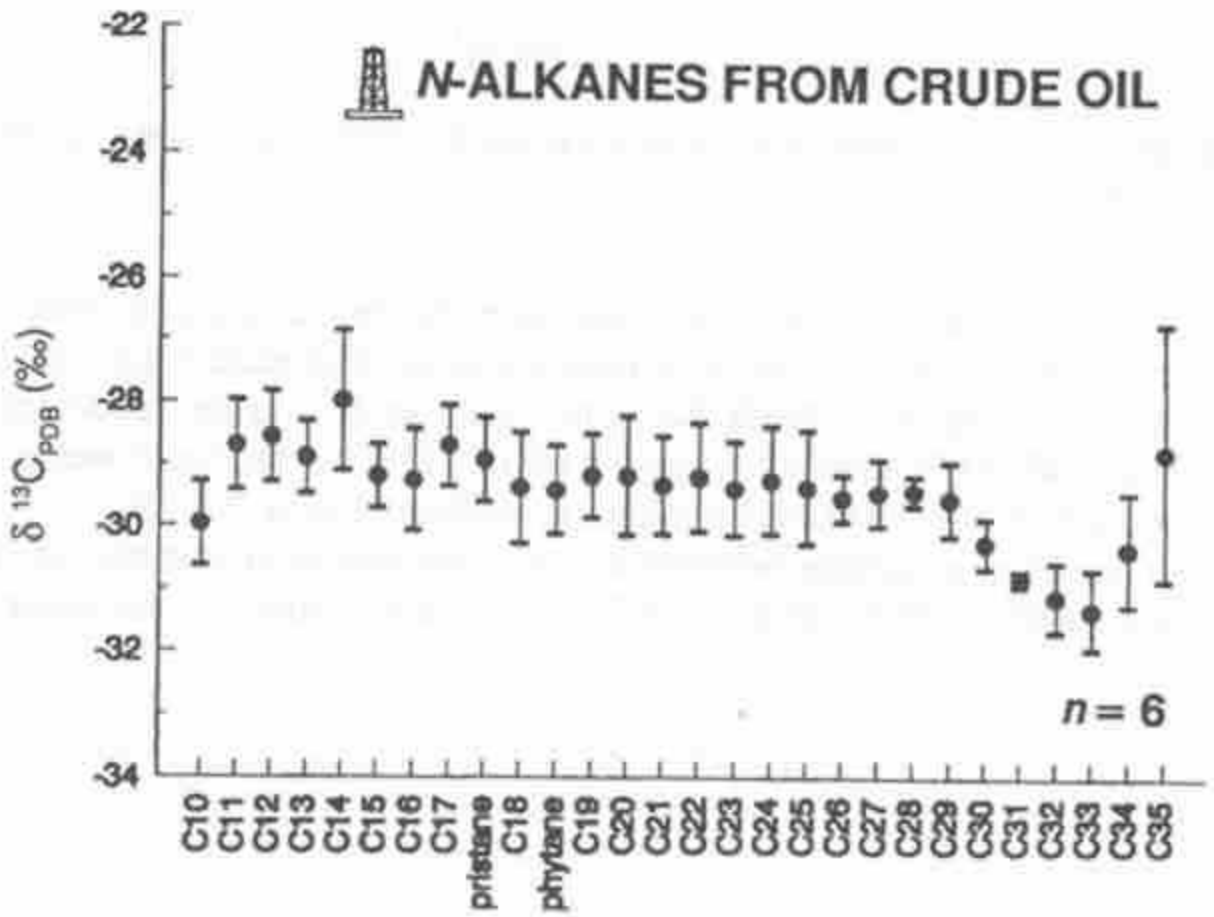

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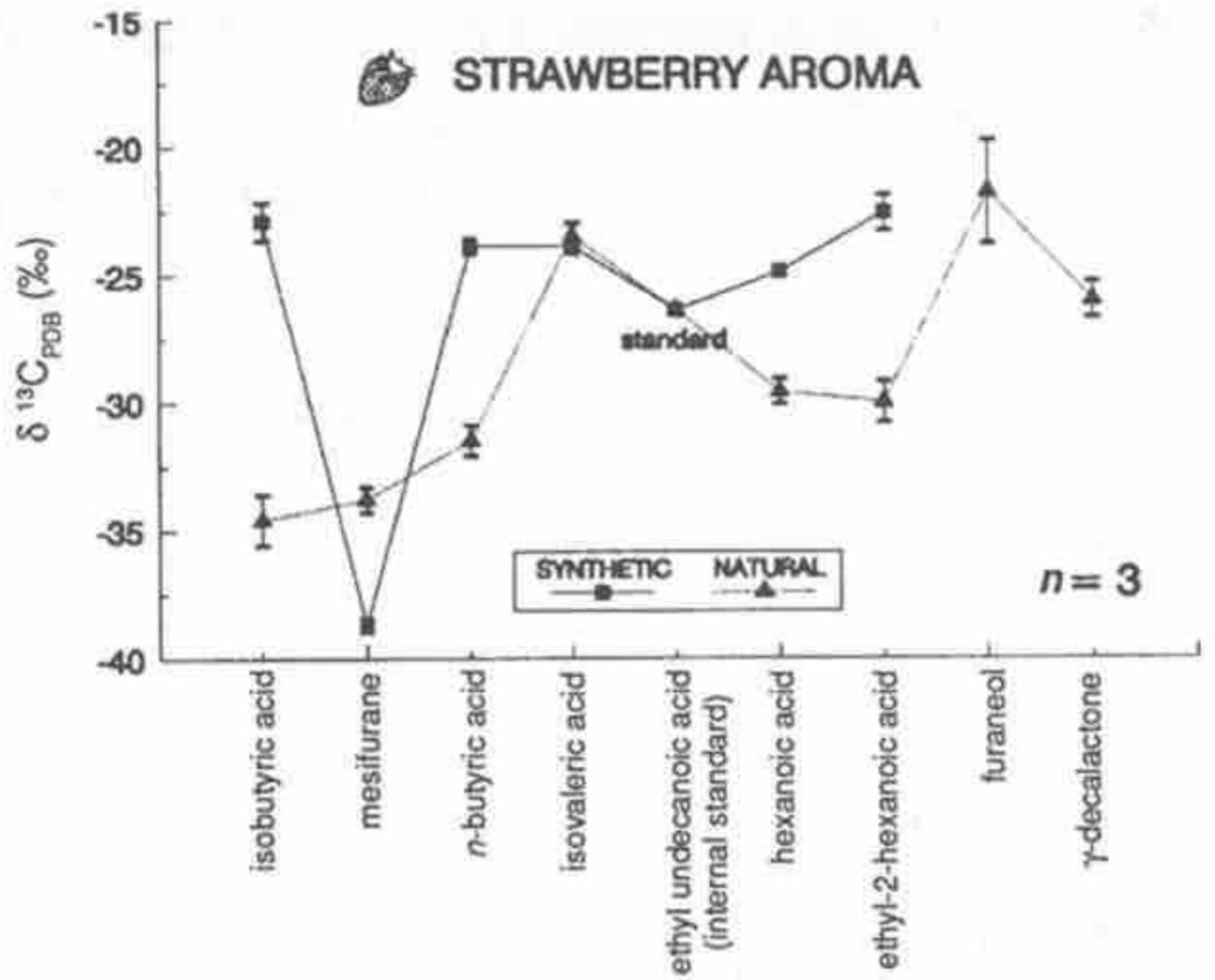

Figure 14.11 Isotopic pattern of compounds in a natural and synthetic strawberry aroma (courtesy of J. Koziet, Centre de Recherche Pernod Ricard, Créteil, France).

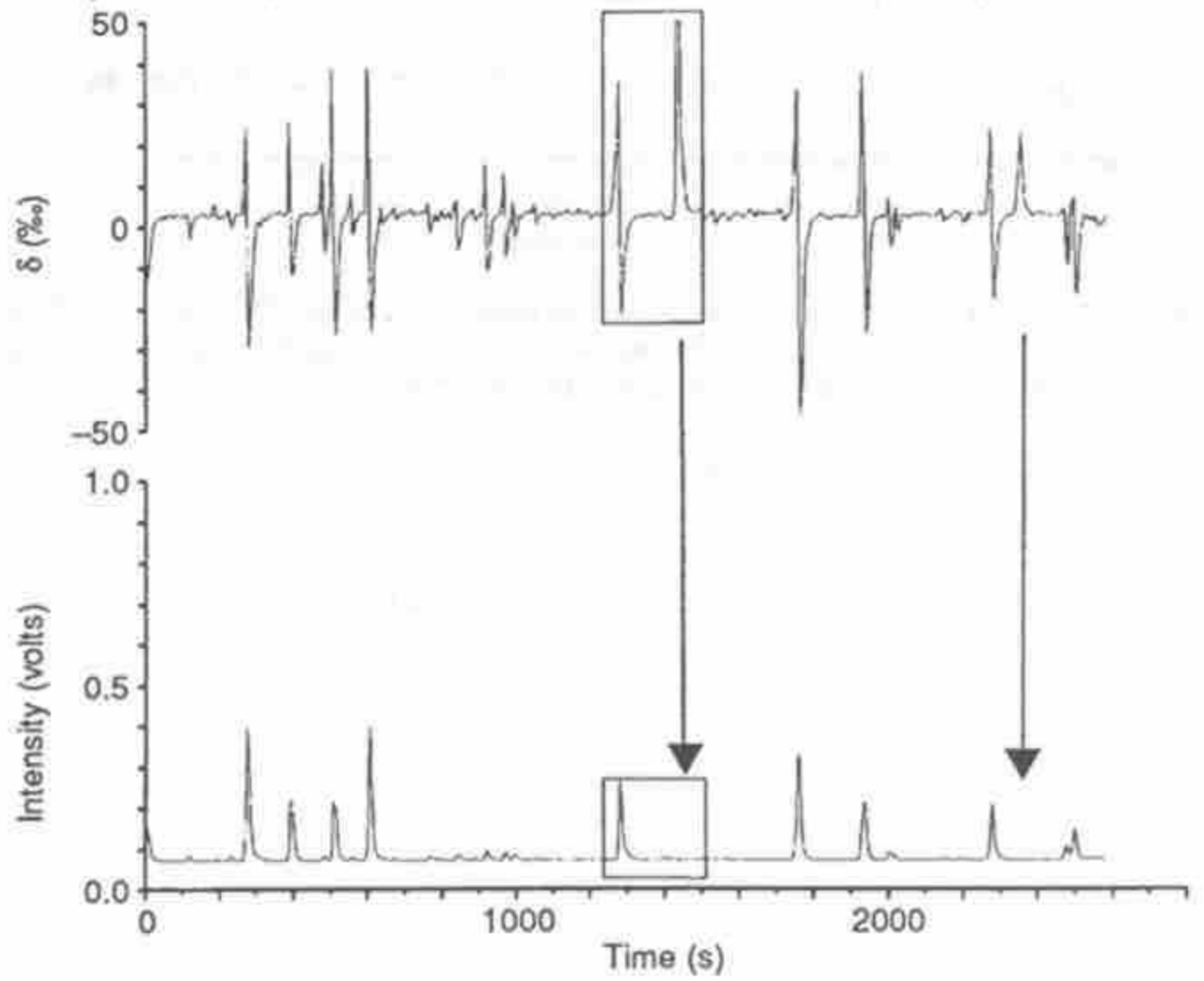

Figure 14.12 Raw data output from a mixture of metabolites. Boxes show area of chromatogram displayed in detail in *Figure 14.13*.