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FOREWORD

The practice of stable isotope ratio determination has undergone profound changes during the past decade. While this analytical task used to be the artwork of a small but very active group of experimentalists, it has now largely shifted to the hands of laboratory operators. Rapid technical developments in the last few years, particularly in the areas of non-mass spectrometric and mass spectrometric analysis of organic and inorganic matter have enabled a multitude of new applications in biogeochemistry and related fields. An IAEA Advisory Group meeting was held in September 1999 to identify and discuss these recent technical advances as well as new scientific opportunities and analytical challenges. There was an expressed need by the scientific community for concurrent development of suitable, high-quality stable isotope reference materials, standardized methods and calibration procedures.

By consensus of participants, the Advisory Group established a list of candidate reference materials that would improve transferability of results and ultimately enable broader application of isotope techniques.

This report includes a summary of discussions at the meeting and contributions on isotope applications in a range of specific biogeochemical fields using the new analytical techniques. It is expected to serve as a useful reference for researchers and laboratory managers who plan to develop or apply state-of-the-art stable isotope techniques.

The papers were reviewed by W. Brand, of the Max Planck Institute for Geochemistry, Jena, Germany. The IAEA officers responsible for this publication were M. Gröning, of the Agency's Laboratories, Seibersdorf and Vienna, and P. Aggarwal, of the Division of Physical and Chemical Sciences.

EDITORIAL NOTE

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SUMMARY

New analytical techniques for stable isotope ratio measurements have become popular over the past decade. These include non-mass spectrometric techniques using laser spectroscopy, and mass-spectrometric techniques with on-line or continuous flow apparatus for sample introduction.

Two non mass spectrometric techniques have emerged to be most promising for high precision stable isotope analysis. The first technique is infrared laser absorption spectrometry applied to small water samples (10 μL) injected into evacuated chambers for the direct and parallel determination of δ^2H, $\delta^{18}O$ and $\delta^{17}O$ in the produced water moisture. In this method the intensities of individual isotope selective absorption lines caused by rotational-vibrational transitions in the water molecule are measured using a tuneable infrared laser system. The precision obtained so far is 0.7‰ for δ^2H, and 0.5‰ for both $\delta^{18}O$ and $\delta^{17}O$. The second technique, called laser assisted isotope ratio analysis (LARA), is suitable for $\delta^{13}C$ analysis of CO_2 samples using two fixed frequency $^{12}CO_2$ and $^{13}CO_2$ lasers by measurement of the electrical response of a gas discharge induced by the laser optogalvanic effect. This technique finally measures an electrical discharge signal being proportional to the concentration of the isotopic species measured. The precision obtained so far is better than 1‰ for $\delta^{13}C$ and about 0.5‰ for $\delta^{18}O$.

The non mass spectrometric methods share the advantage of avoiding isobaric corrections that are inherent in mass spectrometric measurement. Due to the optical character of the measurements, they can be used for the parallel measurement of several samples and standards at the same time, limited ultimately only by space and budget considerations for the installation of multiple measurement chambers. The infrared laser absorption method provides means for the simultaneous measurement of all stable isotope ratios directly from one water sample without any sample preparation effort, but requires expensive laser equipment; costs which could be minimized only by the development of special diode lasers tuneable in the near infrared spectrum. The LARA method is a relatively cheap and robust method for direct CO_2 analysis in gas mixtures with 5% CO_2 in N_2, but of course needs a suitable conversion of the sample material into CO_2 gas as in the case of mass spectrometric methods. Further research is currently carried out for the measurement of other isotopic ratios such as $\delta^{18}O$ with the LARA method. The development of similar systems for $\delta^{17}O$ and δ^2H analysis seems possible.

In view of their demonstrated usefulness and identifiable avenues for improvement, there is likely a promising future for these techniques, which in some cases could replace mass spectrometry by simpler and more robust alternatives. Commercial interest in those techniques will be crucial for their further development.

For now, the majority of stable isotope ratio determinations are still performed by mass spectrometry. The on-line or continuous flow technique already accounts for a majority of these analyses, and covers an area where new applications are invented nearly day by day. The precision for $\delta^{13}C$ and $\delta^{18}O$ analyses using such techniques is in the order of 0.2‰, for δ^2H about 2‰. The terminology, including acronyms describing the technique, has proliferated overwhelmingly and this warrants some clarification. 'On-line' and 'continuous flow' refer to the fact that there is a carrier gas flowing uninterruptedly for sweeping reference gas and sample gas into a mass spectrometer. The term GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) is in wide use for describing the coupling of gas chromatography with isotope ratio MS via a combustion interface. In spite of its wide recognition the technique is rather specific for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ determination from GC eluates. It fails to accurately describe its more recent analytical sibling: the $^{18}O/^{16}O$ and D/H determination from GC eluates via a pyrolysis interface. The same argument applies to GCCMS (gas chromatography/combustion/mass spectrometry) which was introduced at the presentation of the first commercial system in 1988. It also fails to describe the most widely employed technique that couples a conventional elemental analyzer with isotope ratio mass spectrometry via an open split interface.

In addition, CSIA (compound specific isotope analysis) and BSIA (bulk sample isotope analysis) have been used for describing the general coupling of elemental analysers operating in combustion or pyrolysis mode with isotope ratio mass spectrometry.

In spite of all the acronyms (the list is far from complete) the techniques essentially mean that the gas analyte (CO_2, N_2, SO_2, CO, HD etc) enters the mass spectrometer entrained in helium. Hence, all chromatographic techniques that make use of helium as a carrier gas can be used. The mass spectrometer simply follows the isotope ratio of the transient signals by monitoring two or three chromatographic traces simultaneously and providing data handling for extracting precise isotope information from these traces. This has also been referred to as 'isotope ratio monitoring' or 'IRM techniques'.

The technical revolution that happened in stable isotope ratio measurement techniques has suffered from the limited availability of reference materials. Considering that combustion efficiency or pyrolysis efficiency in the preparation step may vary slightly, given the common and known problems of mass discrimination during injection of sample material onto a GC column and bearing in mind that open split couplings can cause isotopic fractionation due to diffusion, it becomes evident that sample and reference material should be closely comparable in nature and that they must be treated equal. Introducing a reference gas from a tank is a fabulous way to monitor and correct for changes in response of the mass spectrometer. However, it does not account for deficiencies in the sample inlet line. To compensate for this effect the standard material must go through the same inlet. This can be referred as the *IT principle* for *I*dentical *T*reatment of sample and reference material during the course of an analysis. From the *IT principle* it follows imperatively that there should be as many chemically different reference materials as there are chemically different sample materials. This evidently is not possible.

How can this dilemma be solved? Clearly, the problem to cross calibrate chemically different reference material has existed in the past and it has created a lot of work followed by controversy and compromise or definition. There are still two scales for $^{18}O/^{16}O$ in use (VSMOW and VPDB) that have been linked together by measurement followed by consensus and definition. Another example is the widespread use of NBS22 rather than NBS19 for standardizing carbon lab reference material for combustion techniques. These problems are not new and they were difficult to solve in the past. Given the expressed need for a diversification of reference materials the problem of accuracy of international reporting standards comes again into question. A prominent example is the use of accepted reference material for $^{18}O/^{16}O$ determination of organic material using high temperature pyrolysis coupled to an isotope ratio MS. The calibration materials are water (VSMOW) and carbonate (NBS19). Both are problematic to pyrolyze, the carbonate for instance due to the fact that the 3rd oxygen is only partly involved in the pyrolysis and in addition because oxygen in NBS19 may also be present in a chemical form different from carbonate. NBS19 is a natural product after all. These oxides can at least partly react in the high temperature pyrolysis step. It does not play a role when NBS19 is reacted with H_3PO_4. Water (VSMOW) suffers from another problem. Here the exchange of oxygen with hot oxygen bearing surfaces prior to formation of CO as well as the availability of carbon as a reaction partner can alter the measured isotopic composition. Hence, these primary materials have variable decomposition pathways during carbon aided reduction at the temperatures used (1400°C) and thus are not ideally suited as reference materials for the pyrolysis technique. Other, secondary materials like cellulose are available but their value on the international scale is not known very precisely. In addition, cellulose is slightly hygroscopic making it less ideal for use as every day or as long term reference.

The need for chemically closer reference materials for strictly applying the principle of identical treatment of sample and reference arises in almost all new couplings of automated sample preparation units with isotope ratio mass spectrometry. In a similar way it is needed for new non mass spectrometric techniques for measuring stable isotope ratios as for instance in laser spectroscopy. It is evident in D/H and $^{18}O/^{16}O$ analysis by high temperature pyrolysis described above. For D/H, two

reference compounds (NBS22 and IAEA-CH-7) can be used, but they are very similar in isotopic composition. Even in case the δD values of both materials can be reproduced in one set of experiments (thus indicating independence of the pyrolysis preparation from compound specific effects), there is still the need for proper scaling in order to compensate for the known and well documented effects of scale contraction (or sometimes even expansion) in the mass spectrometric measurement. Preferably, water samples should also be arranged on the same δ-scale, but this seems to be rather difficult to achieve and requires further experimental investigations.

The need for diversification of reference materials is also apparent in GC applications where organic compounds are separated, combusted (or pyrolyzed) and measured for their isotopic compositions. Here, concomitant injection of a chemically identical reference compound into the GC does not help because the reference compound will elute at the same retention time as the sample. Thus, either referencing needs to be done using a chemically different but similar compound. This requires that the cross calibration actually does not pose analytical problems or that the calibration is based on a scheme of different GC runs which are calibrated on the basis of reference gas pulses providing the link between the sample and reference runs. For moderate precision the referencing could be done only once a day. For higher precision more frequent runs of reference compounds or mixtures will be required. For designing a working referencing scheme in the laboratory, the availability of both dedicated mixtures in glass ampoules as well as single calibrated compounds will be necessary.

Water equilibration among other techniques has been revisited in order to make it faster and extend the measurement range to much smaller sample sizes. The method has been described for both, D/H and $^{18}O/^{16}O$ analysis. Here, the value of the *IT principle* becomes most evident. Due to the temperature dependence of the equilibration constant the experiment must be performed at a constant temperature. Mostly, temperature stability of 0.1ºC can be safely guaranteed for a certain time, e.g. for a day or even a week. In order to measure precisely over long periods of time, the best way to proceed is to include a decent number of internal laboratory water standards into the measurement scheme. This procedure not only allows for automated accuracy, it also provides the opportunity to correct for temperature drift over time, for reference gas drift, if applicable, and for correction of scale contraction by placing a very light reference water together with the ordinary reference water into the carousel or autosampler. Some of the newer systems also allow direct measurement of D/H from organic material, even from methane. Here, the same question arises, how can referencing to international δ-scales be achieved? The ideal case would be the availability of suitable reference material including CH_4 gas (in lecture bottles).

A particularly difficult problem is encountered with air samples. Here, very high precision is often mandatory for reading the faint signatures. Even worse: this precision needs to be maintained on the same δ-scale over decades in order to follow longer term trends in the development of the atmosphere. In general, suitable reference materials in this field must be air, either in sample flasks or in gas cylinders that are routinely exchanged among laboratories in order to calibrate on the same δ-scale. Several years ago, the Australian CSIRO Division of atmospheric research, in cooperation with the IAEA, has produced a set of ten high pressurized gas cylinders filled with air and a well defined concentration of CO_2, CH_4 and N_2O of known isotopic composition. The circulation of those cylinders among four expert laboratories (CLASSIC — circulation of laboratory air standards for stable isotope comparisons) provided the first instance for a close intercalibration of laboratories engaged in studies of the isotopic composition of atmospheric trace gases.

Overall, the meeting was marked by a synergism of discussions among experts from a variety of scientific fields with a common interest in advancing the measurement science of stable isotope ratios. A detailed assessment of needs and wishes of the different user communities are outlined in the individual contributions presented in this volume. The annex contains a master list of candidate reference materials and four working group reports.

OPTICAL SPECTROSCOPIC TECHNIQUES

LASER SPECTROMETRY APPLIED TO THE SIMULTANEOUS DETERMINATION OF THE δ^2H, δ^{17}O, AND δ^{18}O ISOTOPE ABUNDANCES IN WATER

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Abstract. We demonstrate the first successful application of infrared laser spectrometry to the accurate, simultaneous determination of the relative ^{2}H/^{1}H, ^{17}O/^{16}O, and ^{18}O/^{16}O isotope abundance ratios in natural water. The method uses a narrow line width color center laser to record the direct absorption spectrum of low-pressure gas-phase water samples (presently 10 µl liquid) in the 3 µm spectral region. The precision of the spectroscopic technique is shown to be 0.7‰ for δ^2H and 0.5‰ for δ^{17}O and δ^{18}O, while the calibrated accuracy for natural waters amounts to about 3‰ and 1‰, respectively.

1. INTRODUCTION

Quantitative information on the natural variation of isotope abundance ratios in water is invaluable in a wide variety of disciplines, from paleo-climatology, to hydrology, atmospheric chemistry, and bio-medicine. The currently available methods for the measurement of isotope ratios in water are far more cumbersome (as well as inaccurate) than for, for example, CO_2. Traditionally, all methods make use of isotope ratio mass spectrometers (IRMS) designed specifically for the purpose of measuring isotope abundance ratios. Due to the nature of the molecule, water samples are not introduced directly into the mass spectrometer. Instead, the water is chemically converted to CO_2 and H_2, and the ^{18}O/^{16}O and ^{2}H/^{1}H isotope ratios in these molecules are measured. The conventional method for ^{18}O/^{16}O is to bring the water sample into contact with CO_2 of known isotopic composition and to wait several hours for the bicarbonate reaction to establish isotopic equilibrium. The H_2 is most commonly produced by reduction of water over hot (approximately 800°C) uranium or zinc. These sample pretreatments are sometimes hazardous, generally time-consuming, and almost always the limiting factor in achieving the high accuracy demanded in the majority of studies. An additional disadvantage of the traditional IRMS method is the virtual impossibility to measure the ^{17}O/^{16}O ratio in water, due to the mass overlap of ^{17}O^{12}C^{16}O and the much more abundant ^{16}O^{13}C^{16}O.

It is in this light that there is much interest in alternative methods, especially for hydrogen. The use of platinum catalytic equilibration of a water sample and hydrogen gas in combination with an IRMS appears promising [1, 2]. But this method too has its disadvantages: the equilibrium state is accompanied by a very large isotope fractionation (about -740‰ at room temperature), which is also extremely temperature dependent (~6‰/ºC). Furthermore, the gas sample arriving at the IRMS contains approximately one-quarter the original amount of deuterium, considerably decreasing the (already low) mass-3 signal, with obvious consequences for errors due to background correction, amplifier offset, and, above all, the H_3^+-correction. Finally, the amount of water required is rather large (typically 4–5 ml), prohibiting the use of this method in quite a number of possible applications. The latter is also true for electrolysis, a technique that can measure δ^{18}O and δ^{17}O (in the electrolytically produced O_2) [3]. In fact, the only realistic alternative appears to be on-line pyrolysis of water in combination with a continuous-flow IRMS. The latter method is able to deal with very small sample sizes (down to 1 µl), provides a high throughput, and is able to achieve very reasonable precisions (2‰ for δ^2H and ~0.3‰ for δ^{18}O; unfortunately the data presented by Begley and Scrimgeour are insufficient to give an estimate of the overall accuracy) [4].

Here we report on our efforts to use a completely different measurement technique, based on direct absorption laser spectroscopy. For most relatively small molecules the room-temperature, low pressure, gas phase infrared spectrum reveals absorptions due to individual ro-vibrational transitions that each can be uniquely assigned to one of the various isotopic species present. The absorption intensities of these lines, relative to that of a line belonging to the most abundant isotopic species, can

be used to calculate the isotope ratio of interest. The measurement of the absorption intensity is done by recording the attenuation of a laser beam with narrow spectral line width as a function of wavelength.

This technique has some major advantages over the traditional IRMS method: smaller sample sizes and measurement of all isotope ratios directly in the water vapor, avoiding the time consuming and inaccurate sample preparations that are otherwise required. Moreover, with the spectroscopic method it is possible to accurately measure the $^{17}O/^{16}O$ ratio in water (in addition to the $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios), as well as site-selective isotope ratios in more complex molecules. Both of these are impossible or completely impractical with the conventional mass-spectrometer (including pyrolysis and continuous-flow systems), and provide ample opportunities for completely new research. Finally, in contrast to other known methods it provides a "single route" approach towards results for both $\delta^{18}O$ and $\delta^{2}H$ (as well as $\delta^{17}O$): a single measurement on the same amount of water vapor yields at once all isotope ratios of interest.

2. EXPERIMENTAL

The apparatus, depicted in Fig. 1, uses as its light source a tunable near-infrared, single-mode, color center laser (Burleigh FCL-20), pumped with a krypton-ion laser. The laser output is used to record direct absorption spectra in two identical gas cells, equipped with multiple-pass reflection optics (giving an optical path length of about 20 m.). The spectra are corrected for laser power fluctuations and absorptions outside the gas cells.

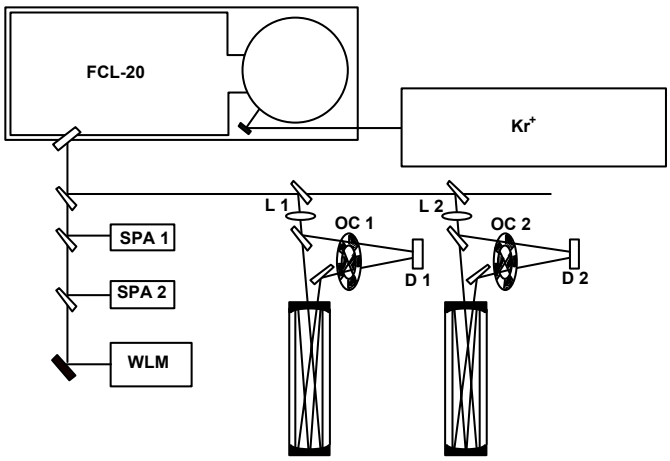

Fig. 1. Schematic representation of the experimental set-up. FCL-20 = color center laser, Kr^{+} = krypton-ion laser, SPA1 = 150 MHz FSR spectrum analyzer, SPA2 = 8 GHz FSR spectrum analyzer, WLM = wavelength meter, L = 500 mm focal length lens, OC = dual frequency optical chopper, D = InAs infrared detector.

For each gas cell, the signal and laser-power beams are amplitude modulated at different frequencies and directed at the same InAs infrared detector. Signal recovery is by means of phase-sensitive detection. A personal computer controls the laser tuning and data acquisition.

The gas cells are filled to a pressure of about 13 mbar (well below the saturated vapor pressure), requiring about 10 µl liquid water. One of the gas cells is filled with reference water with a well-known isotopic composition with respect to the calibration material VSMOW. The isotopic composition of the sample water in the second gas cell is determined by comparing the relative intensities of the selected ro-vibrational transitions in the sample cell to those in the reference cell. A typical (laser power corrected) spectrum is shown in Fig. 2.

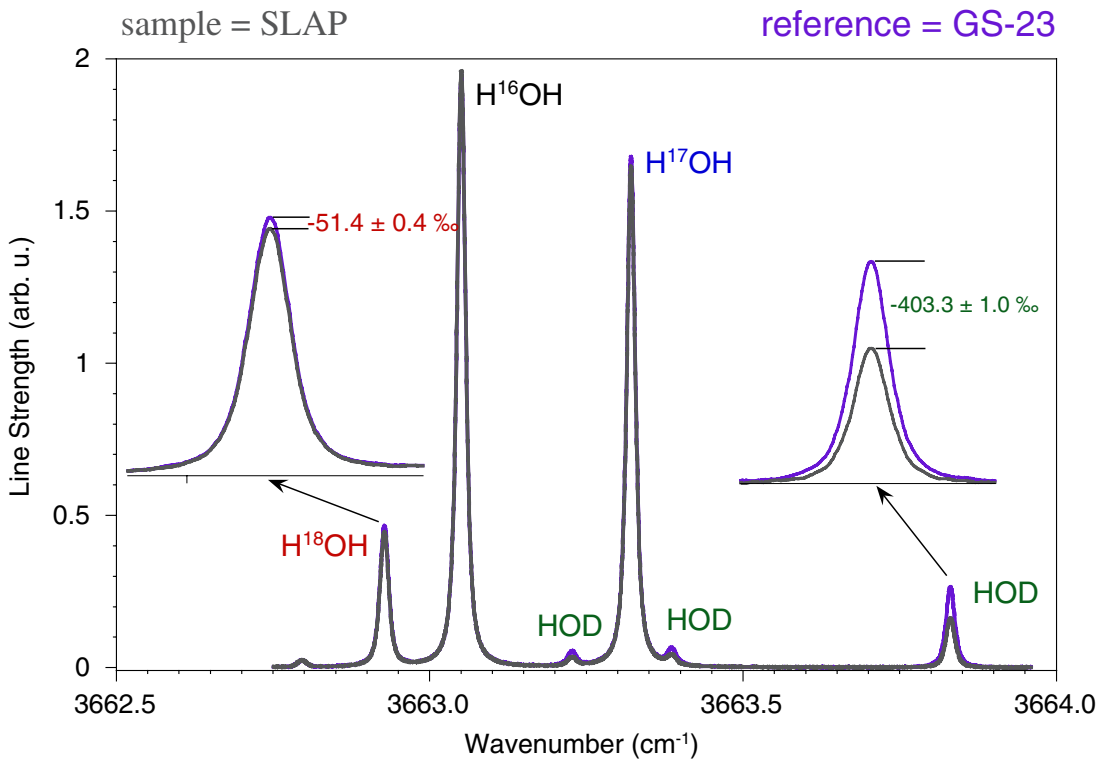

Fig. 2. Typical sample and reference spectra showing transitions due to all 4 isotopic species of interest. The two spectra are scaled such that the most abundant species appears equally intense in both. The ratio of the sample and reference intensities than gives the abundance ratio, which in turn yields the δ-value, after correction for a differential pressure effect.

Provided the temperature is kept constant (and the same for both gas cells), the δ-value can be computed from the measured absorption coefficients α_n, where the subscript *n* refers to the abundant ($a = {}^1H^{16}O^1H$) or rare ($x = {}^1H^{18}O^1H$, ${}^1H^{17}O^1H$, or ${}^1H^{16}O^2H$) isotope:

$$\delta(x) = \frac{R_x^{sample}}{R_x^{ref}} - 1 = \frac{(\alpha_x / \alpha_a)^{sample}}{(\alpha_x / \alpha_a)^{ref}} - 1 = \frac{(\alpha_x^{sample} / \alpha_x^{ref})}{(\alpha_a^{sample} / \alpha_a^{ref})} - 1 \qquad (1)$$

The temperature coefficients of the transitions used in this study are sufficiently small that no special precautions, like active stabilization of the gas cell temperature, have to be taken in order to avoid measurable temperature effects on the δ-value. Which is not too surprising if one realizes that the δ-value is only sensitive to a temperature difference between the two gas cells. In contrast, all experimental data were corrected for pressure differences between the sample and reference cells (which manifest themselves through a different line width in the reference and sample spectra).

A typical measurement series ("run") consists of about 15 individual laser scans over the selected spectral section. The average value of the measurements (after eventual removal of accidental outliers) is reported as the final result, together with its standard error. Including sample introduction and gas cell evacuation between the measurement series, one run takes a little less than one hour.

3. CALIBRATION

In order to calibrate the instrument we measured the (IAEA) reference material GISP, as well as a series of local water standards that are well-characterized with respect to VSMOW by repeated mass-spectrometer analyses in our laboratory (see Table I). The local standard "GS-23" was used as

working standard in the reference gas cell. Consequently, the laser-spectrometer (LS) values are initially referenced to this material. These have been converted to values relative to VSMOW using the laser spectrometrically determined value of GS-23 with respect to VSMOW. This inherently takes care of the zero-point adjustment of the laser spectrometric δ-scale. The ^{17}O-isotope ratios of our natural water standards (for which to date no consensus values have been established internationally) are known through the relation [3]:

$$1+\delta(^{17}O)=\left[1+\delta(^{18}O)\right]^{0.5281} \quad (2)$$

As the ^{2}H-, and to a lesser extend the O-, measurements are afflicted with a large memory effect (the influence of the previous sample on the current measurement), it turned out to be occasionally necessary to inject 3 or more water samples before the measured δ-value reached its final value. To minimize the influence of this memory effect on the calibration procedure, large steps in δ-values between subsequent samples were avoided as much as possible. For the same reason, data were recorded both in increasing and in decreasing order of δ-value.

Unfortunately, the results prior to normalization tend to underestimate (overestimate) the "true", positive (negative) δ-values, or, to put it differently, a scale expansion factor larger than unity is needed. It appears as if the sample is mixed with reference water (but not *vice versa*). Although we have established that the sample introduction procedure cannot be blamed, we have not yet been able to eliminate this residual effect (perhaps caused by memory effects in the vacuum system). As has become apparent over the years in numerous international ring tests, IRMS-based measurements too require scale expansion factors larger than unity. In particular for ^{2}H the deviations found are often much larger than those of the present laser system. A pragmatic approach to this problem, in which the δ-scales are defined by a linear calibration using two different calibration waters (e.g., SLAP in addition to VSMOW), has been generally accepted, and in fact is recommended by the IAEA [5]. The same solution is adapted here.

In Table I the mean of the LS δ-values (referenced to VSMOW), that determined the scale expansion factor, is compared with the IAEA consensus value, for each of the isotopes.

Table II confronts the LS results with the MS results by comparing the current “best” values for a series of 7 water samples (including VSMOW and SLAP, which define the scale expansion factor of the linear calibration).

TABLE I. SLAP δ-VALUES (‰) (REFERENCED TO VSMOW)

Isotope	Laser spectrometer[a]	Consensus value[b]
$\delta(^{2}H)$	-415.47 (0.85)	-428.0
$\delta(^{17}O)$	-28.11 (0.23)	-29.70
$\delta(^{18}O)$	-53.88 (0.37)	-55.5

[a] Based on 11 measurement series (or runs, each consisting of 15 individual laser scans) and acquired over a one-month interval. The standard error is given between brackets.

[b] Consensus value: recommended by the IAEA [6]. The $\delta^{2}H$ value results from a mixture of isotopically pure synthetic waters and is regarded to be correct in absolute terms. The $\delta^{18}O$ is the consensus value of 25 laboratories; the true value is likely somewhat more negative. The $\delta^{17}O$ value is based on the consensus $\delta^{18}O$ value in combination with (2).

The total calibration effort also presents us with RMS values of the residuals, and these give a good indication of the over-all accuracy of the method, including all effects of sample handling. The values are: 2.8‰, 0.7‰, and 1.3‰ for δ^2H, $\delta^{17}O$, and $\delta^{18}O$, respectively. The precision of the method is given by the standard error of the individual results of one series of (typically) 15 laser scans. The current average values of these are: 0.7‰, 0.3‰, and 0.5‰, for δ^2H, $\delta^{17}O$, and $\delta^{18}O$, respectively. In the case of ^{17}O and ^{18}O the precision can still be improved by increasing the number of laser scans in one run (i.e., increasing the measuring time). For δ^2H the minimum standard error has already been reached at this point, indicating that in this case the precision is limited by sample-handling errors, including memory effects and isotope fractionation at the gas cell walls.

The reproducibility of the measurements is rather good, especially considering that the results presented here were gathered over an extended period of time (about two months). This means that the system is now ready to be applied to the bio-medical doubly-labeled water method to measure energy expenditure, as well as to the accurate measurement of natural abundances, for which especially the δ^2H determination is already competitive.

4. CONCLUSIONS

We have shown that laser spectrometry presents a promising alternative to conventional mass spectrometric isotope ratio analysis of water. In particular, the laser based method is conceptually very simple and does not require cumbersome, time-consuming pre-treatments of the sample before measurement. This excludes an important source of errors. Moreover, all three isotope ratios, $^2H/^1H$, $^{18}O/^{16}O$, as well as $^{17}O/^{16}O$ (virtually impossible by means of IRMS), are determined at the same time without requiring different (chemical) pre-treatments of the sample.

The precision of the method is currently about 0.7‰ for $^2H/^1H$ and 0.5‰ for the oxygen isotopes. We have shown a calibrated accuracy of about 3‰, respectively 1‰. Since the calibration data were collected over an extended period in time it is expected that more frequent calibration will enable us to achieve an accuracy closer to the intrinsic precision of the apparatus. In addition, the calibration procedure will be improved by the simultaneous measurement of more than one water standard.

Currently, the throughput is limited to about one sample per hour, comparable to that of the original, conventional methods when both δ^2H and $\delta^{18}O$ are determined and the sample preparation time is added to the actual IRMS time. With modest improvements in the detection (faster amplitude modulation and a shorter lock-in time-constant) this can probably be increased by a factor of two, the final limiting factor being the evacuation and flushing of the gas cells. However, the throughput is most easily increased by the use of multiple gas cells, allowing the parallel measurement on many more than just one sample. Considering the very modest demands on laser power, relative to the output power of the available laser system, the number of gas cells is only limited by budgetary and space constraints.

TABLE II. LASER SPECTROMETRY (LS) COMPARED TO MASS SPECTROMETRY. THE LS RESULTS ARE BASED ON BETWEEN N = 4 AND 11 δ-DETERMINATIONS (OF 15 INDIVIDUAL LASER SCANS EACH), SPREAD OUT IN TIME OVER A PERIOD OF BETWEEN 4 AND 10 WEEKS. ALL VALUES ARE EXPRESSED IN PER MIL POINTS. THE STANDARD ERROR OF THE MEAN VALUES REPORTED FOR THE LS MEASUREMENTS IS GIVEN IN PARENTHESES.

	Mass Spectrometry			Laser Spectrometry			
Standard [a]	$\delta(^2H)$	$\delta(^{17}O)$ [b]	$\delta(^{18}O)$	$\delta(^2H)$	$\delta(^{17}O)$	$\delta(^{18}O)$	N
VSMOW	0.0	0.0	0.0	0.0 (0.4)	0.0 (0.13)	0.0 (0.5)	8
SLAP	-428.0	-29.70	-55.5	-428.0 (0.9)	-29.7 (0.2)	-55.5 (0.4)	11
GISP	-190.0	-13.21	-24.76	-188.8 (0.3)	-13.2 (0.3)	-25.0 (0.5)	8
GS-23	-41.0	-3.36	-6.29	-41.4 (0.8)	-3.3 (0.3)	-6.7 (0.4)	8
GS-31	-257.8	-75.48	-137.3	-260.5 (0.4)	-76.5 (0.9)	-139.3 (0.2)	3
GS-30	-403.3	-127.55	-227.7	-405.4 (0.8)	-128.0 (0.4)	-232.5 (0.5)	8
GS-32	-91.5	--	-56.84	-98.6 (0.5)	-46.9 (0.10)	-58.0 (0.5)	4

[a] The VSMOW and SLAP values for δ^2H and $\delta^{18}O$ are those recommended by the IAEA.[6, 5]. The intercomparison material GISP has the consensus values [6]: δ^2H = -189.7 (1.1) ‰ and $\delta^{18}O$ = -24.79 (0.09) ‰. The Groningen GS local standards have been established by repeated mass spectrometric analysis in our laboratory over a period of several years. GS-23 is a natural water; GS-30, GS-31, and GS-32 are synthesized.

[b] The $\delta^{17}O$ values of those water samples that exhibit a natural relation between the ^{17}O and ^{18}O abundance ratios (i.e., all except GS-32) have been calculated using (2).

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LASER ASSISTED RATIO ANALYSIS — AN ALTERNATIVE TO GC/IRMS FOR CO_2

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Abstract. A new technique for laser based analysis of carbon isotope ratios, with the acronym LARA, based on large isotope shifts in molecular spectra, the use of fixed frequency isotopic lasers, and sensitive detection via the laser optogalvanic effect is reviewed and compared with GC/IRMS for carbon dioxide in specific applications. The possibility for development of new classes of isotope ratio measurement systems with LARA is explored.

1. INTRODUCTION

A new technique for laser based analysis of carbon isotope ratios was first reported in 1994 [1]. The method is based on large isotope shifts in molecular spectra, the use of fixed frequency isotopic lasers and sensitive detection via the laser optogalvanic effect. Subsequent development [2] has demonstrated that laser assisted isotope ratio analysis, given the acronym LARA, is a viable alternative to traditional IRMS for carbon dioxide in specific applications and may make new classes of isotope ratio measurement systems possible.

The optogalvanic effect has been used for atomic and molecular spectroscopy for many years [3]. It is based on the electrical response of a gas discharge to optical perturbation. If a laser of intensity I, and frequency ν, is incident on a weak electrical discharge, the response S, of the discharge can be approximated very closely by:

$$S = nLI(\nu)A\sigma(\nu)C \tag{1}$$

where n is the areal density (molecules/cm^2) of interacting species, L is the length of the interacting region, A the area of the laser beam, σ the interaction cross section and C an optogalvanic proportionality constant.

Fig. 1 is a schematic representation of a basic measurement system used for $^{13}C/^{12}C$ in CO_2. Two or more samples, a standard and one or more unknowns, are maintained in low power radio frequency gas discharges and irradiated by fixed frequency carbon dioxide isotopic lasers. Mirrors M1-M3, combine the laser beams and direct the light through cells to beam stops BB. Pressure sensors PS, are used to measure the pressure in the cells. Electrical discharges are controlled by excitation circuits which also feedback the signals S. A computer with digital signal processing capability DSP, controls the system via analog to digital and digital to analog conversion A/D. The samples are at low pressure controlled by vacuum pumps and adjustable valves.

Many different vibration-rotation transitions can be made to lase in CO_2 [4]. The transitions chosen for $^{12}CO_2$ and $^{13}CO_2$ must be well separated in wavelength and are automatically in resonance with the same molecular transitions in the samples, providing the specificity required for the isotope ratio analysis. Purification or concentration of the samples is generally not required. The laser intensity, I, provides a gain factor so that signals for dilute isotopes can be amplified relative to the majority species. The effective intensity can be further multiplyed by using multiple reflections increasing the apparent length of the discharge, L. Another way to enhance sensitivity is through the coupling parameter C, by choosing optimal discharge conditions. An optimum discharge has been found to consist of less than 5% CO_2 in nitrogen. A mixture of CO_2 in nitrogen greatly enhances the optogalvanic effect due to the almost resonant exchange between electron excited N_2 and the upper laser level of CO_2. This is the same reason a CO_2 laser operates with a majority N_2 gas fill [5].

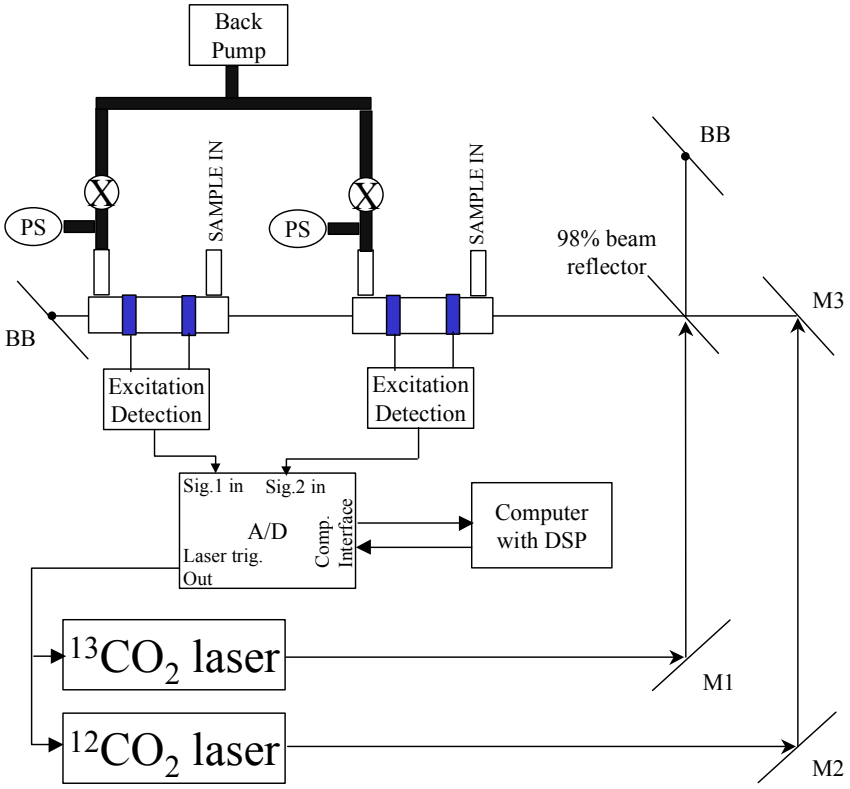

Fig. 1. Schematic diagram of LARA system for $^{13}C/^{12}C$ isotope ratio determination in gas samples containing CO_2.

A negligible fraction of laser power is absorbed in the discharge. In fact, under proper conditions there is a small gain in intensity I, so that multiple samples and a standard can be measured simultaneously in series. The measured signal is electrical. No optical measurement is required eliminating all collection and dispersion optics or light transducers. Optical background, which might otherwise obscure the desired signal, is absent. Using modulated laser beams and lock in detection techniques, extremely high signal to noise ratios are achievable. Using incommensurate modulation rates, multiple signals can be determined simultaneously [6].

A double ratio:

$$\left(\frac{\frac{^{13}S_x}{^{12}S_x}}{\frac{^{13}S_{std}}{^{12}S_{std}}} - 1 \right) \times 1000 \tag{2}$$

directly yields the relative isotopic ratio δ, all parameters of Eq. 1 canceling except for:

$$\frac{\frac{^{13}n_x}{^{12}n_x}}{\frac{^{13}n_{std}}{^{12}n_{std}}}. \tag{3}$$

2. COMPARISON WITH GC/IRMS AND OTHER OPTICAL METHODS

The LARA technology differs both from GC/IRMS and other reported optical techniques [7, 8, 9] for precision isotopic ratio measurements. Table I presents a relative comparison of the measurement systems.

Unlike IRMS, all optical techniques are species rather than mass selective. Hence chemical separation and/or enrichment of CO_2 are usually not necessary. Optical techniques, which rely on deconvolution of spectral line shapes [10], are subject to background optical resonances which may limit sensitivity by obscuring or overlapping the specific isotopic resonances to be analyzed. LARA, however, is only limited by possible differential fractionation effects with other gases in the sample and/or standard.

IRMS analysis begins with an ion source from which ion beams are extracted. The sample is destructively analyzed via ion current collection. The LARA discharge is, in some ways, similar to the ion source discharge; however, the ion density is low and measurements are nondestructively carried out inside the discharge region. Sample gases could be saved and stored for subsequent reanalysis if desired. The obtainable precision and measurement time are, of course, coupled in all systems; however, less sample is required for spectroscopic based measurement due to continuous averaging of an unchanged sample.

TABLE I. CO_2 ISOTOPE RATIO ANALYSIS

	Irms	Ir Optical	LARA
Dispersion	Mass Ion beam Trajectory	Species Tunable laser/Spectrometer	Species Fixed wave-Length lasers
Detection	Ion current	Light Absorption	Electrical Conductivity
Sample Preparation	Extensive	Moderate	Minimal
Gain	No	No	Yes
Sample size	Medium	Large	Small
Speed	Medium	Low	High
Precision	High	Medium	High
Calibration	Sequential	Sequential	Continuous
Limitations	Isobars Fractionation	Resolution	Fractionation

It is interesting to consider ultimate limits to precision and sample size. Most optical measurements are inherently limited to precision of the order 0.5 to a few δ because of the spectral deconvolution algorithm and/or light source used, even when long optical path lengths are employed to enhance weak spectral features. LARA has no such limitation because of the use of fixed wavelength lasers and the gain, via laser intensity, for the more dilute isotope inherent in the optogalvanic measurement. GC/IRMS is limited in ultimate precision by its insensitivity to isobars and possible fractionation effects in the sample preparation, transit and ionization. LARA, too, is limited ultimately by unknown differential fractionation effects in the gas discharge.

3. CALIBRATION AND STANDARDS

Absolute values for unknown isotopic ratios are extremely difficult to achieve [11]. For analytical work a traceable standard is required [12] except for situations such as isotopic tracer studies [13] where relative changes with respect to time of tracer introduction are of interest. Various calibration techniques are used with GC/IRMS; most commonly measurement of standard samples are interspersed with unknown samples. Dual ion sources are often used so that cross contamination of unknown and standard is avoided. Standard gases from national or regional standards laboratories are periodically sent to participating analytical instruments for quality assurance. As is the case with unknown samples, standard gas samples are destructively analyzed, and the change to a new standard can be complicated.

The situation with LARA determination of isotopic ratios is different. Most significantly, the measured parameter is δ directly. The standard to unknown ratio is measured continuously and the standard can be in a sealed cell interchangeable between instruments. Ideally, the standard gas is an identical gas mixture to the unknown, e.g. 3% CO_2 in nitrogen. Otherwise, a calibration must be carried out with a sample of known isotopic ratio in a known gas mixture. As long as a series of unknown samples are of similar origin such as pure CO_2 or air or breath the calibration is straightforward. However, to compare isotopic ratios of CO_2 in different gas mixtures, fractionation effects in the discharge must be carefully considered. Measurements have been carried out with concentrations of CO_2 varying over several orders of magnitude. There is a small concentration dependence to the optogalvanic coupling parameter of equation 1, and further work on calibration for samples of differing concentration and other gas concentrations is required. A standard for CO_2 concentration and gas mixture to intercompare LARA with GC/IRMS would be desirable to cross calibrate between instruments. No correction is required for isobaric species with LARA measurements, so empirical correction factors now in use with GC/IRMS to correct for $^{12}C^{16}O^{17}O$ in determination of $^{13}CO_2$ concentration might be checked using the species sensitivity of LARA isotope ratios.

4. APPLICATIONS

4.1. Laser assisted ratio analysis of human breath

The first commercial application using this technology was developed for clinical use in analysis of human breath after ingestion of ^{13}C labeled compounds [6]. As breath is about 5% CO_2, with nitrogen as the majority gas, it is suitable for direct optogalvanic analysis using a gas mixture typical of normal breath, or the patient's own baseline breath, as the "standard". The only sample preparation required is removal of water vapor, the percentage of which can vary greatly from sample to sample hence causing differential fractionation effects in the discharges [14].

Several instruments have been used in hospitals in the United States, Canada and Europe and several thousand breath tests have been analyzed. Regulatory agencies in the United States (FDA) and Europe (EMEA) have approved the LARA system for the urea breath test for detection of H.pylori infection. Clinicians have been pleased with the high sensitivity and specificity of the system as well as its ease of use [15–18].

In an experiment, designed to compare the LARA system with a GC/IRMS optimized for breath analysis, 139 duplicate breath samples from two volunteers were taken after ingestion of labeled $NaH^{13}CO_3$. One set of samples was analyzed in a clinical LARA and the duplicates in the GC-IRMS (Finigan-Mat Breath-Mat). The excellent correlation obtained is shown in Fig. 2. It should be noted that the measurement time on the GC-IRMS was roughly twice as long as the measurement time on the LARA.

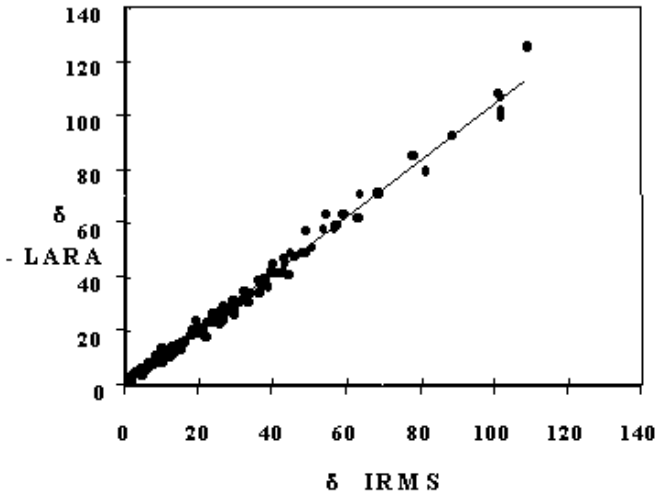

Fig. 2. Correlation of LARA analysis with GC/IRMS analysis after ingestion of $NaH^{13}CO_3$. The slope is 1.038, the intercept is -0.016 and R^2 is 0.992.

4.2. Analysis of atmospheric CO_2

Studies of atmospheric gases, atmospheric chemistry and global carbon balance require accurate and precise measurements of isotopic ratios and isotopic ratio variations with time, particularly for carbon and oxygen in carbon dioxide [19]. Researchers and policy makers seeking to understand the global carbon cycle and place enforceable limits on greenhouse gas emissions need high quality data on carbon fluxes, sources and sinks. While data for the concentration of CO_2 in the atmosphere has been well monitored additional constraints, such as the ^{13}C and radiogenic ^{14}C isotopic data are needed to distinguish between natural (i.e., biogenic) and anthropogenic sources (i.e., fossil fuel combustion). In addition, the question of whether carbon is stored or released from a given area, such as a forests, is key to developing a complete and quantitative inventory of the carbon budget.

Currently, the United States NOAA maintains the largest network of sampling sites for CO_2 concentration and stable isotopic analysis of CO_2 as shown in Fig. 3. Whole air samples are collected from these sites, shipped back to a central laboratory for high precision analysis of CO_2 concentration and isotopic composition. The NOAA flask network involves a large number of nations around the world, includes 4 NOAA baseline observatories, 40 Cooperative Sites, 4 commercial vessels and 2 sites located on existing commercial towers [20]. The flask approach critically limits the number of such samples that can be analyzed and increases the logistic complexity and cost of analysis due to sample transport, alteration during transport, etc.

As preconcentration is not necessary, and air can be continuously flowed through an instrument, the LARA technology may provide unprecedented opportunities to acquire data on the isotopic composition of atmospheric CO_2. In preliminary work at Rutgers University, discharges have been optimized with air flowing through cells so that signals from the unprocessed air exceed noise levels by factors of 1000 with modest (minutes) averaging time. The system can run unattended for days at a time. In Fig. 4, data from a single day illustrates measurements possible with dry air flowing in two cells.

As can be seen, the mean delta, 0 (as unknown and standard are the same air), shows a mean square variation less than 1δ, and apparent concentration variation of a few ppm. The apparent variations with time may be due to temperature swings, which are only partially compensated for, flow variations, wall sticking and other potential fractionation effects. Improvements in precision by an order of magnitude are expected with improved engineering of gas flow, cell temperature and related independent variables.

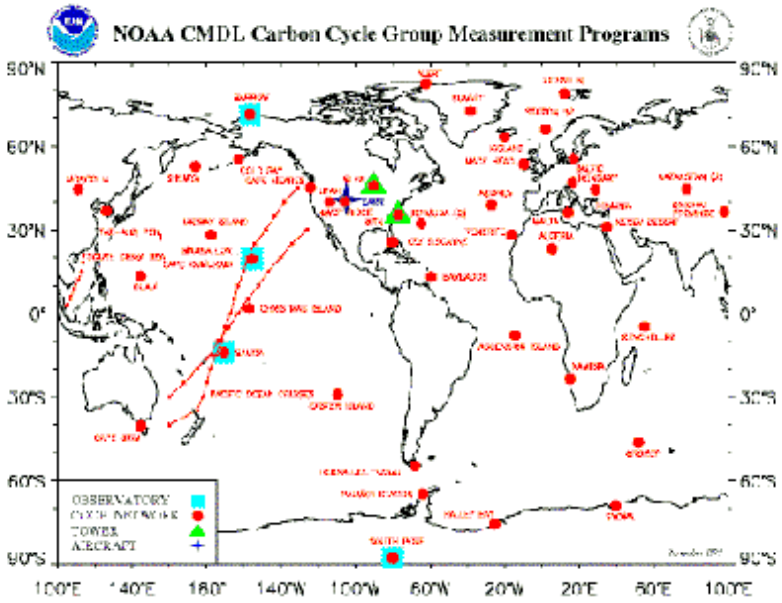

Fig. 3. Global distribution of NOAA CO_2 sampling sites.

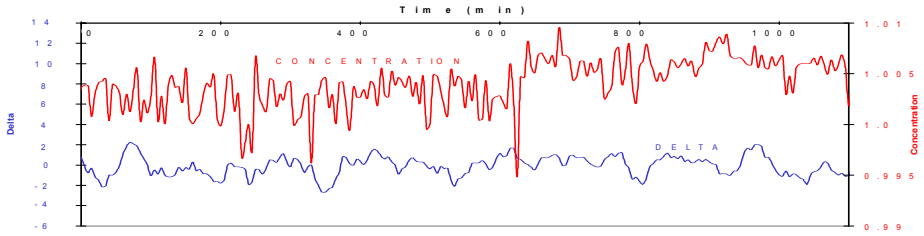

Fig. 4. Long term measurement of CO_2 concentration and $\delta^{13}C$ in standard dry air.

5. RESEARCH DIRECTIONS

As indicated, the LARA technology is quite young and there is more to learn and many potentially fruitful areas to explore. The ongoing development of reproducible standards and the limits to standard comparison precision are related. Atmospheric measurements of CO_2, and time variations with flow through systems are another area already under study. In addition to carbon, other element isotope ratios can also be determined using LARA provided that suitable lasers for the sensitivity and specificity required can be obtained. Research on oxygen isotopes, nitrogen isotopes and D:H ratios is planned.

Work on $^{18}O/^{16}O$ analysis in carbon dioxide based on the molecule $^{12}C^{18}O^{16}O$ is already in progress. In this case, the isotopic laser for the asymmetric molecule is somewhat more complicated, compared to $^{13}C^{16}O_2$, due to isotopic equilibration in the laser discharge which yields a 50% maximum concentration of the desired molecule. The mixed molecule $^{12}C^{18}O^{16}O$ has a high density of molecular states and there is overlap of laser bands with the symmetric molecules. Nevertheless, choosing a suitable transition in $^{12}C^{18}O\ ^{16}O$ near 9.6 μm, a strong optogalvanic effect signal unique to the ^{18}O containing molecule is obtained. Because of exchange effects with ground state $^{12}C^{16}O_2$ in the discharge, the sensitivity to ^{18}O is largest at concentrations less than 2% CO_2 in nitrogen. Results indicate that high precision (<0.5δ) measurements for $^{18}O/^{16}O$ ratios in CO_2 with samples as small as 100 nmoles are possible with measurement times less than 1 minute. Under certain conditions,

especially if water vapor is present, large fractionation effects involving ^{18}O in the electrical discharge cell can be observed. This is not surprising in view of the extremely high fractionation effects which have been observed in upper atmosphere ozone production [21].

The LARA isotopic analysis technology described may, in fact, provide a means for real time study of fractionation effects, as the optogalvanic signals from various discharge species can be studied as a function of time with resolution of tens of seconds, using flow through cells. Systems can be developed to yield comparable precision with a sample orders of magnitude smaller than those studied thus far. Similar systems could be developed for ^{17}O:^{16}O. Discussions at meetings such as this, and the needs of diverse user communities and funding agencies will determine the priorities and rate of progress of this exciting technology.

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CF-TECHNIQUES APPLIED FOR STABLE ISOTOPES IN ORGANIC MATERIALS

ON-LINE MEASUREMENT OF $^{13}C/^{12}C$ AND $^{15}N/^{14}N$ RATIOS BY E/A-DILUTER-IRMS

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Abstract. Efficient food control requires rapid procedures for testing source authenticity. Food is produced inside a closed 'isotopic environment' from where it inherits a specific isotopic composition or fingerprint. Isotope ratio mass spectrometry (IRMS) measures isotopic compositions using simple gases like CO_2 or N_2 exclusively. From food samples these gases may be produced by combustion in a commercial CHN analyser (Elemental Analyser, EA). Following GC separation of the combustion gases the elemental content is determined using a thermal conductivity detector (TCD) . The effluent of the EA is coupled to the mass spectrometer via an open split. Because the relative amounts of the bio-elements vary significantly, (often C/N is 25/1 or larger), the amount of analyte gas produced from a single sample must be adjusted e.g. using a diluter. Our diluter configuration can be adjusted to measure repeatedly the $^{13}C/^{12}C$ ratio of carbon dioxide in mineral waters, as well as to measure $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios from biological or soil samples simultaneously. In the first application different types of carbon dioxide, produced naturally (well) or technically (process), can be distinguished. The second application offers the possibility to trace the fate of a fertilizer in vineyards by determining the isotopic variation of nitrogen and carbon in soil and vines.

1. INTRODUCTION

Food control using natural stable isotope variation is the most modern tool for checking authenticity. In contrast to 'quality' (chemically pure), 'authenticity' refers to the correct origin and natural composition. A particular food may be without risk for consumers, but still may not originate from the region of the production proposed or be of exclusively natural composition. At first glance this may not constitute a disadvantage for consumers. However, it changes competition on the market and it certainly is not favourable for the consumer or the environment. Food production in Europe is the most important factor determining use and hence structure of the landscape. While the European Union spends billions of EC to support the original pattern of local agricultural production, more money is lost by illegal production of food inside and outside the EU.

Modern traffic enables a quick translocation of materials. Open borders and different administrative systems render backtracing of material by registration obsolete. Therefore, the fingerprint of the natural isotopic composition of a product probably is the only tool to reconstruct its individual history. Produced or formed in its local 'isotopic environment' which varies with the individual geographical location food gets a non-changable isotopic fingerprint. Usually control authorities require and enforce a rapid analysis of a product. For such rapid screening the overall isotopic data are sufficient in many cases.

The paper presents two examples of such a screening procedure used in daily routine. First, the $^{13}C/^{12}C$ ratio of carbon dioxide in mineral water is measured to distinguish between gas from wells and from technical processes. German regulations for the declaration of commercially distributed mineral waters require this differentiation. The second application is a study of the transfer of nitrogen from fertilizer to wine through the soil using $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. In a vineyard top soil vegetation and wine are competing for nutrients. Both isotope pairs are measured in sequence within one run.

2. METHOD

2.1. On-line measurement technique

Our general method for stable isotope analysis is the on-line or continuous-flow technique in isotope ratio mass spectrometry IRMS [1]. The material is converted to chemically simple gases by burning

and reducing it in a set of two furnaces inside an elemental analyser (EA). The gases produced are then separated gas chromatographically. The sample effluent outlet is fed into an isotope ratio mass spectrometer via an open split by a small capillary inlet. The diameter and length of this capillary determine the split and thus the amount of gas introduced into the ion source of the mass spectrometer. The arrangement carefully avoids isotopic fractionations which may occur during diffusion processes. No alteration of the flow during measurement is allowed.

As a consequence a different means of varying the sample amount flowing into the mass spectrometer is needed, in our case a diluter modul . This device can divide and thereby dilute the gas flow by connecting the parallel flow lines of carrier gas. One line flushes through the elemental analyser-TCD-GC branch (sample-helium-flow), the other carries the reference gas. Both lines are linked by a capillary (Fused Silica, 10 cm, 150 µm). For samples containing larger amounts of gas, e.g. carbon dioxide in mineral waters, the diluter may be used to reduce the concentration of the sample gas in the carrier stream. An enhanced pressure of carrier gas is employed to transport the gas sample through the tubes. In front of the open split the concentration of the sample gas is reduced by the diluter. In a similar manner, measurements of biological material, e.g. when measuring $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios simultaneously, require the dilution of at least one of the compounds during a single run, mostly that of carbon dioxide.

All isotopic ratios are related to IAEA standards in theory. In practice they are linked to the certified gases *Isotop*® supplied by Messer-Griesheim Krefeld. Each gas sample has its own certificate according to the regulations of the ISO 9000 series. The notations are given as δ-values related to the IAEA standards.

2.2. On-line measurement of carbon dioxide in mineral waters

Fig. 1 schematically shows the arrangement of the head space extraction from a liquid sample together with the continuous-flow EA-IRMS-device. A gas sample for instance from a closed bottle of mineral water can be fed into the continuous flow system of the commercial equipment. The diluter is the central unit of the extraction equipment represented by a programmable valve-switch which can be handled by the IRMS software (VG Isochrom Software 1.67). In general the diluter acts as a „flow divider“ aimed at adjusting the carbon dioxide to the dynamic range of the MS. This is necessary, because nitrogen in contrast to carbon is a minor component in most biological substances (except urea). For diluting and hence adjusting CO_2 the reference helium flow of the element analyser is used. It is directly coupled to the sample helium flow with a capillary so that approximately 10% of the sample helium flow is obtained. While an open diluter valve directs the flow to the main carrier, the closed valve connects the reference helium flow to the MS. For our application the diluter is used in a different way. Its function is to generate carbon dioxide peaks from a continuous carbon dioxide flow. Consequently, the reference gas line is connected to the extraction facility. For carbon dioxide analysis in mineral waters, the pressure in the extraction equipment (V1, V2, V5 open, V3, V4 closed) was allowed to drop to 5 mbar. After piercing the mineral water bottle by a needle, the carbon dioxide flows through the water trap to the gas reservoir GH (V1, V3, V4 closed, V2, V5 open).

With helium added to the gas reservoir the pressure is allowed to rise to 1.1 bar (Valves V1, V2, V4, and V5 closed, V3 open). The pressure enables a continuous transfer of carbon dioxide into the helium carrier (V4 and V5 open). A fused silica capillary (length 150 mm) is used as a flow resistance between the extraction equipment and the helium line. The length of the capillary, the amount of carbon dioxide taken out of the mineral water bottle and the pressure in the gas reservoir regulate the amount of carbon dioxide in the carrier. The technical arrangement enables consecutive measurements from the same sample by switching the valve gear of the diluter (switching time: ~ 2 sec.). A typical run is shown in Fig. 2.

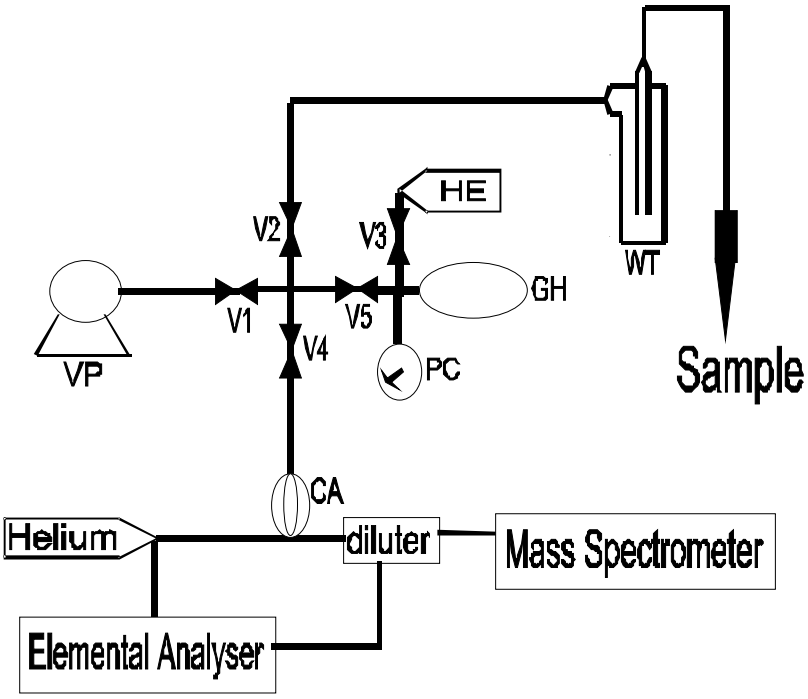

Fig. 1. Schematic representation of extraction equipment (WT – water trap [–90°C]; GH-gas reservoir[250ml]; VP – vac. pump; CA – capillary; V1..5 – valves;HE – helium;PC – pressure gauge.

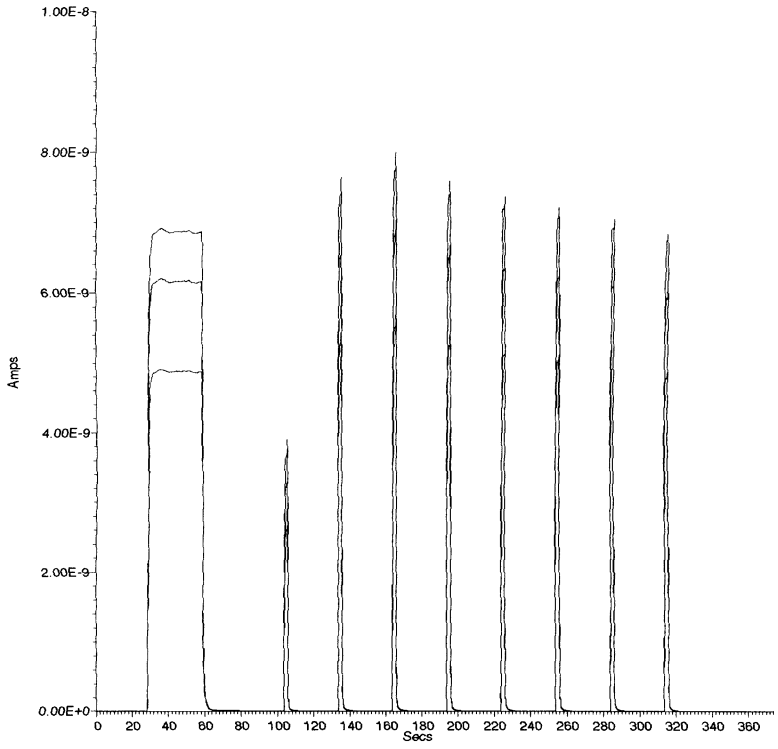

Fig. 2. Plot of a $^{13}C/^{12}C$ run of carbon dioxide extracted from mineral water. The first peak is generated by the reference gas, the subsequent spikes are separate samples gas injections from the gas reservoir (see text).

2.3. Simultaneous measurement of two isotope ratios

The initial intention of the diluter application was to enable a simultaneous determination of more than one isotopic ratio within a single run. For the determination of the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratio of biological material an example is given. The simultaneous measurement of both isotope ratios is possible only when at least one gas is diluted to a suitable range of detection. Systematic effects, in particular isotopic fractionation within the flow system, have to be taken into account.

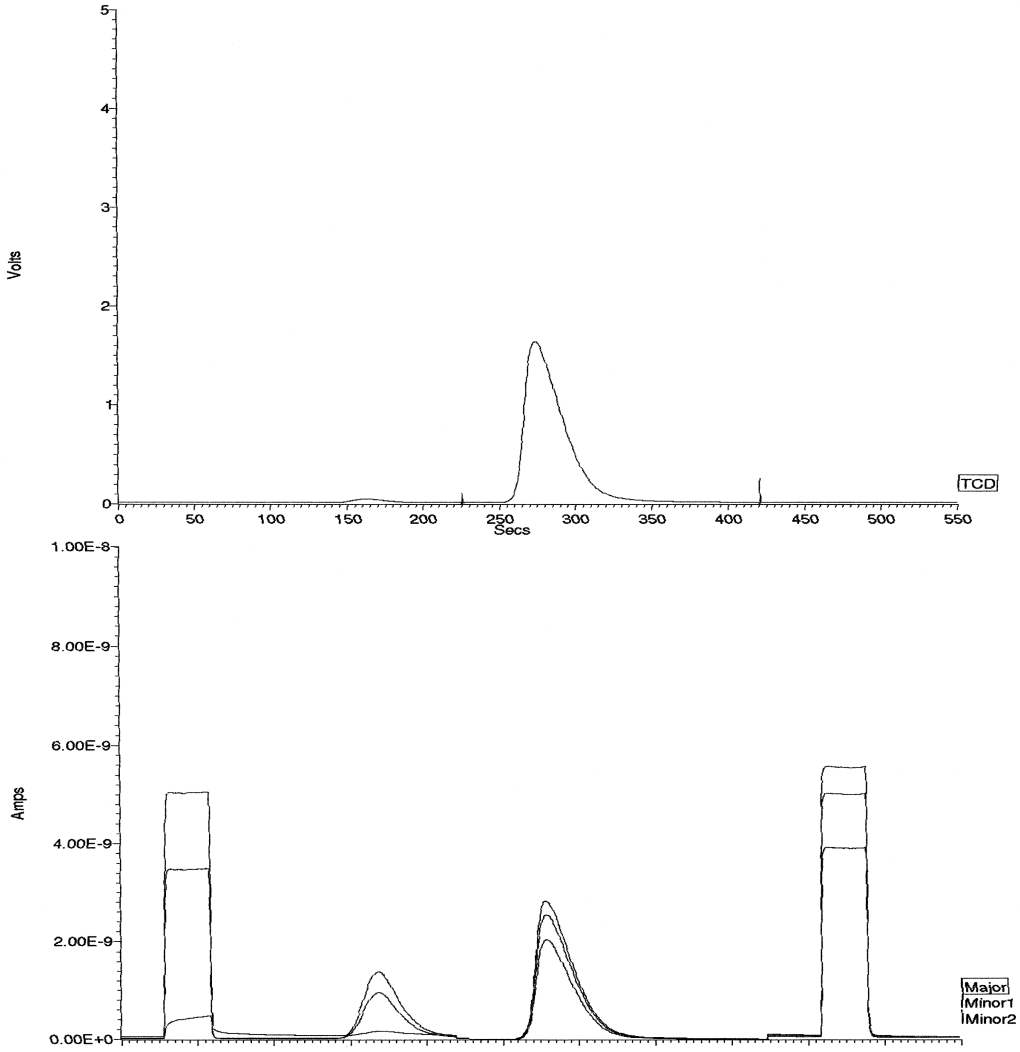

Fig. 3. Run of atropin standard (upper part: TCD signal; lower part: mass spectrometric record of the gases exhausting from the EA., Nitrogen is eluting first, followed by the carbon dioxide) The chromatogram is bracketed by gas injections of N_2 and CO_2 reference gas respectively. Switching of the high voltage and hence the mass position occurs at 225 seconds.

After precise weighing with a Sartorius microbalance samples are wrapped into tin foils . The weight data are fed into the data aquisition system EA 1.67 (Micromass). From an autosampler the samples drop into the hot zone of the EA where they are combusted at 1021° in a pure temporary oxygen environment (purity level 5.8). From here the combustion product gases are swept with helium (purity 6.0) through a reduction furnace filled with copper at 650° . Traces of water are trapped on magnesium perchlorate. The oxidation furnace was packed with tungsten-VI oxide, silver wool and cobalt-II/III oxide coated by silver. The whole packing is kept in place by quartz wool. The filling is cleaned

(removing the ash) or changed after about 600 samples. The copper in the reduction reactor usually is oxidized after 200 samples and has to be reduced by fushing with hydrogen under heating. Each series of samples is controlled by regularly including measurements of a standard substance of known elemental as well as isotopic composition. Following combustion, reduction and water removal the gases are separated gaschromatographically (Porapak Q, 2 m). The concentration of the compounds is determined by a TCD (termal conductivity detector). The retention times under the conditions of operation are 160 s for nitrogen and 275 s for carbon dioxide. A typical run is shown in Fig. 3. The TCD signals of nitrogen and carbon dioxide exhibit a ratio of about 1 : 25, which is typical for plant material. In the case shown the reference substance used is atropin. Table I demonstrates a typical example from a whole sequence run to illustrate the precision of the technique. The dummy samples preceeding each run are not shown.

TABLE I. PART OF A TYPICAL RUN OF ATROPIN STANDARDS AND TWO IAEA-NITROGEN STANDARDS (RAW DATA).

Sample	δ ($^{13}C/^{12}C$) ‰ v.s. PDB	stand. deviation $^{13}C/^{12}C$	δ ($^{15}N/^{14}N$) ‰ v.s. air-N_2	stand. deviation $^{15}N/^{14}N$
Atropin	-3.9		-17.8	
Atropin	-3.9		-17.9	
Atropin	-4.1		-17.4	
Atropin	-3.8		-17.5	
Atropin	-3.9		-17.3	
Atropin	-4.0		-17.5	
Atropin	-3.8		-17.8	
Atropin	-3.9	0.1	-17.5	0.2
IAEA-N1			2.6	
IAEA-N1			2.5	
IAEA-N1			2.6	
IAEA-N1			2.4	0.1
IAEA-N2			22.6	
IAEA-N2			22.2	
IAEA-N2			22.4	
IAEA-N2			22.4	0.2

3. RESULTS

3.1. $^{13}C/^{12}C$ of carbon dioxide in mineral water

Carbon dioxide of commercial mineral waters can vary significantly (Fig. 4). There are two main sources of carbon dioxide as revealed by their different $^{13}C/^{12}C$ ratios. The $^{13}C/^{12}C$ ratios of carbon dioxide from wells range between –4 to –8‰ vs. PDB (‘well carbon dioxide’). The wells are mainly supplied with CO_2 by recent volcanic activity in the Eifel and Black Forest region. The second group of data between –26 and –44‰ represents carbon dioxide mainly manufactured by burning coal or biomass (‘processed carbon dioxide’). This carbon source is dominated by plants using the C_3 photosynthesis pathway, the most common type in plants of our area. German mineral water regulations allow a specific qualification of the mineral water (‘carbon dioxide of its own well’) to be used on the label only when the carbon dioxide originates exclusively from its own well. Usually customers honor this by paying a higher price. The notion behind the declaration suggests that the mineral water in question is a pure natural product. Processed carbon dioxide may not differ in terms of quality but the mineral water is not received as an entirely natural product. The regulation is unique in Europe.

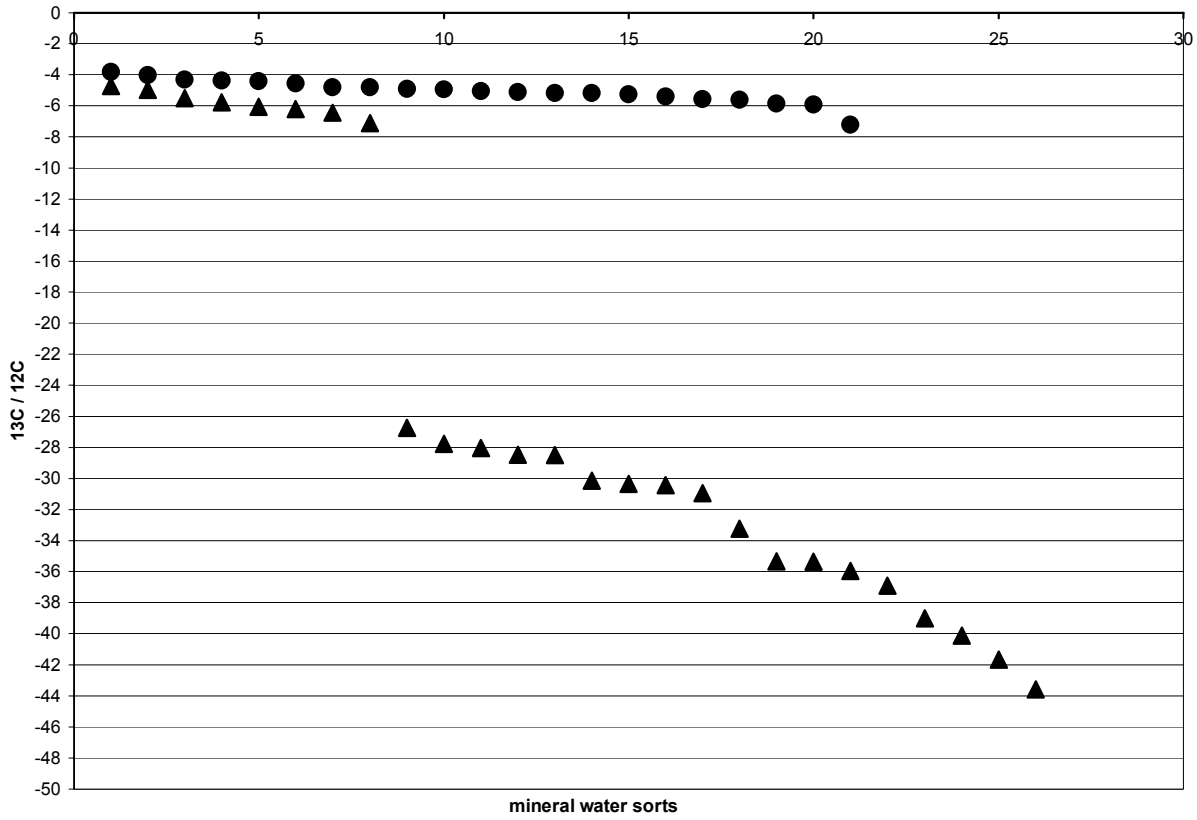

Fig. 4. $^{13}C/^{12}C$ ratio of carbon dioxide in mineral waters (dots: declared as carbon dioxide bottled at its own well; triangle: no declaration, hence technical carbon dioxide and carbon dioxide originating from another well).

Consequently suppliers of mineral water are not allowed to declare their product as carbonified by its own source even if they use well carbon dioxide from an other natural origin. The triangels in Fig. 4 at the upper part of the diagram represent samples which are carbonized by well CO_2 not originating from their own source at the companies source area. The water must be declared as 'carbon dioxide added' neglecting the origin of the gas added, be it from wells or from technical procedures.

3.2. Fertilizer studies using natural $^{15}N/^{14}N$ variation

Vineyards in northern Europe are actively green only from May until October. In wintertime a vineyard consists of resting stems only. During the same period most of the ground water is formed leaching off the nitrate down to the ground water level. In order to keep the nutrients in place the idea was to cover the surface of vineyards with grass or other plants. Legumes would be able to supply the wine plants with air-fixed nitrogen. However, the grass cover also uses the fertilizer nitrogen to built up its own biomass and the fertilizer nitrogen is transported down to the roots of the wine plants in the subsequent year only. Fig. 5 reports results from the biomass of soil covering vegetation and from wine plants as well as from the fertilizer applied and from the soil nitrogen pool. In this case the $^{15}N/^{14}N$ ratio is close to that of the fertilizer applied. The different $^{13}C/^{12}C$ ratios may result from the uptake of carbon dioxide either from the free atmospheric carbon dioxide pool (wine) or from a mixture with the carbon dioxide from soil respiration.

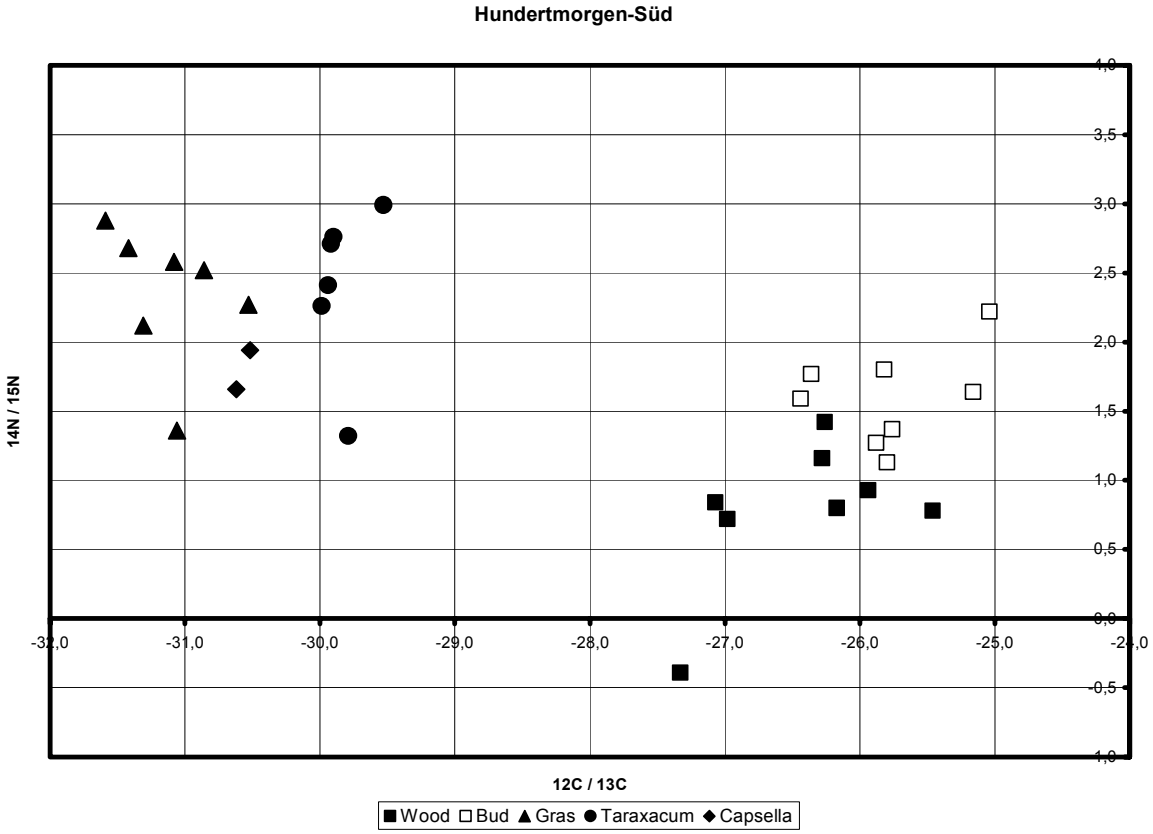

Fig. 5. $^{15}N/^{14}N$ versus $^{13}C/^{12}C$ ratios of soil covering vegetation and of wine plants in a vineyard of Neustadt/Weinstrasse, Palatinate, Germany.

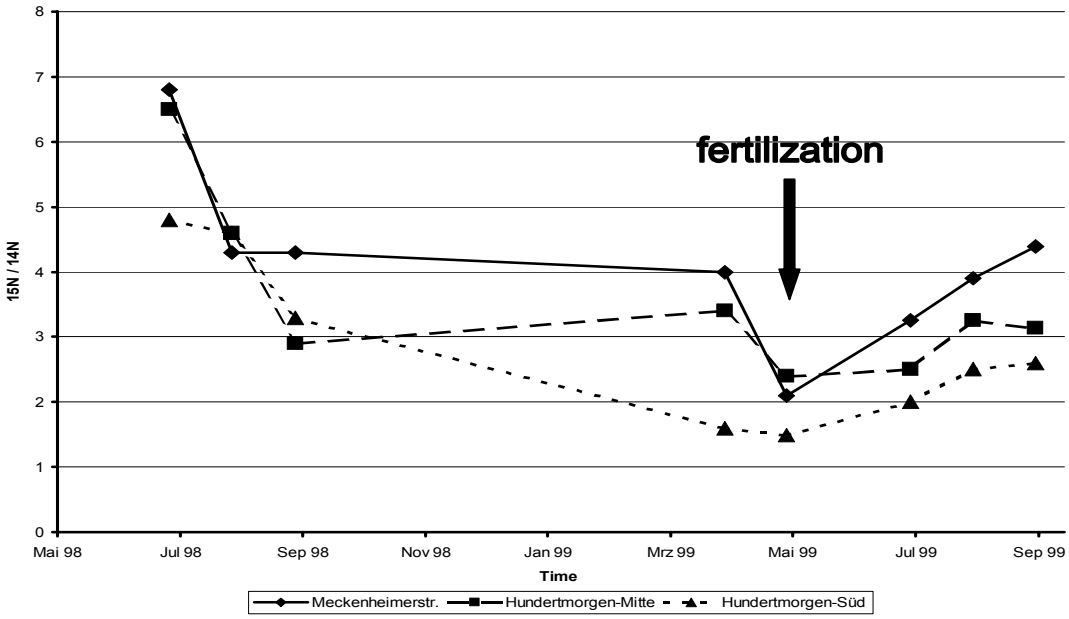

Fig. 6. $^{15}N/^{14}N$ ratio in the soil nitrogen pool and in the corresponding biomass of wine in a vineyard of Neustadt/Weinstrasse, Palatinate, Germany.

Fig. 6 gives an impression of the dynamics of the nitrogen uptake and movement in the soil using the $^{15}N/^{14}N$ data only. Caused by a change in the isotopic composition of the fertilizer the wine plants do not immediately take up the freshly added fertilizer. Rather they mainly utilizes nitrogen from previous fertilizer additions. Until the end of the growth period wine plants get nitrogen from the fertilizer too. Later on they extract nutrients from the soil nitrogen pool again. The soil nitrogen pool tends to more positive $^{15}N/^{14}N$ ratios, possibly caused by an enrichment during previous repeated biochemical turnovers.

4. CONCLUSION

The origin of carbon dioxide added to beverages can be distinguished clearly between natural wells or industrial processes. The relatively large number of products which use carbon dioxide from their own well results from the geographical position of our laboratory which is close to an area of recent volcanic activity (Eifel region, last activity several thousand years ago). A large number of acidic bicarbonate mineral waters points back to the geological history [2–6]. At some places like Maria Laach carbon dioxide is still released continuously to the environment. The method described enables the detection of added industrial carbon dioxide to a product claimed to be of natural origin from a natural well. The method cannot control the use of the carbon dioxide well at the companies facilities until now.

The observation of the $^{15}N/^{14}N$ ratios may become a valuable tool for fertilizer studies without changing the cultivation practice and commercial use. Only the isotopic composition of the fertilizer has to be changed, maybe only the supplier. A convenient label could be set by changing from one fertilizer produced following the Haber-Bosch-procedure (air-N_2) to one of natural origin (e.e. guano). The turnover in a natural ecosystem is a complex process depending on a variety of factors. Therefore conclusions should be drawn carefully. Nevertheless the first summary is that the turnover of fertilizer nitrogen is more complicated than is expected normally, demonstrated by the measurements of the natural variation of the stable isotopes of nitrogen, the $^{15}N/^{14}N$ ratio. “Old” nitrogen seems to be enriched as has been demonstrated previously [7].

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HIGH TEMPERATURE PYROLYSIS: A NEW SYSTEM FOR ISOTOPIC AND ELEMENTAL ANALYSIS

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Abstract. A new method for the automated sample conversion and on-line determination of deuterium, carbon, nitrogen and oxygen isotopes for organic and inorganic substances is presented. The samples are pyrolytically decomposed in presence of reactive carbon in a high temperature pyrolysis system (HTP) at a temperature higher than 1400°C. The method has a great potential for the analysis of hydrogen, carbon, nitrogen and oxygen stable isotopes ratios. The instrumentation and application is very simple and cost effective. The reproducibility of the δ-values is 3‰ for D/H, 0.3‰ for ^{18}O, and 0.2‰ for ^{13}C and ^{15}N respectively. The system is suitable for solid, liquid and gaseous samples. Results are presented for the isotopic composition of international reference materials which show the precision and accuracy of the method.

1. INTRODUCTION

In the past decade on-line techniques have been developed for the automated determination of stable isotope composition of carbon, nitrogen and sulfur using an isotope-ratio-mass-spectrometer (IRMS) connected to elemental analyser systems (EA) [1, 2]. These systems meet the requirements for higher sample throughput and an automated sample preparations modus. The methods are important tools in (hydro-) geology, biology, ecology, (paleo-) climatology and many other fields of isotope research.

The systems consist of an elemental analyser connected to the IRMS. A carrier gas transfers the gaseous combustion products *via* an open split to a mass spectrometer. The efficiency and accuracy of this set up is proved for carbon, nitrogen, and sulphur stable isotope measurements in a number of research works. The advantages of on-line systems are the relatively small sample size, the direct conversion of samples to gaseous products suitable for measurement, and the automatic measurement using an autosampler. The small sample size requires an excellent homogenisation compared to the large sample amounts used in off line systems to obtain representative data. Moreover, the purity of the sample material is important because the elemental analysers are used for bulk analysis. Especially for economic analysis, a simplification of the sample preparation is important. Compared with the traditional double inlet system the number of samples, which can be measured in the same time period number gets increased by an order of magnitude using on line techniques.

In recent years several publications have dealt with on-line techniques for the determination of oxygen isotopes [3, 4]. This paper describes the next step for automated detection of hydrogen, carbon, nitrogen and oxygen isotopes. The High Temperature Pyrolysis system (HTP) is an additional development for the stable isotope measurements with an elemental analyser [5]. This technique allows a parallel determination of hydrogen and oxygen isotopes during one measurement. The pyrolysis is complete at a temperature higher then 1400°C. Therefore, as a key component a new furnace including new materials for the pyrolysis reactor were developed [6].

1.1. Experimental principles

The HTP system consists of a furnace normally kept a temperature higher than 1400°C with a temperature limit up to 1550°C. The samples are pyrolyzed in the presence of reactive carbon and the gaseous pyrolysis products are separated on a gas chromatography column. The column separates the major species H_2, N_2 and CO (CO_2 is not produced at temperatures above 1300°C) [3].

In spite of temperatures in excess of 1400°C some nitrogen bearing organic substances decompose to other volatile reaction products than H_2, N_2, and CO. This was evident due to an observed nitrogen deficiency and an incorrect $\delta^{15}N$ value. These unidentified volatile products can be trapped and removed on an ascarite trap for acidic gases.

The pyrolysis of several substrates is supposed to proceed according to the following simplified equations:

H_2O	+	C	→	H_2	+	CO				
2 KNO_3	+	8 C	→	N_2	+	6 CO	+	[2 KC]		
$BaSO_4$	+	4 C	→	4 CO	+	[BaS]	+	[Ag_2S]*		
$C_{12}H_{22}O_{11}$			→	11 H_2	+	11 CO	+	C	(Sucrose)	
Cellulose			→	x H_2	+	y CO				
Amino acids		→	x H2	+	y CO	+	z N_2	+	unknown N-species**	

The hydrogen, nitrogen and oxygen of the samples are converted quantitatively to H_2, N_2 and CO.

* The samples, rapped in silver capsules, fall into the reactor. Here sulphur may react with the silver forming silver sulphide.

** The nitrogen from the amino acids is not completely converted to z N_2.

1.2. Apparatus

The apparatus uses a new elemental analyser especially developed for the HTP. It consists of a furnace suitable for temperatures up to 1550°C, a carrier gas system, a Poraplot column (5A molecular sieve, CHROMPACK), and an ascarite trap (Fig. 1). The system allows the use of two sample inlets. An autosampler can be connected directly with the reactor for the measurement of solid samples. Alternatively, an injector inlet with a septum for liquid and gaseous samples can be installed.

In total, two different autosamplers , one for solid samples and one for liquids or gaseous samples can be used. The complete system was constructed by HEKAtech (HEKAtech, Wegberg, Germany) under supervision of the UFZ. The system is controlled by the standard software of the mass spectrometer. The layout of the reaction tube depends upon the sample amount which should be measured. For solid samples the layout is shown in Fig 2. The reactor consists of an ceramic tube (Al_2O_3, o.d. 17 mm, i.d. ≈ 14 mm, length ≈ 470 mm, HEKAtech, Wegberg). Within this tube, a glassy carbon liner (o.d. ≈ 13 mm, i.d. 9 mm, length ≈ 380 mm, HTW, Thierhaupten, Germany) is installed. The liner is positioned in the reaction tube at the upper end. The position is fixed with a quartz wool plug, with silver wool layer underneath to trap the sulfur outside of the heating zone and glassy carbon grits at the bottom. Important for the construction of the reactor is a small gap between the ceramic tube and the glassy carbon liner in the hottest zone of the furnace, because the reaction between the glassy carbon and the oxygen from the ceramic results in an increase of the background (CO).

All parts of the reactor are commercially available. The inner tube is filled with glassy carbon grits up to the hottest zone in the furnace with a small layer of reactive nickelized carbon (ca. 10 mg) on top. The sealings (O-ring, viton) at the upper and the lower ends of the ceramic tube can be used to a maximum temperature of 150°C and requires cooling by special fans. Downstream the gas flow of the pyrolysis reactor is an ascarite trap to remove acidic gases and a GC-column for the separation of the pyrolysis products.

2. RESULTS AND DISCUSSION

The samples analysed were internationally distributed reference materials and laboratory standards from different laboratories. Solid samples were weighed in silver capsules (4 × 6 mm, HEKAtech, Wegberg, Germany). In first instance liquid samples were weighed in silver capsules for liquids (2 × 5 mm) as well. Later liquid and gaseous samples were directly injected with a 1 µl syringe through a septum into the reactor.

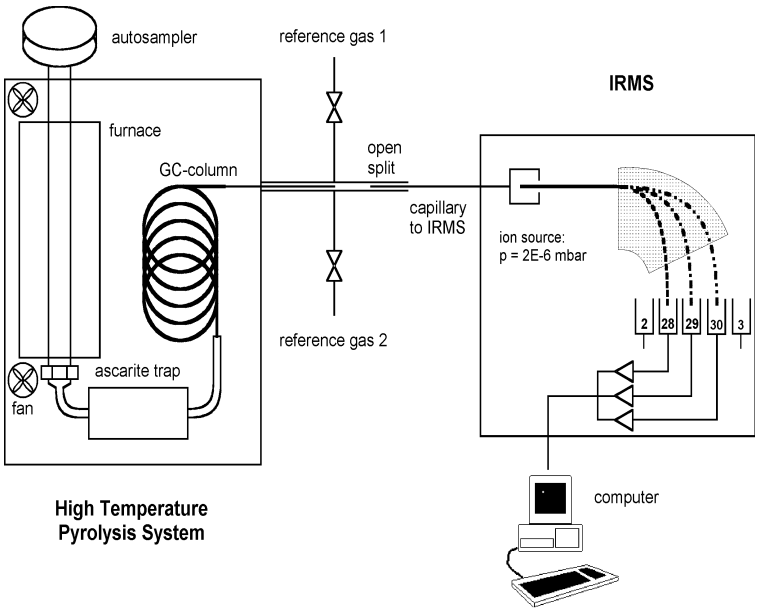

Fig 1. Schematic diagram of the HTP-Elemental Analyzer, ConFlo II-Split and IRMS.

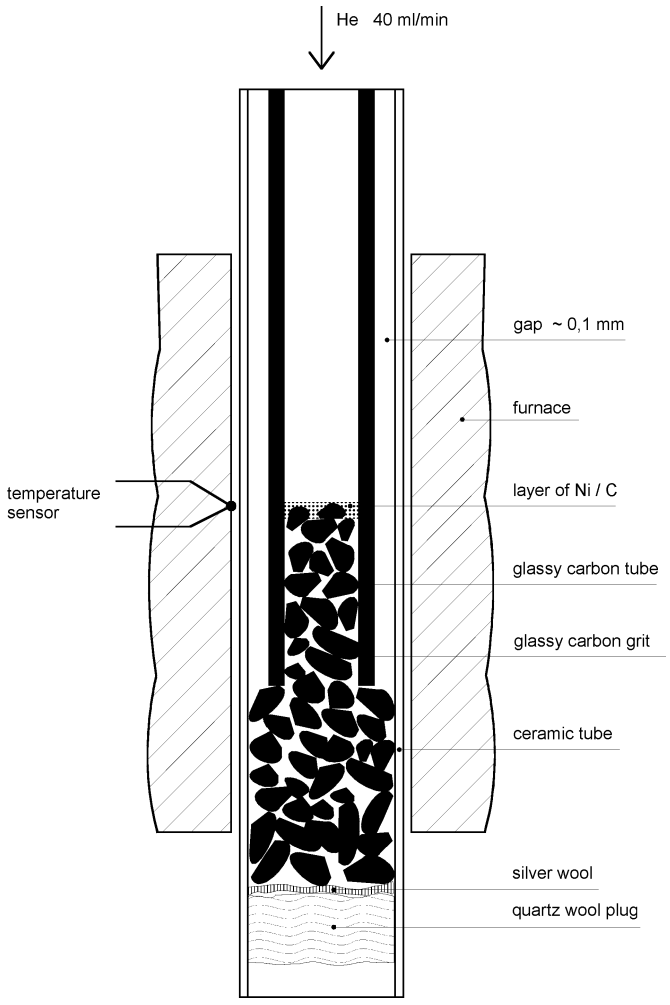

Fig. 2. Schematic diagram of the HTP reactor. The reaction furnace is heated to 1450°C and the system is flushed with a stream of carrier gas (He 5.0, AGA, Germany) at 40 ml/min. The gas flow was set to this relatively low flux to avoid cooling the reaction zone by the carrier gas.

The solid samples were dropped with the auto sampler Eurocup 40 (HEKAtech, Wegberg, Germany) into the reaction tube. Liquid samples were injected with a CTC A200S auto sampler (CTC Analytics, Zwingen, Switzerland) through a septum into the reaction tube. The pyrolysis products are transported with the carrier gas and separated on the GC-column into distinct H_2, N_2 and CO peaks. The infrastructure of a ConFloII interface with open split, 2 different reference gases, and He dilution was applied to transfer the products into a delta plus XL mass spectrometer (both Finnigan MAT, Bremen, Germany). The pressure in the ion source is 2 × E-6 mbar.

The ion currents of masses m/z 2 and 3 to measure hydrogen isotopes and m/z 28 to 30 for the determination of oxygen isotopes were recorded by the ISODAT software (Finnigan MAT, Bremen) and the results were calculated relative to H_2 and CO reference gases, respectively. Results of cellulose measurements demonstrate the measurement of three isotope values i.e. δD, $\delta^{13}C$ and $\delta^{18}O$ from a single pyrolysis experiment.

In connection with the measurement of nitrates a special problem arises. The ion source of the MS shows a memory effect on the mass trace m/z 30 from the measurement of nitrogen. The background is significantly increased after the nitrogen peak and drops only slowly. This prevents a precise measurement of the CO isotopes (mass 30, possibly formation of NO at the filament, personal communication, Finnigan MAT) for a few minutes. The $\delta^{15}N$ value can be detected precisely, but the $\delta^{18}O$ value shows a positive shift. With a modified arrangement, for example a better gas chromatographic separation or a more inert ion source the isotopic composition of oxygen in nitrogen containing substances might be possible. Presently two separate measurements are necessary to achieve correct results.

The typical sample size for the determination of $\delta^{18}O$ is 2-6 µmol oxygen (O). After about 150 samples the silver residues from the sample capsules in the reactor have to be removed. Commonly, the glassy carbon filling has to be exchanged after 300 measurements because metals and other solid residues from the samples precipitate on the surface of the carbon grids.

Table 1a-d presents some examples for the measurement using the high temperature pyrolysis. The experiments slightly differ for each sample types. The duration of one sample run was 800 s in all cases. All samples were measured with 4 repetitions (n = 5).

Unfortunately for H-isotopes a small memory effect in the furnace is found. The memory for deuterium is observed as ≈ 2% of the difference of the delta values from two different samples measured in turn. This effect is only noticeable in case of very small standard deviation. An improvement of this problem can be achieved by inserting one or two “blanks” containing the next sample to improve the performance of the reactor before the next sample is measured (especially helpful by water samples).

In spite of this problem the HTP yields an important simplification for many samples, which up to this day can only be prepared with a rather laborious fashion. This new method promises a large potential that may only be fully explored in the future with new applications.

3. CONCLUSIONS

The HTP method for on-line determination of stable isotopes (HCNO) provides fast, accurate and precise analysis for water samples as well as organic and inorganic substances. A comparison of the isotopic composition of international reference substances measured with accepted off-line techniques prove the accuracy and reliability of the new HTP method. The automated HTP technique is less time consuming and safes a lot of manpower compared to other methods. Another advantage is the small amount of sample material needed for the analysis.

TABLE 1A. NORMALIZED $\delta^{18}O$ VALUES OF REFERENCE MATERIALS IN [‰] VS. VSMOW [9]

Sample ID	Sample material	Reference value	HTP
IAEA C-3	cellulose	32.5 [10], 31.85 [8]	32.6 ± 0.1
MKC	cellulose		28.1 ± 0.1
IAEA NBS 127	$BaSO_4$	9.3 [8]	9.3 ± 0.2
IAEA SO-5	$BaSO_4$		12.7 ± 0.2
IAEA SO-6	$BaSO_4$		-10.2 ± 0.2
IAEA NO-3	KNO_3	22.7 [7]	22.3 ± 0.3
Nit-HAL 1	KNO_3	12.0	11.8 ± 0.3
IAEA-NBS 19	$CaCO_3$	28.6 [8]	28.9 ± 0.3
IAEA-GISP	Water	-24.8 [8]	-24.9 ± 0.3

TABLE 1B. NORMALISED δD VALUES OF REFERENCE MATERIALS IN [‰] VS. VSMOW (WATER NORMALISED VS. VSMOW-SLAP SCALE) [7]

Sample ID	Sample material	Reference value	HTP
IAEA-CH7	PE-foil	-100.2 [8]	-100 ± 3
IAEA-GISP	Water	-189.8 [8]	-188 ± 3
MKC	cellulose	- 45	- 45 ± 3

TABLE 1C. NORMALISED $\delta^{13}C$ VALUES OF REFERENCE MATERIALS IN [‰] VS. VPDB

Sample ID	Sample material	Reference value	HTP
IAEA C-3	cellulose	-24.9 [8]	-24.8 ± 0.1
MKC	cellulose	-24.5	-24.7 ± 0.1

TABLE 1D. NORMALISED $\delta^{15}N$ VALUES OF REFERENCE MATERIALS IN [‰] VS. AIR

Sample ID	Sample material	Reference value	HTP
IAEA NO-3	KNO_3	4.6 [8]	4.7 ± 0.1
Nit-HAL 1	KNO_3	-27.2	-27.2 ± 0.1
NH_4 -	$(NH_4)_2SO_4$	-0.4	-0.4 ± 0.1

ACKNOWLEDGEMENTS

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ISOTOPE METHODS FOR THE CONTROL OF FOOD PRODUCTS AND BEVERAGES

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Abstract. The measurement of the stable isotope contents provides useful information for the detection of many frauds in food products. Nuclear magnetic resonance (NMR) and isotopic ratio mass spectrometry (IRMS) are the two main analytical techniques used for the determination of stable isotope contents in food products. These analytical techniques have been considerably improved in the last years offering wider possibilities of applications for food analysis. A review of the applications for the control of food products and beverages is presented. The need for new reference materials is discussed.

1. INTRODUCTION

For several decades the measurement of the natural abundance of stable isotopes has been mainly used in geochemistry and environmental research. Recently, isotope techniques have seen a growing interest in many other fields of research. Due to the improvement of the techniques numerous applications have been published in biomedical science, ecology, pharmacy and also within the field of consumer protection for detection of frauds in food and beverages. The measurement of the various isotope ratios $^{2}H/^{1}H$ (D/H), $^{18}O/^{16}O$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ in different fractions of a product often provides information about the origin of starting materials. Isotope methods have therefore shown to be a major tool for checking the compliance of food products with national and international regulations. The technological progress of the last years has led to an increased use of hyphenated techniques for measuring isotope ratios using isotope ratio mass spectrometry (IRMS) coupled to a continuous flow elemental analyser (EA-IRMS), a pyrolysis unit (Py-IRMS) or a gas chromatograph (GC-IRMS). Nuclear magnetic resonance of deuterium (SNIF-NMR) has also demonstrated to be useful for site-specific measurement of intramolecular $^{2}H/^{1}H$ ratios of some organic molecules. These applications and techniques require establishment of new international standards to ensure comparability of isotope analyses of food products at an international level. This paper does not try to exhaustively review the application of isotope techniques in food analysis. It aims at presenting some basic aspects of isotope fractionation in plants and introduce analytical techniques used for the determination of isotope ratios as a tool for authenticity control of food products. The various possibilities offered by isotope techniques for food analysis are illustrated by selected applications for some of the major products of interest for consumers. Furthermore, implications for choosing new reference are also discussed.

2. STABLE ISOTOPES AND ISOTOPE FRACTIONATION

In nature, all of the major organic bio-elements (C, H, N, and O) are mixtures of two or more stable isotopes. The mean isotopic abundances observed for C, H, O and N are presented in Table I. It has been observed that the isotope ratios of a given molecule vary depending on its origin. This variability is linked to the isotope abundance of the starting pools and to the isotope fractionation associated with the various physical processes, chemical reactions and/or biochemical pathways involved during the formation of the molecule. In the water cycle, a well-known isotope fractionation takes place during the evaporation of the water from the oceans where depletion in heavy isotopes is observed in the vapour with respect to that of the liquid state. A similar isotope fractionation occurs in the transpiration of water from plants. The isotope ratios observed in plant water are positive relative to those of the corresponding ground water. Plants can be classified in three categories according to their photosynthetic pathways [1–3]. Plants belonging to the first category fix the atmospheric CO_2 by carboxylation of ribulose 1,5-diphosphate leading to two molecules of phosphoglycerate (chain of three carbon atoms, hence the name C_3 plants). This RuBisCo reaction is accompanied by a strong ^{13}C isotope effect causing a large depletion in the carbon-13 content of the plant (carbohydrate $\delta^{13}C$

TABLE I. ISOTOPE RATIOS OF STANDARDS AND TECHNIQUES USED FOR ISOTOPE DETERMINATION IN FOOD PRODUCTS

	Hydrogen	Carbon	Oxygen	Nitrogen
Isotope Ratio	$^{2}H/^{1}H$	$^{13}C/^{12}C$	$^{18}O/^{16}O$	$^{15}N/^{14}N$
R x 10^{6}	155.76	11237.2	2005.2	3676.5
Standard	V-SMOW	V-PDB	V-SMOW	Air
(molecule)	H_2O	$CaCO_3$ *	H_2O	N_2
Technique	IRMS, NMR	IRMS	IRMS	IRMS

* PDB (Pee Dee Belemnite carbonate) is exhausted. It has been replaced with NBS 19 (V-PDB scale). Other international standards available from IAEA include: IAEA-CH-7 (polyethylene, ex PEF1), NBS22 (oil), IAEA-CH-6 (sucrose).

values of these plants are ranging from –28‰ to –23‰). Most plants belong to this group (e.g. grape, rice, barley, wheat, soybean, potato, rye, sugar-beet). The second category are the so-called C_4 plants. They fix CO_2 by carboxylation of phosphoenolpyruvate (PEP-Carboxylase reaction) leading to four-carbon product, oxaloacetic acid. The PEP-Carboxylase reaction shows almost no isotope fractionation with respect to the carbon-13 content of atmospheric CO_2. Only the diffusion of CO_2 into the intercellular space exhibits a small fractionation of about 4‰. Products derived from C_4 plants show higher carbon-13 contents than analogous products from C_3 plants ($\delta^{13}C$ values of carbohydrates from C_4 plants are generally around –10‰). Cane, sorghum, millet and maize are the most important representatives of this group from the agro-economical point of view. The third category are CAM (Crassulacean Acid Metabolism) plants which have an intermediate metabolism. As a result intermediate carbon-13 contents are found for the products derived from these plants (carbohydrate $\delta^{13}C$ values range from -18‰ to -12‰). Pineapple, vanilla and agave are the more important plants from this group from an economic point of view.

Following photosynthesis secondary metabolisms which transform carbohydrates into proteins and lipids are accompanied by a further ^{13}C depletion leading to a large variety of $\delta^{13}C$ values in a given organism.

3. THE TECHNIQUES

Two analytical techniques are mainly used for the measurement of stable isotope ratios in food and beverages. These are the Isotope Ratio Mass Spectrometry (IRMS) for $^{2}H/^{1}H$, $^{18}O/^{16}O$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios and the deuterium Nuclear Magnetic Resonance (^{2}H-NMR) for the intramolecular distribution of deuterium.

$$\delta D(‰) = \frac{R_{Sample} - R_{VSMOW}}{R_{VSMOW}} \times 1000$$

IRMS has a constant magnetic field which separates the different isotope species of the measuring gas (generally CO_2, H_2 or N_2) introduced into the ion source where they are ionised. The determination of the deuterium abundance in an organic compound requires conversion of the hydrogen from the original chemical form to molecular H_2. The H_2^+ (m/z = 2) and HD^+ (m/z = 3) species are then separated by the magnetic field of the IRMS and their corresponding ion currents (i) are measured on two different collectors leading to the ratio $R_{sample} = i_3/i_2$ (i_3 has to be corrected for the contribution of H_3^+ species formed from H_2 and H_2^+ in the source) [4]. The ratio obtained for the sample is compared to that of the International Standard V-SMOW (Vienna-Standard Mean Ocean Water) and the content in deuterium can therefore be expressed in ‰ on the δD scale [5].

Similarily, the carbon-13 content is determined on carbon dioxide gas resulting from the combustion of the sample. The various possible combinations of the ^{18}O, ^{17}O, ^{16}O and ^{13}C, ^{12}C, isotopes are found at mass 44 ($^{12}C^{16}O_2$), mass 45 ($^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$) and mass 46 ($^{12}C^{16}O^{18}O$). The mixed isotopomer species $^{13}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can often be neglected due to their low abundance. The corresponding ion currents are determined on three different collectors. The ion current measured for mass 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the intensity current measured on the detector for mass 46 by considering the relative abundance of ^{18}O and ^{17}O (Craig correction) [4]. The comparison with a reference calibrated on the international V-PDB scale allows the precise calculation of the carbon-13 content in $\delta^{13}C$ units. The nitrogen-15 content is determined against that of N_2 in air. The results are expressed in $\delta^{15}N$ units.

One method for the determination of oxygen-18 in water fractions has been originally published by Epstein and Mayeda. It is widely used for the control of wines and fruit juices [6]. Its principle is based on the isotope equilibration of the liquid water sample with CO_2 gas. Through equilibration, the ^{18}O information of the water is transferred to the gas phase. The ^{18}O abundance, expressed in $\delta^{18}O$ units, is determined by IRMS against that of the reference water V-SMOW which defines the international scale.

During the past decade, the analytical capabilities of IRMS have been considerably enhanced owing to the development of on-line techniques which for instance couple elemental analysers or gas-chromatographs with isotope ratio mass spectrometers. These techniques have increased the productivity of isotope laboratories considerably. They allow to carry out a large number of analyses per day or to analyse the isotopic profile of several organic compounds extracted from the same initial matrix in a single chromatographic run.

Oxygen isotope ratio measurements in organic matter are usually carried out using time-consuming off-line pyrolysis techniques not suitable for analysis of a larger number of samples. More recently, several groups have introduced on-line techniques based on high-temperature pyrolysis of organic samples. Here, carbon monoxide needs to be produced in a quantitative manner from the original sample. CO is directly used as the analyte gas [7–11]**.**

The NMR technique is used for the determination of the site specific deuterium content of an organic molecule [12–15]. In spite of a considerable lack in sensitivity which implies the use of relatively large sample sizes (minimum of 1 mmol), deuterium NMR presents interesting features for the characterisation of organic compounds. It provides a " fingerprint" of the deuterium content within the molecule which is difficult to mimic while maintaining a sizeable profit from adulteration on an industrial scale. The deuterium content measured by NMR is generally expressed in absolute ratio D/H in ppm units. Here, 1 ppm corresponds to 6.4‰ on the V-SMOW scale.

4. APPLICATION OF ISOTOPE TECHNIQUES FOR FOOD AND BEVERAGE ANALYSIS

4.1. Wine

Wine has always been one of the products steadily analysed either for improvement of quality or for detection of possible frauds. Wine is obtained by fermentation of grape must and its alcohol grade is proportional to the initial sugar concentration of the must. An increase of the alcohol grade of wine can be obtained by addition of foreign sugars before or during the fermentation. In the European Union this practice, called chaptalisation, must be in compliance with the European regulations that stipulate maximum levels of enrichment for the various European vine growing areas [16]. The main botanical sources of sugar being used are cane (C_4) and beet-sugar (C_3). As it was shown by Bricout, the chaptalisation with cane sugar is easily detectable by IRMS because of the significant increase of the carbon-13 content of the ethanol resulting from the fermentation of the mixture of C_4 cane and C_3 grape sugar [17]. On the other hand, because the C_3 metabolism of grape and beet being the same, the chaptalisation with beet sugar can not be detected by the same carbon-13 IRMS method. By using

quantitative deuterium NMR, Martin showed that the internal distribution of deuterium in ethanol, measured by the ratio R which represents the D/H ratio of the methylene site against that of the methyl site, is very different for grape and beet [12]. It can be used to quantify mixtures of ethanols from these two botanical origins and therefore to detect chaptalisation with beet sugar [13]. It was also shown that this technique enables the detection of chaptalisation with cane sugar. Further development of the methodology improved the precision considerably [14, 18]. The method was adopted by the European Community as the official method for the detection of chaptalisation of wine (and grape must) which was followed by the decision to establish a E.U. wine database [19, 20]. More examples of using isotope techniques for the analysis of wine and other beverages include:

- $^{18}O/^{16}O$ IRMS for the detection of addition of water and for the characterisation of the origin of wines [21]. Further to O.I.V.[1], the European Community has adopted the determination of oxygen-18 as an official method for analysis of wines and has included this parameter in the E.U. Wine Database [22, 23].
- ^{2}H-NMR for detection of edulcoration.
- Carbon-13 IRMS for the characterisation of natural gaseification of sparkling wines [24].

Isotope ratios have also been used for the characterisation of the geographical origin of wines [25–28]. Finally on-going research using isotope ratios is made in order to trace possible addition of glycerol [29].

4.2. Sugar

Since the two major economical sources of sucrose belong to two different groups of plants (C_3 for beet and C_4 for cane) it is relatively easy to discriminate the sugars from these two botanical origins by measuring their carbon-13 content by IRMS [17]. The same distinction is possible by using the deuterium NMR on the ethanol obtained by fermentation of sugar: ethanol derived from sugar of C_4 origin has a higher deuterium content in the methyl group.

However, additional information is also given by the deuterium content which concerns the physiology of the plant from which the sugar originates [30, 31]. It has been found that C_3 aerial plants like grape or apple tree and C_3 cereals like wheat, rye or barley exhibit a higher deuterium content than beet which grows underground. The high deuterium content of carbohydrates from C_4 plants, such as cane or maize sugars, allows an easy recognition of these botanical origins. Adulteration of grape sugar products (e.g. concentrated rectified must) with exogenous sugars, in particular beet sucrose, can be evidenced by isotope methods used together with compositional analysis of polyalcohols [32].

4.3. Alcohol and spirits

Spirits and more generally alcoholic drinks are derived from fermentation of sugars. Thus, isotope methods used for ethanol also allow to control fraud in such beverages. It has been shown that the deuterium content $(D/H)_I$ of the methyl site of ethanol depends mainly on the deuterium content of the fermented sugar while that of the methylene site $(D/H)_{II}$ is governed mainly by the deuterium content of the fermentation water [33, 34]. In other words, $(D/H)_I$ bears the information about the botanical origin while $(D/H)_{II}$ holds information upon the fermentation process. It is therefore possible to check the botanical origin of a variety of alcoholic products. High $(D/H)_I$ ratios as well as high carbon-13 contents are found for rum and tequila which should originate from cane and agave respectively. More negative isotope ratios are encountered for spirits derived from a C_3 origin (plum, cherry, apple, grape barley or potato) [35]. An example of rums adulterated with beet alcohol (C_3 plant) is shown in Fig. 1.

[1] Office International de la Vigne et du Vin — 18 rue d'Aguesseau 75008 Paris (France).

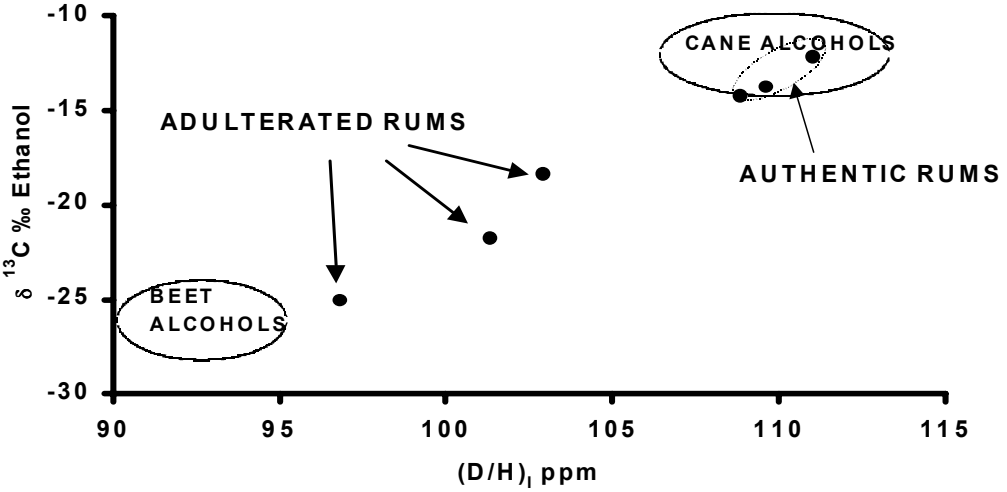

Fig. 1. Control of authenticity of spirits: rum. The absolute deuterium ratio of methyl site of ethanol $(D/H)_I$ is measured by ^{2}H-NMR.

Maize is widely used in blending with malt (barley) for the elaboration of some commercial whiskies. Since maize is a C_4 plant it can be easily distinguished from the C_3 barley on the basis of the isotope ratios which have therefore been proposed for the determination of the percentage of maize in commercial whiskies [36].

The isotope techniques are particularly interesting for the control of extra-rectified neutral alcohol that does no more contain the characteristic "impurities"(esters and higher alcohols) usually analysed by gas-chromatography for checking the origin (grape or cereal) of alcohol. In this case, only the combined use of NMR and IRMS allow for the determination of the botanical origin of the raw material (e.g. beet, cane, maize, potato). Synthetic alcohol is easily identified by its very high deuterium contents on both methyl and methylene sites [12].

4.4. Vinegars

Vinegar is often used as ingredient in many food products. Methods for identification of its botanical origin have been proposed based both on IRMS and on NMR techniques [37, 38]. Similarily to what is observed in the isotope filiation sugar-alcohol the isotope information is also kept after oxidation of ethanol to acetic acid (vinegar). This provides a convenient tool for control of the authenticity of the "expensive" vinegars derived from fruits. It could also be useful for the characterisation of some particular origins which produce "special" vinegars according to particular traditional processes. It may be emphasized that many canned products contain vinegars that should be of natural origin and sometimes claimed to be from one single botanical origin (generally wine). In 1993 AFNOR[2] adopted a method for the control of the vinegar used for canned macquerels [39].

4.5. Fruit juices

Many studies using isotope techniques have been carried out on fruit juices since about 20 years. One of the most widely known application is the distinction between the direct juices and the juices made from concentrates by redilution with "tap" water on the basis of the IRMS determination of deuterium and oxygen-18 content of the water of the juice [40]. An example of recognition between these two categories of fruit juice is shown in Fig. 2. The fruit juice industry has taken into account the possibilities of control offered by the isotope techniques and has published indicative ranges of values for isotope contents of genuine fruit juices [41].

[2] Association Française de Normalisation-Tour Europe , 92049 Paris, La Défense (France).

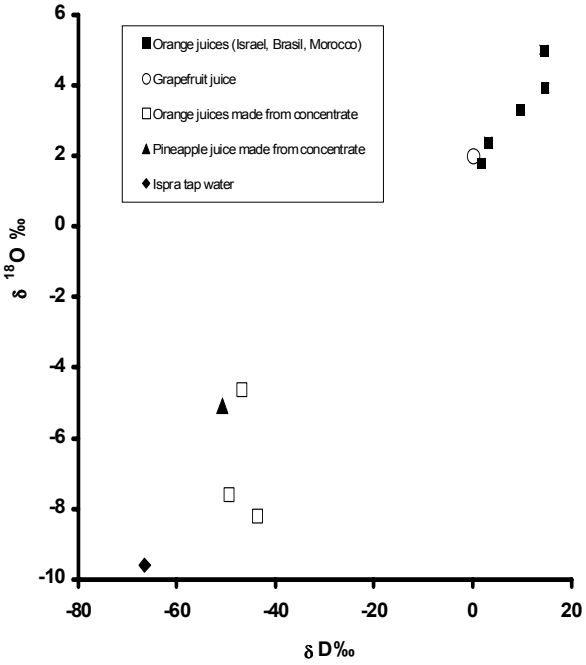

Fig. 2. Discrimination between direct fruit juices and juices made from concentrates based upon $\delta^{18}O$ and δD values of the juice. Isotope values of our laboratory tap water (Ispra tap water) are shown for comparison.

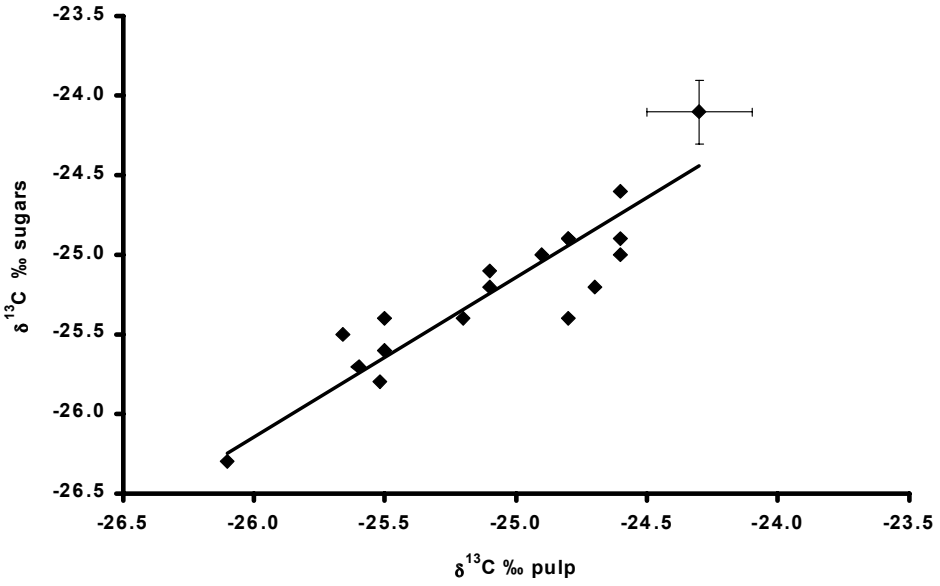

Fig. 3. Comparison of $\delta^{13}C$ ratio of the pulp and sugar fraction in orange juices from Sicily. A representative uncertainty of measurement (single standard deviation) is indicated for one sample.

Different isotope techniques have also been proposed for the detection of adulteration of fruit juices by addition of foreign sugars. For the detection of addition of beet sugar, Bricout and Doner have proposed the derivation of sugars as nitrate esters prior to their determination of their (D/H) ratio by IRMS [42], [43]. The detection of addition of cane or maize sugar is carried out by measuring ^{13}C abundance of the sugar fraction. For orange juices, a better sensitivity is obtained when an internal standardisation against the pulp fraction is carried out [42]. In Fig. 3 we present values for $\delta^{13}C$ of pulp and sugar fractions of genuine orange juices from Sicily (I) measured in our laboratory.

An indirect method has been studied by Brause et al. in the case of fruit juice concentrates adulterated by syrups. The principle is determining the ^{18}O abundance of the residual water from the concentrate: typically a pure fruit juice concentrate should show more positive $\delta^{18}O$ values (> +12 ‰) because of the concentration process which enriches the residual water in heavy isotopes [44].

The simultaneous use of deuterium NMR and carbon-13 IRMS has been shown to be very powerful for the control of authenticity of fruit juices [45, 46]. Due to the natural variation of isotope ratios, the development of databases containing the isotope ratios of authentic products is needed and a better precision would be achieved for the determination of adulteration when the precise geographical origin of the product is known.

More recent studies have shown that the $\delta^{15}N$ value of pulp could be a parameter indicative of the geographical origins of fruit juices [47].

4.6. Edible oils and lipids

The low $^{13}C/^{12}C$ ratio of lipids is shown to result from isotope fractionation during the oxidation of pyruvate to acetyl coenzyme A [48, 49]. It has been demonstrated that a large difference exists in the isotope content between the methyl and carbonyl carbon atoms of acetyl coenzyme A and in the carbon atoms of deriving lipids. This was confirmed by a positional carbon-13 isotope analysis of pyruvate and acetate by stepwise quantitative degradation [49].

Carbon isotope ratios of edible seed oils have been reported in literature, regarding in particular C_3 plants oils (sunflower about -27 ‰, soybean -28 ‰, palm -27 ‰, coconut -25 ‰, peanut -28 ‰) and C_4 plant oils (maize -12 ‰) [50, 51]. Due to its C_4 botanical origin, maize oil is easily recognised by measurement of the $^{13}C/^{12}C$ isotope ratio. GC-IRMS determination of $\delta^{13}C$ values of individual fatty acids have been proposed as a method to detect adulteration of maize oil with other vegetable oil [52]. Although not sufficient for an unambiguous recognition of C_3 oils this technique provides information that can be used in conjunction with other oil analyses to detect adulterations [53].

Concerning olive oil a study was carried out on the ^{13}C abundance of oil and some of its classes of compounds [54]. The isotope values for the bulk oil, aliphatic alcohols, sterols and glycerol were those expected given their biosynthetic origin, but distinctly different for each class of compounds. Based on those differences a further study was carried out to detect the adulteration of olive oil with pomace oil [55].

The characterisation of the geographical origin of virgin olive oil from various producing countries of the Mediterranean basin has been studied by measuring the $^{18}O/^{16}O$ ratio of bulk oil by pyrolysis-IRMS technique [11, 56]. Another study for determination of the geographical origin and of the purity of extra virgin olive oil has been performed by measuring the $^{13}C/^{12}C$ ratios of fatty acids by GC-IRMS [57].

A few studies on edible oils and fatty acids have been carried out using deuterium NMR, providing new information on isotope fractionation caused by biochemical, physiological and natural environmental effects. In particular, the site specific deuterium distribution in the fatty acids has been found to be related to the mechanism of fatty acids biosynthesis [58, 59].

4.7. Honey

Adulteration of honey by addition of syrups seems to be a frequent and widespread practice. An official method of analysis using the carbon-13 IRMS technique has been early adopted by the A.O.A.C. for the detection of the addition of High Fructose Corn Syrup (HFCS) [60].

However due to the variety of botanical origins, a large range of $\delta^{13}C$ variation may be expected in some cases (e.g. catsclaw, citrus). It results a lack of sensitivity of the method and a large "gray area" where the adulteration of a commercial product cannot be clearly established. In order to reduce this "gray area" the method was further improved by considering an internal standardisation of the $\delta^{13}C$ of honey against that of its protein fraction [61, 62]. Fig. 4 presents the application of this method using data published by White and Winters [61].

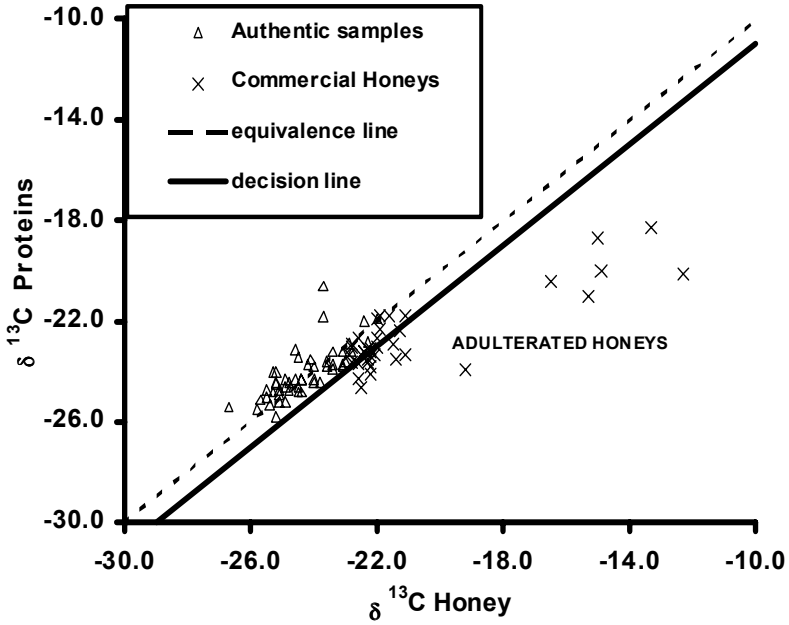

Fig. 4. Internal standardisation for the detection of adulteration in honey products with C_4 sugar (data from White and Winters [61]).

4.8. Flavours

Isotope ratios of flavour compounds have been studied in great detail since many years [63]. The isotope techniques have demonstrated their ability to distinguish between natural products extracted from plants or obtained by biogenesis from their cheaper synthetic or hemisynthetic homologues. The modern hyphenated IRMS techniques, in particular GC-IRMS, allowing the isotope analysis of several components of an essential oil, permit to obtain valuable information regarding the authenticity and the origin of the product. The number of applications of GC-IRMS will certainly increase in the next years. We will not further extend our discussion on the possibilities offered by this technique but present some typical examples of characterisation of flavour compounds by stable isotope ratio analysis.

Vanillin is the most widely used flavour compound in the food industry. A strong incitement for frauding vanillin exists because of very big price differences between natural vanillin extracted from vanilla beans, vanillin from synthetic origin from eugenol or guaiacol and vanillin from hemisynthetic origin derived from lignin or curcumin. The isotope ratios of vanillin have been extensively studied in order to detect the possible adulteration of this flavour. Vanilla is a CAM plant and consequently the first method proposed for detection of adulteration of vanillin was the determination of its $\delta^{13}C$ value. The values for $\delta^{13}C$ of vanillin extracted from vanilla-beans should be in the range -17 ‰ to -21 ‰ while other sources show much lower values (generally < -26 ‰) [64-66]. This method was successful until vanillin appeared on the market which was slightly enriched in ^{13}C on either methoxy or carbonyl sites in order to mimic the global carbon-13 content of natural vanillin extracted from vanilla-beans.

In order to detect this sophisticated fraud it has been proposed to chemically degrade vanillin prior to the determinating the isotope ratio of the carbon corresponding to these positions [66-68]. Information about the origin of vanillin is also obtained from the site specific deuterium content measured by NMR [69-70]. Now the development of the GC-IRMS technique allows the determination of the carbon isotope ratios of a series of components in a 'vanilla extract'. This 'isotope profile' can be used to assess the authenticity of the extract [71].

Anethole is another molecule that has been studied extensively. This compound enters in the composition of some popular aniseed spirits like Pastis and Ouzo. According to the European regulation about spirits, the anethole should originate from star anise or green anise [72]. Like in the case of vanillin, the site specific deuterium contents of anethole determined by NMR allow a clear distinction of the various possible synthetic or botanical origins [15].

The major compound responsible for the flavour of raspberry is 4-(4-hydroxyphenyl) butan-2-one and is called raspberry ketone. In nature, it occurs only at very low concentrations in plants so that its extraction is not economically feasible for its use by food industries. Alternatively it can be obtained by bioconversion of natural precursors, or by chemical reactions (chemical catalysts) using natural precursors. In the first case it can be labelled 'natural'. On the other hand, the use of chemical synthesis and/or precursors from petrochemical origin permit to produce this flavour at lower cost but in this case it cannot be labelled 'natural'. The site specific deuterium contents of raspberry ketone and its precursors have been studied by ^{2}H-NMR in order to identify the origin of these molecules and the processes that have been used to produce them. It has been found that the deuterium distribution on the H-atoms of the aromatic ring gives information about the natural or synthetic origin of one precursor (para-hydroxybenzaldehyde). The deuterium content of the methylenic positions could inform about the process (catalytic hydrogenation or fermentation) used to reduce the double bond of the precursor [73]. Further determinations of the D/H, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios of raspberry ketone extracted from *Taxus baccata* and obtained by oxidation of plant extrative betuligenol has been carried out to study the isotope pattern of extracted or biogenerated natural raspberry ketone [74].

4.9. Coffee and tea

The isotope contents of caffeine from various geographical origins have been studied by Dunbar in 1982 [75]. Interestingly, the first aim of this work was not the characterisation of the caffeine as a component of a food product but rather the establishment of a new methodology for the determination of the geographical source of illegal drugs such as morphine and cocaine. Indeed, because of the difficulty to legally obtain samples of morphine or cocaine the authors chose to take caffeine as a model alkaloid for testing their C, H, O isotope fingerprinting method by IRMS. Their results showed that both organic oxygen-18 and deuterium abundance in caffeine provide information about the geographical origin of tea or coffee. Further experiments using ^{2}H-NMR confirmed that the geographical origin of coffee can be checked by the determination of its isotopic contents [76].

5. CONSIDERATIONS ABOUT REFERENCE MATERIALS

Most of the calibration standards used for isotope analysis were first established taking into consideration the needs of biogeochemistry. Few of the International Standards available by IAEA are relevant for $\delta^{13}C$ analysis of food products with an elemental analyser-IRMS. However, none of these materials is really suitable for GC-IRMS techniques now used for control of flavours in food products. Moreover, these international standards are generally covering the $\delta^{13}C$ determinations but leave gaps for $\delta^{15}N$ and in particular for $\delta^{18}O$ determinations in organic compounds [77]. It is necessary for the scientific community that these gaps are filled in the near future. Analysts need international standards suitable for on-line stable isotope analysis in order to ensure the best traceability of isotope determinations against the primary international standards. For GC-IRMS as many applications as those already established for gas chromatographic separations may be envisaged. It is certainly not realistic to establish international standards for all possible applications. It seems more reasonable to concentrate the efforts on a selection of few candidate compounds. These selected compounds should cover most of the chromatographic conditions taking into account the polarity of columns and volatility of analytes. Moreover they should preferably be calibrated for several isotope ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, D/H, $^{18}O/^{16}O$ and possibly $^{34}S/^{32}S$. The establishment of these new international standards could be carried out by the scientific community in collaboration with IAEA and other institutions experienced in reference materials like NIST, USGS and the Institute of Reference Materials and Methods (IRMM) of the JRC-Geel.

TABLE II. REFERENCE MATERIALS PREPARED IN THE FRAME OF THE PROJECT REFMAT (SMT 4 CT96-2086)

	REFERENCE MATERIAL				
Parameters to be certified	Ethanol E (96%, vol)	Sugar S	Synthetic Wine W1 7% alc vol	Synthetic Wine W2 12% alc vol	E + water Mixture W3 12% alc vol.
	CRM 656	CRM 657	CRM 658	CRM 659	CRM 660
$\delta^{13}C$ of ethanol (IRMS)	X				X
$(D/H)_i$ of ethanol (^{2}H-NMR)	X				X
$\delta^{13}C$ of sugar (IRMS)		X			
$\delta^{18}O$ of water (IRMS)			X	X	
$(D/H)_w$ of water (IRMS)					X
Alcoholic grade	X				X

Regarding NMR some certified reference materials have been available for a few years and are routinely used by laboratories as working standards (e.g. tetramethylurea) for calculating D/H values and for quality control monitoring of NMR determinations (NMR sealed tubes) [78, 79].

It is worth mentioning that five reference materials, dedicated to the authentication of wines and sugars, are currently in preparation within the European project REFMAT [80]. These reference materials presented in Table II have been chosen to cover the main applications of isotope techniques used to analyse these products and will be applied to control the isotope ratio determinations by IRMS and NMR and also preparation steps such as the distillation. These materials have been subjected to stability and homogeneity testing. They will be proposed soon for certification and should then be available as Certified Reference Materials by IRMM.

6. CONCLUSION

Isotope methods have proven their ability to characterise the authenticity of a variety of food products as well as of alcoholic or non-alcoholic drinks since a long time. Thanks to technical improvement in instrumentation it is likely that many other applications will be developed in the coming years. The continuous flow techniques now widely used in IRMS allows for high sample throughput with a high reliability of results. This is convenient for constituting isotope ratio databases of authentic samples as well as for the routine control of many marketed products. Moreover the GC-IRMS technique developed in the decade has brought a very powerful tool to the food analyst. It is also probable that multi-isotope fingerprinting will lead to a better characterisation of the (geographical) origin of a substance in question. Internal isotope ratio standardisation as proposed for honey and some fruit juices may be extended to other food products and should improve the sensitivity for the detection of fraudulent practices.

Finally authenticity control of food and beverages is only one of many possible uses for isotopic techniques: many studies have shown that nutrition problems as well as efficiency tests of pharmaceutical products can be tackled with success. Applications for recognising the origin of illegal drugs or detecting doping in sports have also been developed.

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METHODS IN THE USE OF GC/C/IRMS FOR THE ANALYSIS OF BIOCHEMICAL AND POLLUTANT ISOTOPE SIGNATURES OF COMPOUNDS

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Abstract. The potential for application of GC/C/IRMS analysis to any multitude of environmental, ecological or biochemical research areas is only beginning to be realized. Extension of compound-specific isotope analytical data derived from modern organisms and settings to yield interpretations of ancient depositional environments certainly appears possible. Further application of GC/C/IRMS approaches to understand the cycling of carbon and nitrogen, the identification and alteration of pollutants, or resolve metabolic relationships between compounds in living or extinct organisms are all within the scope of future research.

1. INTRODUCTION

Stable isotopic determinations made on bulk materials are the weighted averages of the isotopic compositions of mixtures of hundreds to thousands of chemical compounds, each of which having its own isotopic abundances. The relative contribution of each of these materials to the isotopic content of the bulk material could theoretically be quantified through mass-balance or isotopic-mixing equations. The stable isotope analysis of individual molecular components holds great potential as a method of tracing the source, biochemistry, diagenesis or indigeneity of a material. Stable carbon and nitrogen isotope analysis of bulk organic materials is a well-established method for tracing biosynthesis as well as the sources and history of organic matter in the geosphere. For example, nitrogen isotopes have been used to assess trends in early diagenesis and to elucidate conditions on the early Earth, and to assess the origins of organic nitrogen in extraterrestrial materials as well as to establish trophic orders in modern and fossil food chains. This paper endeavors to present a perspective on recent research on isotope analyses of individual compounds, or compound specific isotope analyses, that are of biosynthetic or environmental interest. The compounds studied to date include hydrocarbons, tetrapyrroles (chlorophyll derivatives), fatty acids, carbohydrates and amino acids. In recent studies, nitrogen and carbon isotope analyses of components of petroleum and hydrocarbon extracts of sediments have indicated the preservation of original source materials. Isotope analyses of individual amino acids using both carbon and nitrogen isotopes have been useful in detailing indigeneity of organic matter in meteorites and fossils, and helping to understand diagenesis. Inscribed in the isotopic signature is an indication of the biosynthetic pathway used in the formation of the compound. The transfer of nitrogen and carbon within the organism forming the component is thus able to be better understood. These pathways in turn imprint the signature of the organism in the rocks and sediments from which the compounds can later be isolated.

Over the years, numerous attempts have been made to isolate individual molecular components using liquid or gas chromatographic (GC) techniques in order to better interpret or trace the source of history of an organic material. The possibility of comparative biochemistry in modern or fossil organisms has been suggested through the assessment of the isotopic differences between compounds of a family of components. Such differences are the result of enzymatic fractionation effects during synthesis or metabolism of the compound, an example of such an effect has been clearly seen using the enzyme transaminase, with nitrogen isotopic fractionations being observed in acetyl-glucosamine and in the amino acids asparagine and glutamine (and others) in both cultured and natural populations of organisms [1]. Isotopic compositions of individual hydrocarbons have the potential for establishing sources for the

materials, bacterial or otherwise, and have been useful in correlation techniques both in the petroleum industry and in pollution assessment. Individual carbohydrate isotope compositions also show great potential in metabolic and diagenetic studies. Depletions in the isotopic compositions of the products of reactions permit calculations to be done which quantify use and production of new organic materials and resolve them from native materials, even though the chemical compositions of the substances are identical. For non-GC applications for isotope analysis, in addition to establishing separation techniques to provide sufficient material for stable isotope analysis (usually milligram quantities for liquid chromatographic separations), the analytical scheme needs to produce little or no isotopic fractionation of the compound and is labor intensive and time consuming. Large differences in isotopic composition of a single compound exist across the chromatographic peak, owing to chromatographic isotopic fractionation effects [2]. Further, addition of carbon (or nitrogen) to an isolated component is possible through column bleed, or eluent used in the separation of the compound. Certain compounds may require unique separation schemes owing to close similarities in chemical structure. However, the studies to date have yielded important information regarding the source and history of the compounds characterized. Through recent technological advancements, gas chromatographic effluents can be combusted and the resulting carbon dioxide directly introduced into a stable isotope ratio mass spectrometer (IRMS). This modification, GC/C/IRMS (Fig. 1), allows for rapid analysis of the carbon and nitrogen isotopes on components in a mixture, and with increased sensitivity, on the order of 0.5 nmol of each compound [3]. Gas chromatographic-based systems are presently constrained by the volatility of the components investigated. Compounds which do not have this constraint include hydrocarbons, the analyses of which have already clearly demonstrated the power of such technology and measurements in the assessments of source and the history of organic materials [4–7].

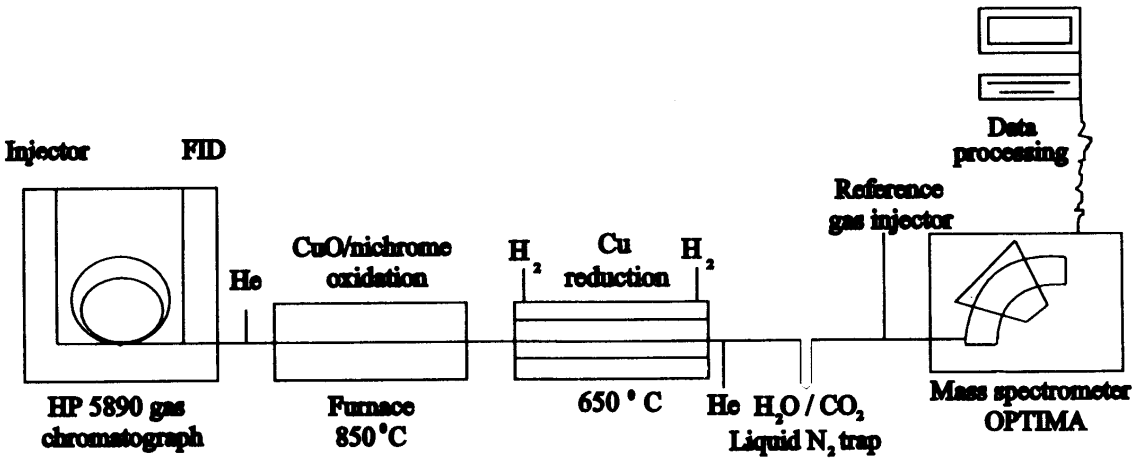

Fig. 1. Schematic of the GC/C/IRMS systemincorporating both oxidation and reduction furnaces [20].

Nonvolatile, multifunctional molecules, including carbohydrates, fatty acids and amino acids require derivatization prior to gas chromatographic analysis to increase volatility. Through the derivatization, additional carbon (but not nitrogen) is thus added to the parent compound. This addition, as well as fractionations associated with derivatization procedures involving bond rupture and formation in, for example, esterification and acylation [8] need to be corrected for in order to ascertain the original isotopic composition of the compound. The original carbon and nitrogen isotopic compositions of individual amino acids and their stereoisomers have been able to be computed, however, through analysis of standards prepared in a similar fashion [8]. Using derivatives, enrichments of ^{13}C and ^{15}N in amino acids and for both stereoisomers of the same amino acid from a meteorite analyzed using GC/C/IRMS, have confirmed the extra-terrestrial origin of those components and supported the lack of contamination by terrestrial compounds in the absolute concentrations and stereoisomer relationships [9, 10].

2. INDIVIDUAL AMINO ACID ISOTOPE ANALYSIS

The foundation for the recent advancements in the use of a combination of molecular techniques in conjunction with stable nitrogen and carbon isotopic compositions may be ascribed to the initial work by Abelson and Hoering [11] on algal cultures. Using a liquid chromatographic separation procedure for the isolation of sufficient material for stable carbon isotope analyses of individual amino acids and their carboxyl groups, these authors strengthened our understanding of natural biosynthetic pathways and the effects of decarboxylation during the diagenesis of organic matter. Studies on nitrogen metabolism and biosynthesis have also been attempted using stable isotopic compositions of individual amino acids [12–14] using similar separation approaches and subsequent isotope analysis. Amino acid isotopic compositions have also continued to be investigated, with pathways for nitrogen incorporation and intermolecular transfer [2] and comparative biochemistry in fossil materials [15] being elucidated. Nitrogen isotope abundances in the individual amino acids appear to be related to kinetic isotope effects associated with transamination reactions during synthesis [16]. Because the process of amino acid racemization has little apparent effect on the isotopic compositions of stereoisomers [17] the possibility of an absolute criterion for determining the indigeneity of amino acids in fossil or extraterrestrial materials has been suggested [18, 19]. Unlike hydrocarbons, the application of GC/C/IRMS to amino acid analysis is complicated by the fact that amino acids are non-volatile, multifunctional molecules that require derivatization prior to gas chromatographic analysis. Although the derivatization procedure introduces additional carbon (but no nitrogen) and an apparent fractionation during the esterification and acylation [8], the carbon isotopic compositions of individual amino acids and/or their stereoisomers can be computed through analysis of standards prepared in a similar fashion, using mass-balance equations which incorporate fractionations inherent to the derivatization procedure. The isotopic composition of the nitrogen in the derivative is that of the original compound [20]. Replicate stable isotope analyses of the trifluoroacetyl (TFA)/isopropyl (IP) ester derivatives, determined by both conventional isotope ratio mass spectrometry (IRMS) and GC/C/IRMS, indicate that this procedure is highly reproducible (standard deviations typically 0.3–0.4‰) and that isotopic difference between the original, underivatized amino acid and the amino acid corrected for derivatization effects is better than 0.5‰.

Extreme enrichments in amino acids and similar enrichments in both stereoisomers of the same amino acid from a carbonaceous chondrite analyzed using GC/C/IRMS, the Murchison meteorite, have confirmed the extraterrestrial origin of those components and supported the lack of contamination by terrestrial compounds in the absolute concentrations and stereoisomer relationships [9, 10]. Such a study required that the technology for the isotope analysis have the sensitivity for determinations on only fractions of nanomols of the individual stereoisomers. Applications of this technology (GC/C/IRMS) to interpret fossil organic matter, to establish its indigeneity or to suggest modern biosynthetic relationships have recently been explored [1, 21]. The molecular isotope approach in the analysis of individual amino acids also appears to be able to resolve bacterial contributions from higher plant additions to organic materials preserved in sediments [22]. Few studies have analyzed the isotopic compositions of chemical components other then hydrocarbons or amino acids.

3. ISOTOPIC COMPOSITIONS OF INDIVIDUAL LIPID COMPONENTS

The earliest application for this technique has been in the analysis of lipids and hydrocarbons with sources of petroleum derived materials [23–25], or food chain origins of hydrocarbon natural products determined through isotope analysis of individual alkanes [26]. Further refinements in the isotopic characterization of potential biological sources of petroleum-related hydrocarbons are presently being attempted. The direct interfacing of the gas chromatograph through a combustion furnace to an isotope ratio mass spectrometer or GC/C/IRMS, requires much less of a compound for analysis [3] allowing for much more cost- and labor-effective analysis to be done on these types of compounds. Petroleum-derived or related components appear to show a relationship to the original biological metabolism prior to deposition [27–31]. Extraterrestrial origins of certain hydrocarbon components which were extracted from the Murchison meteorite were analyzed using this new technology [32]. Origins of sedimentary

lipids derived from tree waxes were also documented using GC/C/IRMS [33]. Lipids that originated from tree waxes were clearly more depleted than those derived from phytoplankton. And thus resolution of chemically indistinguishable sources is now possible. Gas chromatographic based systems are presently constrained by column and component resolution considerations and the volatility of the components investigated. For this reason, hydrocarbons appear to be ideal components for direct GC/C/IRMS analysis, owing to their excellent resolution using capillary gas chromatography and little need for derivatization to increase volatility. Additionally, pollutants, including PCBs and PAHs, are also ideally suited for the source and history analysis through the GC/C/IRMS analysis of extracts of natural materials [34]. The isotope ratio of the element (for example, as carbon dioxide, mass 45/mass 44. or 2/1) changes dramatically across a single peak. As a result, integration of the entire peak along with background and column bleed corrections need to be incorporated in the data analysis; there are also corrections for the addition of carbon or nitrogen to the actual peak. Hydrocarbon components from fossil fuels have isotopic compositions which are readily resolvable from those of 'natural' lipids, and thus have potential in the analysis and tracking of pollutants. Atmospheric contaminants from biomass burning has been able to be tracked to source materials because of the selective isotope signals of both the PAHs produced and the volatilization of FAs [35].

A more recent development in the field of lipid-related materials has been the utilization of a separate isolation scheme using liquid chromatography for the purification of chlorophylls, chlorophyll-derived pigments and other tetrapyrroles, followed by isotopic characterization [36–41]. This separation allows for the determination of both carbon and nitrogen isotopic compositions of the pigments. Analyses of this sort enable a strictly biochemical basis for interpretations of inputs and preservation of primary production in sediments to be addressed in both modern and ancient depositional environments. These compounds hold promise for future analyses and use with the advancements in the high temperature technologies now available for GC.

Few early attempts at the assessment of the isotopic compositions of long chain fatty acids exist [42, 43]. Those studies observed depletions in the individual fatty acids which were consistent with fractionations associated with lipid synthesis, and could yield clues as to the origins of long-chain hydrocarbons in sediments and fossil fuels, as well as the sources of the fatty acids themselves. The analysis of fatty acids using GC/C/IRMS technology has been attempted through analysis of the methyl esters of the fatty acids [35, 44–48] and has indicated that the identification of source carbon for specific organisms, plant types and depositional environments is possible.

4. ISOTOPE ANALYSES OF CARBOHYDRATES

The largest reservoir of carbon which can be chemically characterized is that of carbohydrates. Degens et al. [49], through isolation and isotope analysis of major biochemical fractions of phytoplankton which included different groupings of carbohydrates, concluded that the most labile materials were carbohydrates and proteins. Utilizing an ion exchange technique, others [50] isolated N-acetyl-D-glucosamine, the monomeric unit of chitin. The study of this monosaccharide has suggested a use in palaeoenvironmental and palaeoclimatic reconstruction by the determination of $\delta^{13}C$ on a single marine organism-derived compound [51]. Stable isotope analysis enabled others [52] to study the isotopic compositions of carbohydrates isolated from individual organisms and sediments. In general, individual sugar isolates from organisms or polymers including glucans. galactans and chitins all have carbon isotopic compositions similar to but, typically, depleted by approximately 2‰, from the organism for which they were isolated. The monosaccharides essentially maintain the isotopic signal of the organism and reflects the primary source of carbon entering the organism, i.e., from the Calvin cycle (C3 pathway), Hatch-Slack cycle metabolism (C4 pathway) or from marine bicarbonate. Carbohydrates may be derivatized to acetates and analyzed for their carbon stable isotopic compositions using GC/C/IRMS following a correction for the isotopic effect of derivatization and the addition of the acetate carbon [53]. The similarity in isotopic composition of the monosaccharide to the organism is not maintained in the nitrogen isotopic compositions of the N-acetylglucosamine isolated from chitin. An approximately 9‰ depletion is observed in samples of the polymer and pure compound relative to the organism [50, 52]. As

sources of nitrogen for growth and metabolism, amino acids donate nitrogen for cellular biosynthesis. The isotope ratios of the carbohydrate which receives a donated nitrogen reflects the action of enzymatic isotopic fractionation which occurs during the transfer of nitrogen. Aspartic acid has been observed to be depleted by up to 9‰ in $\delta^{15}N$ relative to glutamic acid in the transamination of glutamic acid to aspartic acid [16]. Transaminase enzymes are likely influential in the transfer of nitrogen from glutamine to fructose in the initial synthesis of glucosamine, the precursor of N-acetylglucosamine. The consequences of not considering the above depletions in the nitrogen isotopic signature, in environments impacted by inputs of chitin, could include inappropriate assignment of sources of organic nitrogen. Marine environments receive large amounts of chitin from the exoskeletons of marine invertebrates. An incorrect assessment of the amount of nitrogen from more ^{15}N enriched sources could be possible under these circumstances. In an investigation on a peat, it was observed that the hexose, mannose was isotopically similar to the whole peat and extracted carbohydrate [1]. However, xylose showed a distinct depletion in ^{13}C by over 7‰ when compared with other carbohydrates or the bulk carbon of the peat. This fractionation is consistent with a direction which indicated production of new material [2, 16]. Similar isotopic trends have been noted in *Sphagnum*, with certain sugars remaining constant (rhamnose, arabinose) whereas other sugars (xylose, galactose and glucose) become increasingly depleted in ^{13}C with depth. In general, plant carbohydrates are isotopically similar to plant bulk carbon; isotopic depletions indicate that these lighter sugars are being newly produced, and when compared with the changes in the chemical compositions, gives an indication of the lability and rates of utilization and turnover. The constancy of the arabinose and rhamnose isotopic compositions with the relative increases in the molar concentrations of these materials (three- to fourfold) may reflect the amount of the plant mass which has been lost during decomposition reactions.

5. TECHNIQUES

5.1. Amino acids

Solutions (0.05 M) of amino acid enantiomers and racemic amino acids are prepared by dissolving appropriate amounts of crystalline amino acids (Sigma, St. Louis, MO) in distilled 0.1 N HCl. Acidified (2.8 M HCl) 2-propanol was prepared by the addition of 250 μL of acetyl chloride (99+%, Aldrich, Milwaukee, WI) per mL of 2-propanol (HPLC grade, Fisher Scientific, Fairlawn, NJ). The acidified alcohol should be used within 48 h of preparation. Trifluoroacetic anhydride (99+%, Pierce Chemical Co., Rockford, IL) is used for acylation. Reagents of the same lot numbers are used for all derivatizations.

Two hundred nL aliquots (10 nmol) of the amino acid solutions are dispensed into individual 4-mL screw cap vials with Teflon cap liners. The samples are evaporated to dryness under a stream of N_2 at 40°C. For GC/C/IRMS analysis, 100 nL aliquots of a standard solution is prepared in an identical manner. The dried samples are esterified with 0.5 mL of the acidified 2-propanol for 1h at 110°C. After 1 h the reaction is quenched by placing the vials in a freezer. Next, 0.25 mL of each sample is pipeted into a 20 cm × 7 mm-i.d. Pyrex tube. The solvent is removed by evaporation under a gentle stream of N_2 at 25°C. Two successive 0.25-mL aliquots of CH_2Cl_2 are placed in each tube and evaporated to remove excess 2-propanol and water. The remaining portions of the esterified samples are evaporated to dryness under nitrogen, redissolved in CH_2Cl_2 and dried again.

The amino acid isopropyl esters are acylated with 0.5 mL of trifluoroacetic anhydride (TFAA) and 0.5 mL of CH_2Cl_2 for 10 min at 110°C. Next, the vials are chilled in a freezer and then placed in an ice bath where the excess TFAA and CH_2Cl_2 are removed by evaporation under N_2. The derivatives are redissolved in 0.25 mL of CH_2Cl_2 and evaporated at 0^oC to remove residual traces of TFAA and trifluoroacetic acid. The derivatives are then dissolved in 0.5 mL of CH_2Cl_2 and transferred to Pyrex tubes, and evaporated to dryness under N_2.

The TFA isopropyl esters of the individual enantiomers and racemic amino acids are analyzed directly for their stable carbon or nitrogen isotope compositions by using the Micromass Isochrom

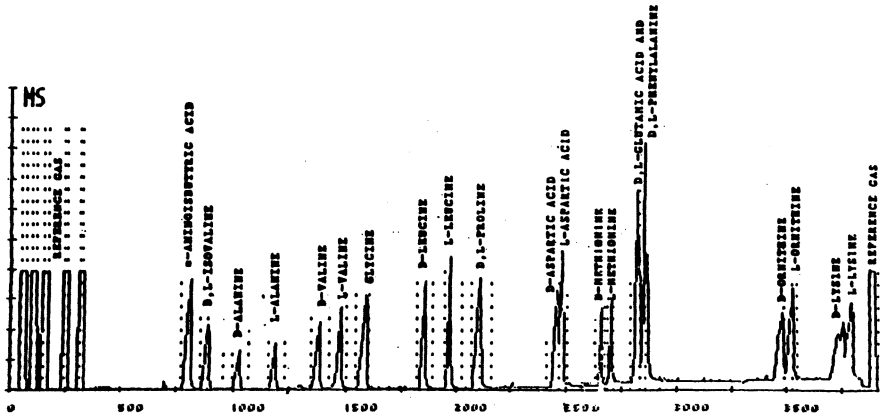

Fig. 2. Chromatogram of amino acid separation for $\delta^{13}C$ analysis of stereoisomers [8].

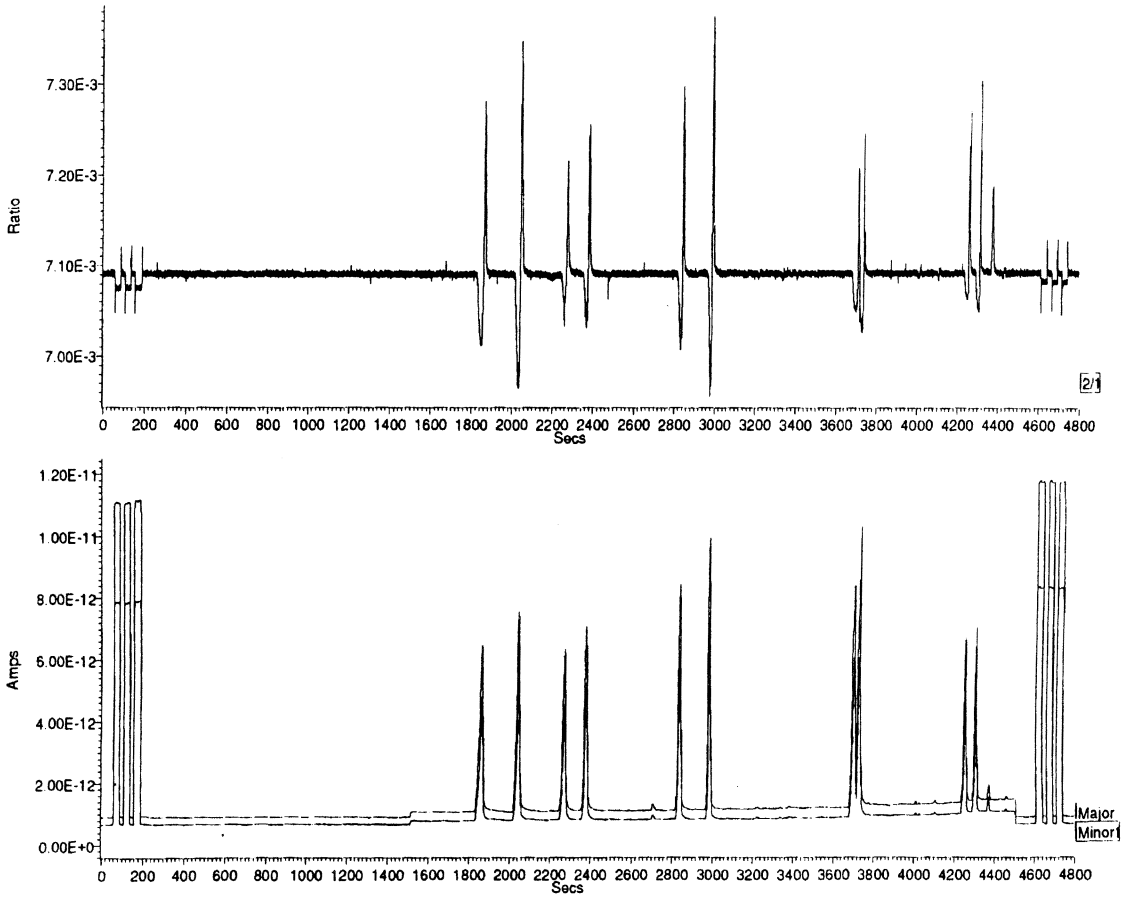

Fig. 3. Chromatogram of amino acid separation for $\delta^{15}N$ analysis of stereoisomers [20].

GC/C/IRMS, Optima GC/C/IRMS or Isoprime GC/C/IRMS systems. The present GC/C/IRMS system at the University of Virginia consists of a Hewlett-Packard 5890 gas chromatograph interfaced to a Micromass Isoprime isotope ratio mass spectrometer via a combustion furnace/water trap. For amino acids, the gas chromatograph is equipped with a 50-m × 0.25-mm-i.d. fused silica capillary column

coated with an optically active stationary phase (Chirasil-Val; Alltech Assoc., Deerfield, IL) capable of resolving the TFA isopropyl esters of amino acid enantiomers. The CO_2 or N_2 combustion products of the compounds eluting from the capillary column are introduced directly into the mass spectrometer ion source: this instrument configuration permits stable carbon or nitrogen isotope analysis at nanomol levels (Figs 2 and 3).

The GC conditions are as follows: splitless injection (~l–2 nmol of each enantiomer derivative is injected, combusted, and subsequently introduced directly into the source of the MS); the carrier gas is ultrapure He (99.9999%) at a head pressure of 80 kPa; the injector temperature is 200°C, and the temperature of the interface between the GC and the oxidation furnace is 350°C; the GC temperature program is 45vC for 3 min, 45–90°C at 45/min, 90°C isothermal for 15 min, 90–190°C at 3C/min, and then 190°C isothermal for 30 min. The solvent (ethyl acetate) peaks are removed from the effluent of the GC through a heart split valve which is open to the FID at the time of injection. The valve is programmed to close at 1500 s to allow the column effluent to be directed to the oxidation furnace. Calibration of the stable nitrogen or carbon isotope composition of each component is accomplished by comparison to three reference gas pulses (each of 30 s duration) introduced at the start of the run and following the opening of the heart split valve at the end of each run, i.e., after 4500 s.

5.2. Carbohydrates

Alditol acetates of the individual sugars (Sigma Chemical Co., St. Louis, MO) are prepared following a method by Fox et al. [54] in which the sample is reacted with sodium borohydride for 1hr at room temperature to reduce the aldehyde group. Following neutralization with acetic acid, resulting in the destruction of the carbohydrate-borate complex, and vacuum rotary evaporation, the carbohydrate mixture is reacted with acetic anhydride in pyridine for 15 min at 100°C. The acetylated products are then rotary evaporated and subsequently washed with methanol and chloroform and lastly, filtered prior to analysis by GC on a SP2330 30 m × 0.25 mm (i.d.) column (Supelco, Inc., Bellefonte, Pennsylvania; film thickness 0.20 μ). Conditions for the GC separation (Hewlett Packard 5890) were isothermal at 180°C for 5 min with a helium inlet pressure of 75 kPa, then oven ramping to 250°C at 3°C/min, and maintenance at the final temperature for 30 min. The injector temperature is 220°C and the detector temperature was 240°C. Stable isotope compositions of the alditol acetates (Fig. 4) are assessed using the combined GC/C/IRMS system which has a combustion furnace at 850°C and a water trap at –90°C [8, 55].

5.3. Fatty acids and hydrocarbons

All the solvents and reagents (Fluka, Switzerland) are of analytical grade or higher purity. The organic solvents are glass distilled, and all the glassware is thoroughly washed, rinsed with deionized water (×4), and ashed (450°C, 12 h) before use. The samples are subjected to alkaline hydrolysis by heating (70°C, 3 h) with 10 mL of aqueous ethanolic (95 volume %) potassium hydroxide (1 N) solution. After cooling, the neutral fraction is extracted with hexane (1 × 10 mL and 2 × 5 mL). Acidification of the hydrolysate with 6 N HCl to pH 1 liberates the fatty acids which are extracted with hexane (1 × 15 mL and 2 × 5 mL). The excess hexane is removed by rotoevaporation at 30°C and dried in a vacuum desiccator. The fatty acids are derivatized to fatty acid methyl esters (FAMEs) with BF_3 in methanol (60°C, 8 min), extracted with 10 mL hexane, and washed with saturated KCl solution (2 × 5 mL). The excess solvent is gently evaporated, and the FAMEs are stored with 0.5 mL of hexane in 2 mL vials with PTFE-lined cap at 4°C until gas chromatographic analysis. The injector temperature is 200°C, to avoid transmerization of the unsaturated FAMEs. For isotope analysis, a Micromass Isoprime IRMS is interfaced to a HP5890GC with a combustion furnace (Cu/Nichrome wire, 850°C) and a cryogenic trap (–90°C). The GC is equipped with an J &W Scientific fused silica capillary column (30 m × 0.25 mm i.d.) DB-FFAP as stationary phase) and operated at the following conditions: splitless injection; helium flow rate mL/min; injector temperature 200°C; initial temperature 150°C; initial time 5 min; temperature ramp rate 10°C/min; final temperature 220°C; final isothermal period 12 min (Fig. 5). The performance of the GC/C/IRMS system, including the

combustion furnace, is evaluated every 10 analyses by injection of a laboratory standard (deuterated naphthalene-dg, Cambridge Isotope Laboratories, MA 01810) of known isotopic composition (working value = –26.2 +/–0.4‰, $\delta^{13}C$). For all runs, background subtraction was performed using the parameters supplied by the GC-OPTIMA software. Two to five replicate GC/C/IRMS runs are performed for each sample. The reproducibility ranged between 0.1 and 0.5‰ (1 SD). The accuracy of the samples' analyses was monitored by co-injection of a FAME laboratory standard (methyl dodecanoate, Supelco) of known isotopic composition (working value = 30.4 +/–0.2‰, $\delta^{13}C$). The isotopic shift due to the carbon introduced in the fatty acids methylation is corrected by the following relationship [56, 57]:

$$\delta^{13}C(FAME) = f(FA)\delta^{13}C\ (FA) + f(MeOH)\ \delta^{13}C(MeOH)$$

where $\delta^{13}C(FAME)$, $\delta^{13}C(FA)$, and $\delta^{13}C$ (MeOH) are the carbon isotope compositions of the fatty acid methyl ester, the fatty acid, and the methanol used for methylation of the fatty acid, respectively; and f(FA) and f(MeOH) are the carbon fractions in the fatty acid methyl ester due to the underivatizad fatty acid and methanol, respectively.

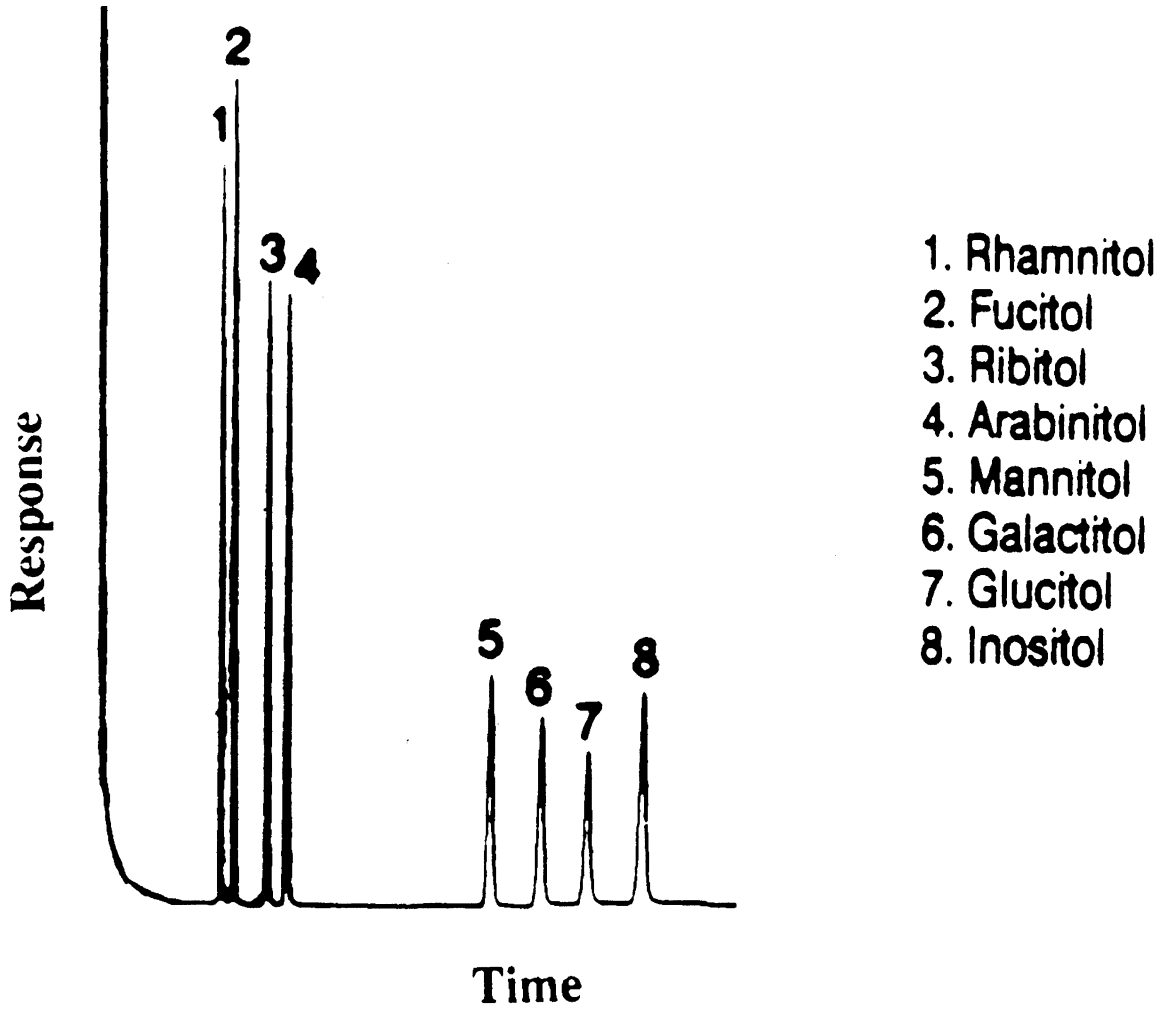

Fig. 4. Chromatogram of carbohydrate separation for $\delta^{13}C$ analysis [53].

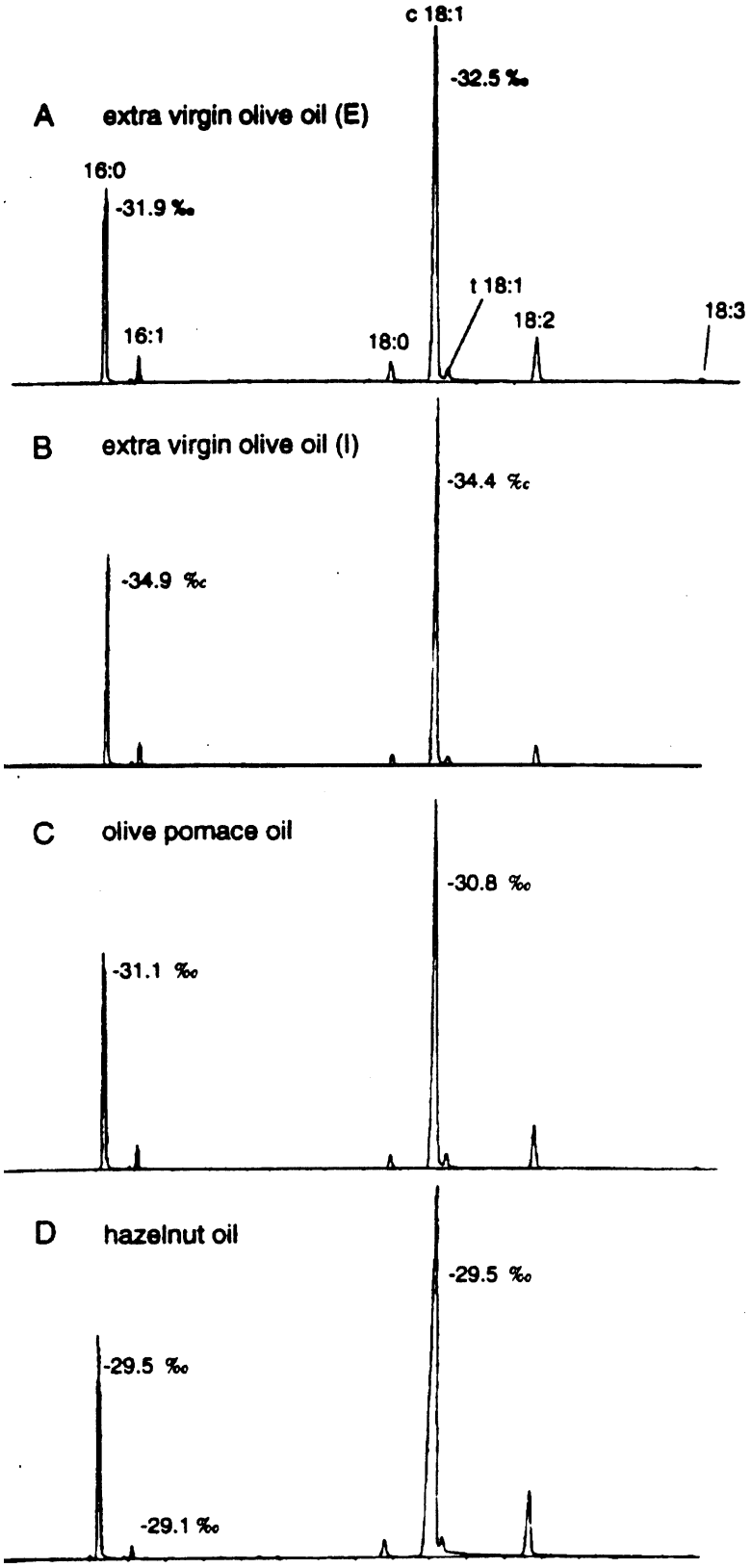

Fig. 5. Chromatogram of fatty acid separation for $\delta^{13}C$ analysis [48].

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STABLE CARBON ISOTOPE RATIOS OF LIPID BIOMARKERS AND THEIR APPLICATIONS IN THE MARINE ENVIRONMENT

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Abstract. Studies on the distribution of lipid biomarkers in the environment help elucidate biogeochemical processes, but recent findings have significantly reduced the specificity of some biomarkers. The analytical development of Gas Chromatography-Combustion-IRMS (GC-C-IRMS) allows the determination of the $\delta^{13}C$ of specific biomarkers, thereby improving the veracity of source apportionment. In this report, we present a brief description of the analytical approach for sample preparation and carbon isotope measurements of individual biomarkers. Selected examples of the applications in the use of GC-C-IRMS for biomarker source elucidation in the marine environment and potential applications to paleoclimatological studies are reviewed.

1. INTRODUCTION

Biomarkers, or molecular markers, are compounds with structures that can be related to specific biological sources due to their own biosynthesis [1, 2]. An important fact in considering their fate is that the transformation of dissolved, suspended and deposited lipids in any environment (oxidizing or reducing) tends towards a selective preservation of low-polar compounds, including hydrocarbons, fatty acids, and sterols. Hence, organic biomarker compounds are often used as tracers of sedimentary organic matter sources [3]. Long-chain n-alkanes, n-alkanols and n-fatty acids are used as biomarkers for terrigenous input; unsaturated alkenones and dinosterol as tracers for marine production and branched-chain fatty acids of the *iso*- and *anteiso* series as bacterial markers [1, 2, 4]. Other biomarkers of high thermodynamic stability, such as terpanes and sterane isomers, are commonly found in petroleum and are widely used to identify anthropogenic contamination sourced from petroleum [5].

The use of lipid biomarkers has permitted useful perspectives in organic biogeochemical studies, but some care about the assumptions used in the different relationships is required. Over recent years, it has been apparent that some biomarkers are more widely distributed in the environment than previously thought, and thus, their specificity has been reduced. Sterols are a good example of this; some of them have been found only in a few classes while others are now known to be quite widely distributed [6]. Certain general markers are still considered unambiguous, but the usefulness of those derived from a variety of sources needs to be explored. The advent of compound specific isotopic analyses (CSIA) through the development of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) [7, 8] allows the determination of the $\delta^{13}C$ composition of individual compounds. Consequently, $\delta^{13}C$ of specific biomarkers should be useful to improve the veracity of source identification, thereby allowing the origin of ubiquitous compounds with multiple potential sources to be determined.

Stable isotope ratios can also be used to distinguish between the photosynthetic pathways of terrestrial species (C_3 vs C_4 type) [9, 10]. Photosynthesis in the marine environment occurs via the C_3 pathway. However, $\delta^{13}C$ values of photosynthetic organisms in the ocean do not always resemble $\delta^{13}C$ values of terrestrial C3 plants. The reasons are that aquatic plants can also utilize bicarbonate, which is known to be ^{13}C enriched relative to dissolved CO_2, and the slower diffusion of CO_2 in water may reduce the extent of fractionation by the enzyme RuBP carboxylase [11]. Other factors that may influence $\delta^{13}C$ values of marine photosynthetic organisms include the salinity, temperature, phytoplankton growth rate and CO_2 availability [12, 13]. All these natural variations in $\delta^{13}C$ can be used for the identification of sedimentary carbon sources and for the differentiation between marine and terrestrial sources.

This report presents a brief description of the analytical approach for carbon isotopic analyses of individual biomarkers. It also provides selected examples of the applications of $\delta^{13}C$ biomarkers to the environment, including the elucidation of biological sources and palaeoclimate studies.

2. SAMPLE PREPARATION AND MASS SPECTROMETRIC MEASUREMENTS

An excellent gas chromatography performance, including baseline separation and complete peak integration, are extremely important for achieving accurate $\delta^{13}C$ measurements [14]. Thus, isolation of the target compounds from the co-eluting compounds that typically comprise a background matrix or unresolved complex mixture (UCM), is essential. Care should be taken, however, during the isolation of the 3 compounds from the complex sample matrix to ensure that no isotopic alteration of the products occurs. The quantitative collection of the compounds during the extraction and purification processes is required [15, 16].

Applications of size-exclusion techniques (e.g., urea adduction and molecular sieving) have been shown to improve the accuracy of the n-alkyl lipid profiles from complex organic mixtures. These techniques, which show no measurable isotope fractionation effect, work effectively in removing the UCM [17].

Some of the pitfalls encountered in the GC-IRMS technique have been discussed by Meier-Augenstein [18]. Depending on the polarity of the stationary phase used in the chromatographic column, isotopic fractionation for some families of compounds might occur. In general, chromatographic isotope effects are minimized when the polarity of the stationary phase matches the polarity of the sample [19]. However, the use of stationary phases of high polarity have a relatively low maximum operating temperature (250^{o}C or less) and show considerable column bleed that might adulterate isotope ratio measurements.

The combustion interface must also be closely monitored. Its performance might vary during the chromatographic run, giving good accuracy for low molecular weight compounds but compromising the accuracy and precision for the high molecular weight compounds due to an incomplete combustion (non-quantitative conversion of the analyte to CO_2). It can also be temporarily overloaded, particularly with the use of derivatization agents, which results in non-quantitative combustion. When derivatives are to be used for obtaining a good chromatographic performance (e.g. carboxylic acids, sterols, aminoacids), the possibility of isotope fractionation processes should be also considered [20]. The additional imprecision of delta values of compounds for derivative groups must be considered before conclusions are drawn.

In order to overcome these problems, the use of internal standards that have the same functional group as the sample compound is an advisable way to check for possible isotope effects. Standardization of the internal standards should be determined using conventional techniques (off-line combustion).

3. BIOMARKER SOURCES AND BIOGEOCHEMICAL PROCESSES

Carbon isotopic compositions of individual biomarkers have been interpreted in terms of biogeochemical processes in ancient depositional environments [7, 21–27]. The isotopic composition of individual lipid biomarkers can indicate the isotopic composition of the parent organism and infer the carbon source utilized by the producer, and thus its position within the ancient ecosystem.

Refined estimate of marine and terrigenous contributions to sedimentary organic carbon have also been provided by using the $\delta^{13}C$ of a marine organic biomarker, such as C_{37} alkadienone [28]. A great number of studies using the GC/C/IRMS technique has demonstrated its potential to support the simple molecular approach clarifying the source of some enigmatic biomarkers. Only some examples from selected lipid class will be presented below and discussed.

A separate analysis of isoprenoid-hydrocarbons, pristane and phytane, for which a common origin from chlorophyll had been generally assumed, seemed to indicate that these two components have different origins in sedimentary rocks from the Eocene Messel Shale [21]. The $\delta^{13}C$ for pristane fitted

with that expected for algal lipids (–25‰) and that of phytane (–31.8‰) with those from methanogenic bacteria.

The $\delta^{13}C$ of the regular C_{18} to C_{25} isoprenoids tentatively assigned an origin from halophilic archae agreed with the enrichment by up 7‰ compared to phytoplanktonic biomarkers of the same sediment [26]. The precursor of the C_{40}-isoprenoid hydrocarbon, lycopane, has yet to be identified but its carbon isotopic composition from water column and sediment samples (between –23.6‰ and –32.9‰) confirm its source from photoautotroph organisms [29].

By comparing the $\delta^{13}C$ of individual n-alkanes and n-alkanols from leaves of lakeside trees with those from the lake's sediments, it was possible to discriminate between the fresh-water algae and terrestrial plants [30]. Carbon isotopic ratios of n-alkanols from a saline sediment enabled to distinguish between the terrestrial long-chain n-alkanols (n-C_{24}, n-C_{26}: from –30 to –32‰) and the marine short-chain n-alkanols (n-C_{16} to n-C_{22}: 18 to –23‰) [31].

Resolution of biological sources of individual fatty acids in modern sediments were also provided by GC/C/IRMS. The isotopic compositions of long-chain fatty acids ($C_{20:0}$-$C_{26:0}$: from –31.0 to –30.7‰) reflect their sources from higher-land plants, whereas the $C_{16:0}$ and $C_{18:0}$ acids (–28.7‰ and –27.7‰) were from marine plankton, and $C_{14:0}$ and $C_{15:0}$ acids (–38.7‰ and –37.2‰) originated from bacteria [32].

Other classes of biomarkers, such as the hopanes, are also not always derived from a common precursor [33]. Stable carbon isotopic compositions for diploptene in sediment cores from the Japan Sea has inferred a cyanobacteria origin (–25‰) for the first section of the sediment and a methanotrophic source (–53.1‰) for the older section [34].

Alternatively, the $\delta^{13}C$ obtained for biomarkers commonly associated with terrestrial sources (e.g., long-chain n-alkanes, n-alkanols and $C_{29}\Delta^5$ sterol) have elucidated sources other than higher plants. The isotope profiles of the long-chain n-alkanes in Arctic surface sediments (no shift with chain length and a systematic difference between the odd-and even carbon numbered compounds) favored a non-continental source [35]. Other possible algal origin of long chain odd n-alkanes in immature sediments was revealed by distributions and carbon isotopic composition [36]. Similarly, the light $\delta^{13}C$ of long-chain n-alkanols in modern sediments from the China Sea area indicated sources (marine and bacterial) different of terrestrial [37]. Another example was recently presented for the commonly terrestrially derived sterol (24-ethylcholest-5-en-3β-ol) where its enriched $\delta^{13}C$ inferred a marine and not a terrestrial origin in saline sediments from Spain [31].

All these studies have claimed that in the future the molecular approach in carbon cycling should be pursued using combined biomarker and stable isotope techniques. However, it has also been shown that lipids synthesized by the same biosynthetic pathway might not have the same isotopic composition.
A striking example was shown by Summons et al. [38] where the isotopic compositions of squalene and hopan-29-ol produced by methanotrophic bacteria differed by more than 10‰. Others instances have recently been presented in different photosynthetic organisms [31, 39]. Small variations within and between biosynthetically related compound classes were observed in cyanobacteria. In algae, e.g. diatoms and dinoflagellates, remarkable differences between the average $\delta^{13}C$ composition of fatty acids and sterols were observed (7.5‰ and 2‰, respectively). Similarly, differences between biomarkers with linear carbon skeletons and those with steroid carbon skeletons may differ by up to 8‰.

Isotopic variations among homologues of the same lipid class were also observed. In diatoms, variations were up to 5‰ within each class of fatty acids and sterols and in the dinoflagellate species, these variations were lower than 3‰. In studies of modern leaf tissues, biosynthetically related n-

alkyl lipids (n-alkanes, n-alkanols and n-fatty acids) in epicuticular waxes have been shown to have similar carbon isotopic compositions, but variations of up to 6‰ were noted for homologous n-alkanes extracted from a single leaf [10].

Significant variations and temporal fluctuations in $\delta^{13}C$ of biogenic hydrocarbons were also documented in spring bloom samples of the NW Atlantic waters. These findings highlighted the influence of growth rate and timing of synthesis on the $\delta^{13}C$ of biomarkers over the course of phytoplankton blooms [40].

All these differences, in particular the intra-specific shifts in $\delta^{13}C$ lipid composition render the assignment and deconvolution of biomarker sources more complicated. It is important that more studies of the compound-specific isotopic composition of modern environments take place to ensure the correct interpretation of biomarkers signatures. Alternatively, the significant progress that occurred in GC/IRMS recently enabling the determination of H/D ratios in individual molecules, will probably provide a better understanding of all these processes. This might help facilitating the interpretation of the carbon isotopic biomarker record.

4. PALAEOCLIMATE APPLICATIONS

Applications of the isotope techniques to palaeoenvironmental studies have been explored in recent years due to the observed trends between dissolved CO_2 in water and fractionation of carbon isotopes during photosynthetic fixation of CO_2 [41–43]. More recently, it has been shown that this relationship is only reliable when typically autotrophic C_3 phytoplankton dominate the particulate organic carbon [44].

In this sense, investigations of the potential use of some marine biomarkers for the recovery of the CO_2 related signal, which would provide much better estimate of the palaeopCO_2 reconstruction for all sea-surface waters, have been done. Analyses of phytoplankton grown in chemostat cultures have shown the importance of many environmental variables (e.g., [CO_2 (aq)], cell growth rate, cell size, cell geometry) on the isotopic composition of phytoplankton [13, 45–47]. However, the long-chain C_{37}–C_{39} n-alkenones, derived exclusively from primary producers (Prymnesiophyte algae), are particularly suitable as recorders of CO_2 levels due to their constant isotopic fractionation [48]. Their isotopic analyses have allowed determination of the glacial-to-interglacial variation in the isotopic composition of the total biomass of the source organism and the recovery of a CO_2 related signal covering ~100 kyr [49]. Alternatively, its low concentration in the particulate matter from low productivity regions has fostered the study of other more abundant biomarkers, such as cholesterol, which has a general planktonic origin with a large proportion typically derived from zooplankton inputs. A significant correlation between $\delta^{13}C$ cholesterol of the suspended particulate fraction from the water column in the Indian Ocean and dissolved CO_2 concentrations was found [31]. Additional investigations are required because calibrations for the isotopic fractionation-[CO_2(aq)] relationship may vary significantly from one environment to another [50].

The development of carbon isotope signatures of biomarkers as a palaeoceanographic tool has also been considered. Interestingly, Schoell et al. [51] have demonstrated that $\delta^{13}C$ of organic constituents such as C_{35} hopanes and C_{27} steranes provided palaeoclimatic information from Monterey-type sediments that complemented palaeoclimatic studies from open ocean sediments. Alternatively, the $\delta^{13}C$ values of terrestrially derived n-alkanes in the marine environment have been used to asses basin-wide vegetation changes in adjacent river catchments on geological timescales [52]. More recently, the $\delta^{13}C$ of specific aromatic plant markers (e.g. cadalene) have contributed to interpreting the climatic conditions in ancient sediments [53]. Future work by biogeochemists will probably demonstrate that other biomarkers also have a similar potential to provide information about the paleoclimate and CO_2 exchanges between the ocean and atmosphere.

5. CONCLUSIONS

CSIA is a powerful tool allowing a more accurate assessment of the sources of organic carbon. The use of this technique in combination with biomarker studies can yield additional important environmental information on specific sources and palaeoclimate studies. Care must always be taken, however, in the interpretation of the carbon isotopic composition of individual biomarkers since isotopic variations within and between compound classes metabolized by the same organism can be substantial.

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CHALLENGES OF $^{13}C/^{12}C$ MEASUREMENTS BY CF-IRMS OF BIOGEOCHEMICAL SAMPLES AT SUB-NANOMOLAR LEVELS

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Abstract. Recent refinements to instrumentation and methodology have facilitated measurement of $^{13}C/^{12}C$ of individual compounds within complex biogeochemical mixtures by CF-IRMS. Specific challenges such as instrument performance, effective compound partitioning, efficient combustion, effective water removal, and isotope effects during online preparation of gases are discussed and practical improvements are presented.

1. INTRODUCTION

Since the advent of isotope ratio mass spectrometers (IRMS) in the 1940's to measure stable isotope ratios in natural substances, it was recognized that variations in the distribution of isotopes in organic compounds, especially with lighter elements, e.g., H, C, O, N, S, can provide diagnostic information. It was confirmed that the distributions of the different isotopes and their ratios, such as $^{2}H/^{1}H$, $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in various phases are controlled by isotope effects, including equilibrium isotope effects (EIE's) and kinetic isotope effects (KIE's). Physicochemical factors and principles based on mass, such as vapour pressures, bond energies, or diffusional velocities, result in isotope effects that partly partition the heavy and light isotope pairs between phases.

Prior to this decade, i.e., before the availability of online combustion, or Continuous-Flow Isotope Ratio Mass Spectrometry (CF-IRMS, [1–3]), most stable isotope measurements were made on either bulk or single constituent samples, e.g., carbonates, leaves, water or methane. In some laboratories compound fractions, such as fatty acids, aliphatics, vitrinite, etc., were isolated from mixtures for isotope ratio determinations, but the isotopic measurement of individual compounds in a complex mixture during these earlier days was generally difficult and non-routine.

Typically the IRMS instruments, prior to CF-IRMS had sufficient resolution and sensitivity to measure isotope ratios in individual compounds, rather the limitation was due to constraints in the sample inlet configuration. Traditional IRMS instruments employ a dual inlet with a viscous leak into the source. Following ionization, acceleration, focussing and magnetic mass separation, the actual isotope ratio measurement is a direct detection of the collision frequency of the target ions. Essentially, the IRMS simultaneously compares the currents (i) generated in the Faraday cups of the different masses, e.g., $^{12}C^{16}O^{16}O$ (mass 44 amu) vs. $^{13}C^{16}O^{16}O$ (mass 45 amu) producing the isotope ratio R where

$$R = {}^{45}i \,/\, {}^{44}i \qquad (1)$$

The isotope measurements are made in the IRMS on simple, stable compounds (e.g., H_2, CO_2, N_2 or SO_2) that are quantitatively formed from the original sample material prior to measuring. These simple molecules are formed by "offline" preparation systems. For example, for C-isotope measurements of a hydrocarbon mixture, the sample is typically combusted offline in a furnace to CO_2 and H_2O. The CO_2 is then admitted under controlled pressure conditions into the IRMS source. The ${}^{45}i \,/\, {}^{44}i$ response of the IRMS is dependent on several instrumental factors, some of which may change within the measurement period. As a consequence, the ratios (R_{sa}) measured on their own are not calibrated absolute abundances. Because of these difficulties and the need to compare results between analyses and instruments, the isotope ratio of the sample is constantly compared in alternation with a standard which has known or accepted isotopic abundances or ratios (R_{st}), e.g. PDB, SMOW, CDT. Thus suitable international standards became and remain a critical requirement of convertible and comparable isotope ratio measurements.

The differences in isotope abundances and hence isotope ratios between samples and standards are relatively small. These small numerical differences are inconvenient to express and recall. As a result the delta notation (δ) is commonly used to report the magnitude of the difference or excursion in isotope ratio, given in permil (‰), between the sample and the appropriate standard, according to:

$$\delta_{sa}\ (‰) = (R_{sa}/R_{st} - 1) \times 10^3 \quad (2)$$

where for carbon the PDB standard (R_{st}) is 0.0112372 ± 0.0000029 [4].

The dual inlet system simplified this calibration, by alternating measurements of sample and standard gases. This approach permitted optimization of beam intensities, pressure balancing between the two gas reservoirs and maintained relatively good vacuum in the source. Generally, samples for a dual inlet are prepared and purified offline. With offline preparation it is difficult and non-routine to quantitatively separate and collect the individual compounds in a complex mixtures. This presented the major limitation for compound specific isotope measurements.

2. DEVELOPMENT OF CF-IRMS

2.1. Basic instrumentation

The development of CF-IRMS[1], initiated by Matthews and Hayes [1], created a minor geochemical revolution. The ability to measure the isotope ratios of individual compounds merged the fields of molecular organic geochemistry and isotope geochemistry. In addition, the online configuration of CF-IRMS means that proportionately more sample material enters the source than by conventional dual inlet, even with cold finger focussing (ca. 1 µl•atm CO_2).

Table I gives an indication of the relative amounts of sample required for CF-IRMS compared with conventional dual inlet systems, using the example of atmospheric methane.

TABLE I. COMPARISON OF SAMPLE REQUIREMENTS OF CONVENTIONAL DUAL INLET VS. CF-IRMS CONFIGURATIONS

Typical Minimum Sample Requirements for Light Gas Isotope Ratio Mass Spectroscopy			
SYSTEM	µl atm CO_2	Nmol CO_2	Equiv. Air (ltr) for atm CH4 (1.8 ppmv)
1.Conventional Inlet (min. 15 mbar)	ca. 50 – 600	2 400 – 28000	30 – 340 (off-line)
2. Micro-coldfinger	ca. 5 – 50	240 – 2 400	3 – 30 (off-line)
3. GC/C/IRMS (inj.)			
(a) CH4 in He	ca. 0.001	0.05	-
(b) CH4 in air	ca. 0.009	0.4	0.005 (on-line) (= 5 ml)
$^{13}C/^{12}C$ (as CO_2 on masses 44, 45, 46: 0.5 to 7 V; 1‰ precision, PDB)			

CF-IRMS shares the basics of the conventional IRMS (ion source, flight tube, detectors, etc.). However, the mode of sample introduction is radically different. Instead of the dual inlet, CF-IRMS has a continual leak of gas into the source, generally from an online preparation line. For example,

[1] The term CF-IRMS encompasses several forms of on-line IRMS analyses, including Elemental Analyzer-IRMS (EA-IRMS, e.g., [12]) and Gas Chromatograhy-Combustion-IRMS (GC-C-IRMS, e.g., [1], [2], [5], [6]. Other terms have been used to describe the approach such as GC-IRMS, GC-irm (GC-isotope ratio monitoring), CSIA (Compound Specific Isotope Analyses), but CF-IRMS might become the more common term.

TABLE II. LIST OF COMPOUNDS IDENTIFIED IN GC-FID CHROMATOGRAM OF FIG. 13A

Cpd#	abbr.	chemical name
1	iC5	i-pentane
2	nC5	n-pentane
3	22DMC4	2,2-dimethylbutane
4	CYC5	Cyclopentane
5	23DMC4	2,3-dimethylbutane
6	2MC5	2-methylpentane
7	3MC5	3-methylpentane
8	nC6	n-hexane
9	22DMC5	2,2-dimethylpentane
10	MCYC5	methylcyclopentane
11	24DMC5	2,4-dimethylpentane
12	223TMC4	2,2,3-trimethylbutane
13	Benz	benzene
14	33DMC5	3,3-dimethylpentane
15	CYC6	cyclohexane
16	2MC6	2-methylhexane
17	23DMC5	2,3-dimethylpentane
18	11DMCYC5	1,1-dimethylcyclopentane
19	3MC6	3-methylhexane
20	1c3DMCYC5	1-cis-3-dimethylcyclopentane
21	1t3DMCYC5	1-trans-3-dimethylcyclopentane
22	1t2DMCYC5	1-trans-2-dimethylcyclopentane
23	nC7	n-heptane
24	1c2DMCYC5	1-cis2-dimethylcyclopentane uncertain
25	MCYC6	methylcyclohexane
26	22DMC6	2,2-dimethylhexane uncertain
27	EtCYC5	ethylcyclopentane
28	25DMC6	2,5-dimethylhexane
29	24DMC6	2,4-dimethylhexane
30	1t2c4TMCYC5	1-trans-2-cis-4-trimethylcyclopentane
31	33DMC6	3,3-dimethylhexane
32	1t2c3TMCYC5	1-trans-2-cis-3-trimethylcyclopentane
33	223TMCYC6	2,2,3-trimethylcyclohexane
34	Tol	toluene
35	2MC7	2-methylheptane
36	3MC7	3-methylheptane
37	1c4DMCYC6	1-cis-4-dimethylcyclohexane
38	nC8	n-octane

CF-IRMS analysis of most liquid or gaseous organic compounds generally involves a gas chromatographic (GC) separation to temporally partition the mixture into the individual constituents (Fig. 1). Because the isotope ratio of these compounds can not be measured as such in the IRMS, i.e., directly as hydrocarbons or fatty acids, they are first converted by either oxidation or reduction after partitioning into the IRMS target gas, e.g., CO_2, N_2, SO_2. This conversion is commonly performed in a high temperature micro-combustion tube (850°C–1100°C) filled with combinations of (oxidized) Cu, Pt and Ni wires. In the case of hydrocarbons, this combustion results in the generation of both CO_2 and H_2O. As discussed below, the introduction of water into the source is highly undesirable, and dramatically influences the stability of the source and the mass abundances. To alleviate this problem,

the combustion water is removed after the micro-combustion tube by either cryofiltering (dry ice trap) or more commonly with a Nafion™ tube from Perma Pure (FIG. 1). Following water removal, the CO_2 in the He carrier gas stream is directed to a splitter where a predetermined fraction is "sipped" by a controlled capillary leak into the source of the IRMS.

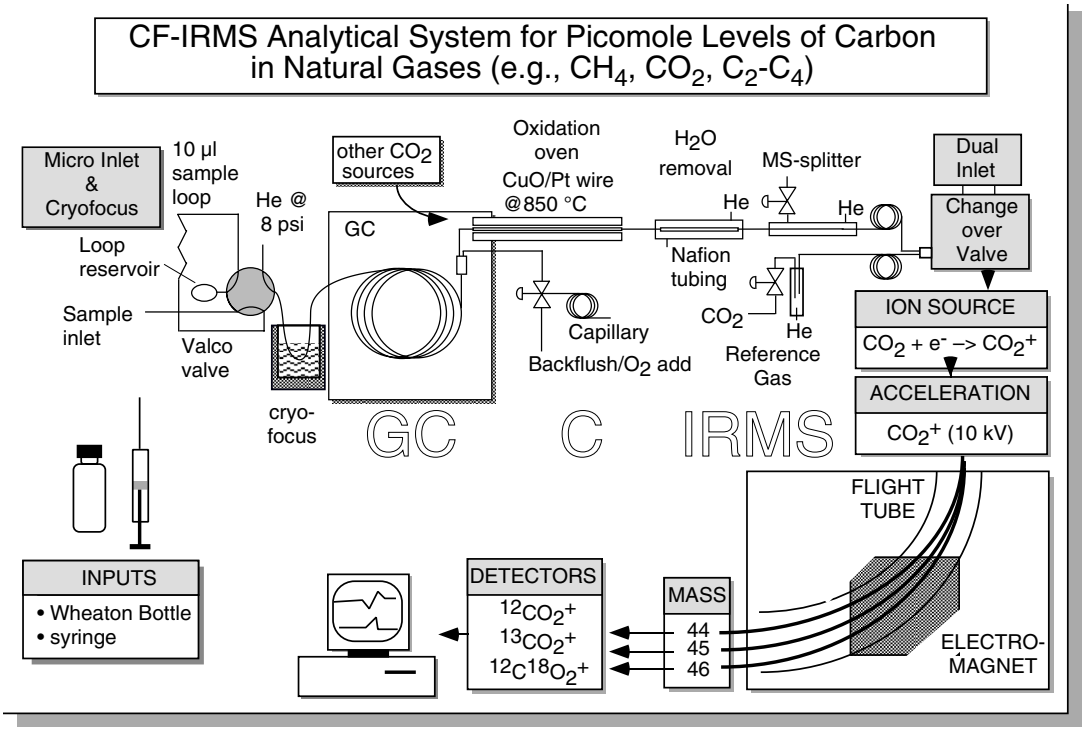

Fig. 1. Schematic of continuous flow-isotope ratio mass spectrometer configuration for measuring low level carbon isotope ratios.

2.2. Instrument challenges

The early challenges of CF-IRMS included dealing with higher pressures in the source (10^{-6}–10^{-5} torr), and introducing a reference gas standard. Much of the gas pressure in the source is the He carrier gas. Differential pumping with a larger turbomolecular pump on a semi-sealed source removes most of the residual gas, and improved and stabilized ion optics in the source can accommodate the elevated pressures. The separately pumped analyzer attains pressures of ca. 10^{-7} torr. The question of external standard introduction was elegantly solved by running a second capillary together with the sample gas stream capillary into the source via a two-hole ferrule. The reference gas is then introduced without a pressure pulse, as shown in Fig. 2 by changing the composition of the gas sipped by the reference capillary. By simply moving the position of the reference gas capillary higher and lower in the mixing tube, the IRMS sipper capillary either sees the reference gas (e.g. CO_2 + He flush gas), or only the He flush gas.

Further complications in the development of CF-IRMS are the limited dynamic range of the IRMS and the limited sipping rates permitted by the IRMS capillary and pumping capacities (ca. 0.2 to 0.5 ml.min^{-1}). The first means that the amount of the individual compounds prepared online must be tailored to the measurement limits of the IRMS. In part, this involves appropriate choice of sample amounts introduced, e.g., injected volume or weight, into the preparation system such as a GC or EA. The use of injector splitters and cold-on-column in GC's are widely used to divide down the amount

of material on column and eliminate excessive solvent peaks, but care must be exercised to avoid molecular and isotopic fractionation by this hardware. A post-combustion splitter offers additional control of the sample amounts by varying the proportion of gaseous sample sipped by the IRMS capillary. Most GC columns optimally require carrier gas flow rates higher than the IRMS sipper capillary, i.e., 1–5 ml•min^{-1} vs. 0.5 ml•min^{-1}. Again the splitter accommodates these flow rate differences.

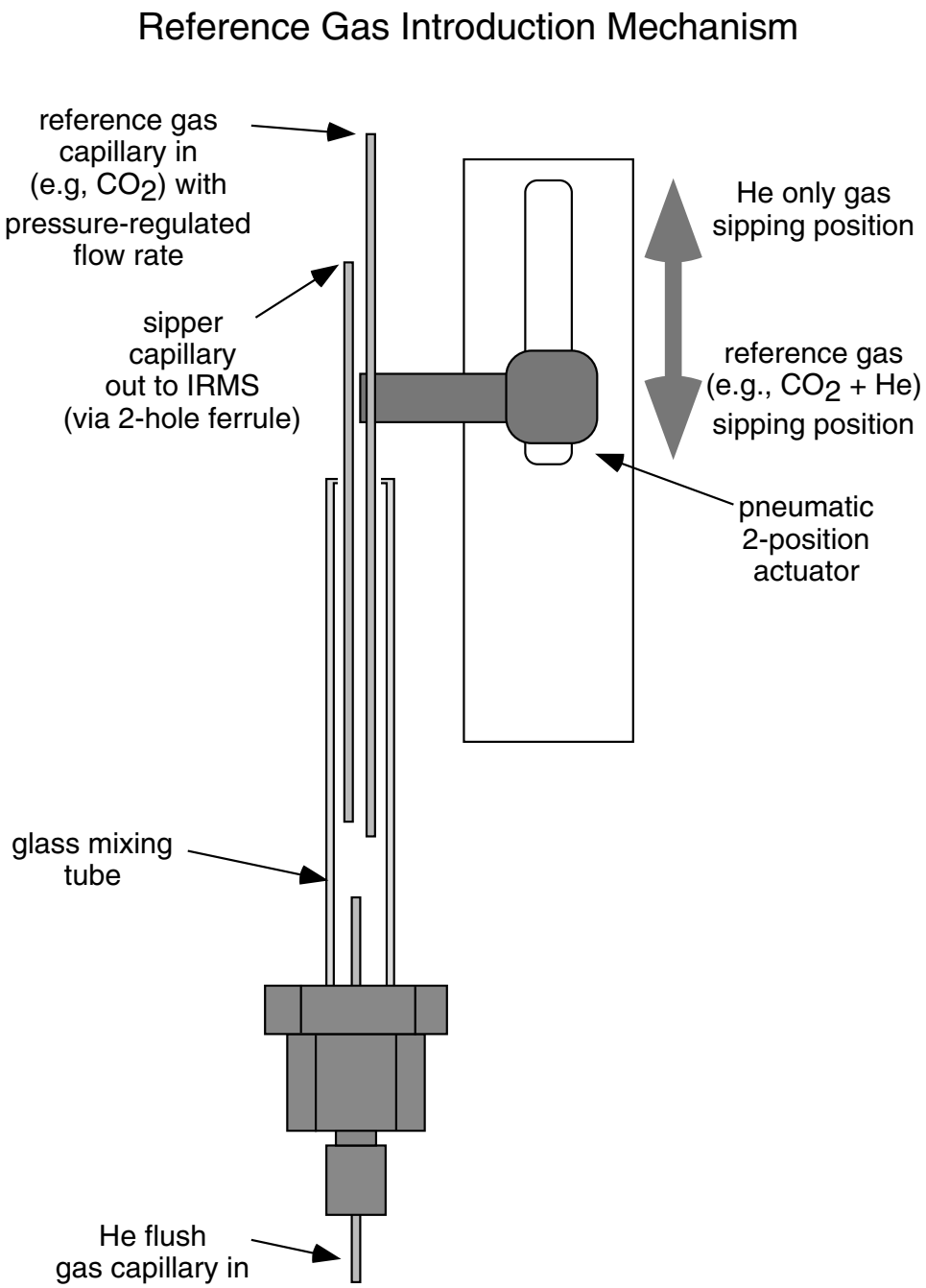

Fig. 2. Schematic of reference gas injection assemble for pulse-free introduction of standard gas into CF-IRMS.

As we gain experience with CF-IRMS several observations have been made to improve the performance of the instrument. These include the requirement for effective compound partitioning, efficient combustion, and effective water removal. Contamination by other sources of the measurement molecule, such as CO_2 can critically affect the measurements. For example, a rising background from increased GC column bleed at higher program temperatures can contribute unacceptable amounts of material that is co-combusted to CO_2 and measured. Above all, a major challenge with CF-IRMS is resolving leak problems in the system. These can provide endless sources of grief. The following is a treatment of some of these CF-IRMS analytical challenges, especially encountered with sub-nanomolar concentrations of compounds.

3. SPECIFIC CHALLENGES WITH CF-IRMS

3.1. Shot noise limit of IRMS

The precision of the isotope ratio measurements is largely controlled by the signal to noise ratio (S/N) of the ion currents for the individual detector cups. This includes the background or "dark" currents of the detectors and the associated electronics, but also the noise related to the shot of the ion beams noise, also termed the ion-statistical limit [5]. In a companion paper Merritt and Hayes, [6]) derived an expression for the maximum IRMS performance, namely,

$$(\sigma/R)^2 = (1 + R)^2/I_{eff}nNR \quad (3)$$

where $(\sigma/R)^2$ is the standard deviation at the shot noise limit, R as Eq. 1, I_{eff} is the ionization efficiency of the gas in question, n is the number of moles of sample gas inlet to the source, n is Avogadro's number. Equation 3 can be re-expressed in terms of the delta notation (Eq. 2) such that,

$$\sigma_\delta^2 = 2\times10^6(1 + R)^2/I_{eff}nNR \quad (4)$$

and simplified in terms of ^{44}i the integrated signal at the detector (^{44}A; Vsec) as:

$$\sigma_\delta^2 = 0.00446\ (1/^{44}A_{sa} + 1/^{44}A_{st}) \quad (5)$$

Fig. 3a shows the influence of shot noise on the isotope ratio as a function of the integrated ion count (peak area) for mass 44 (^{44}A; Vsec) according to Eq. 5. The peak area range is typical of that expected for most analyses. The solid line demarcates the envelope of the first standard deviation $\pm1\sigma$, and the dashed line the $\pm5\sigma$. Fig. 3b expands the scale of Fig. 3a, illustrating the limits of IRMS performance at low peak areas. The shot noise rises rapidly below peak areas of 0.5 Vsec, but for some scientific questions with large isotope excursions, the large error may still be acceptable.

3.2. Sample limits of CF-IRMS — Dilution experiments

To test the response and precision of the CF-IRMS at low sample quantities, we conducted experiments using an exponential dilution flask (Fig. 4). This device allows continuous dynamic dilution of a sample over the entire measurement range of the IRMS. The diluter was connected to a 6-port sample valve, such that samples from the flask could be taken at fixed intervals and analysed by CF-IRMS. The chosen analyte gas for the experiment was methane and the dilution gas was helium. At any point in time (t) the concentration of the analyte gas (C_t) can be described by Eq. 6:

$$C_t = C_i \exp(-tf/v) \quad (6)$$

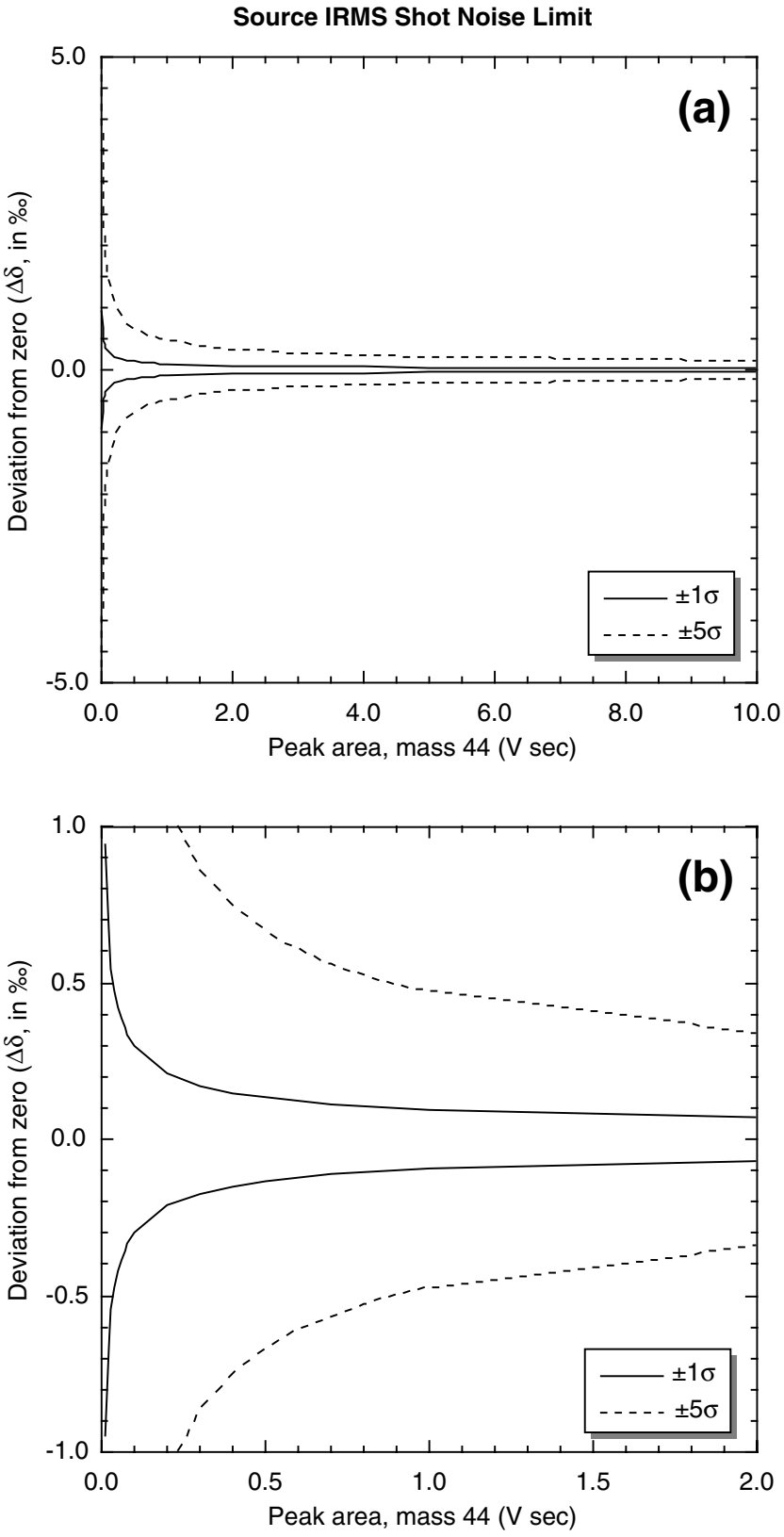

Fig. 3. Theoretical limit of precision of CF-IRMS measurement based on shot noise of IRMS (source, detector).

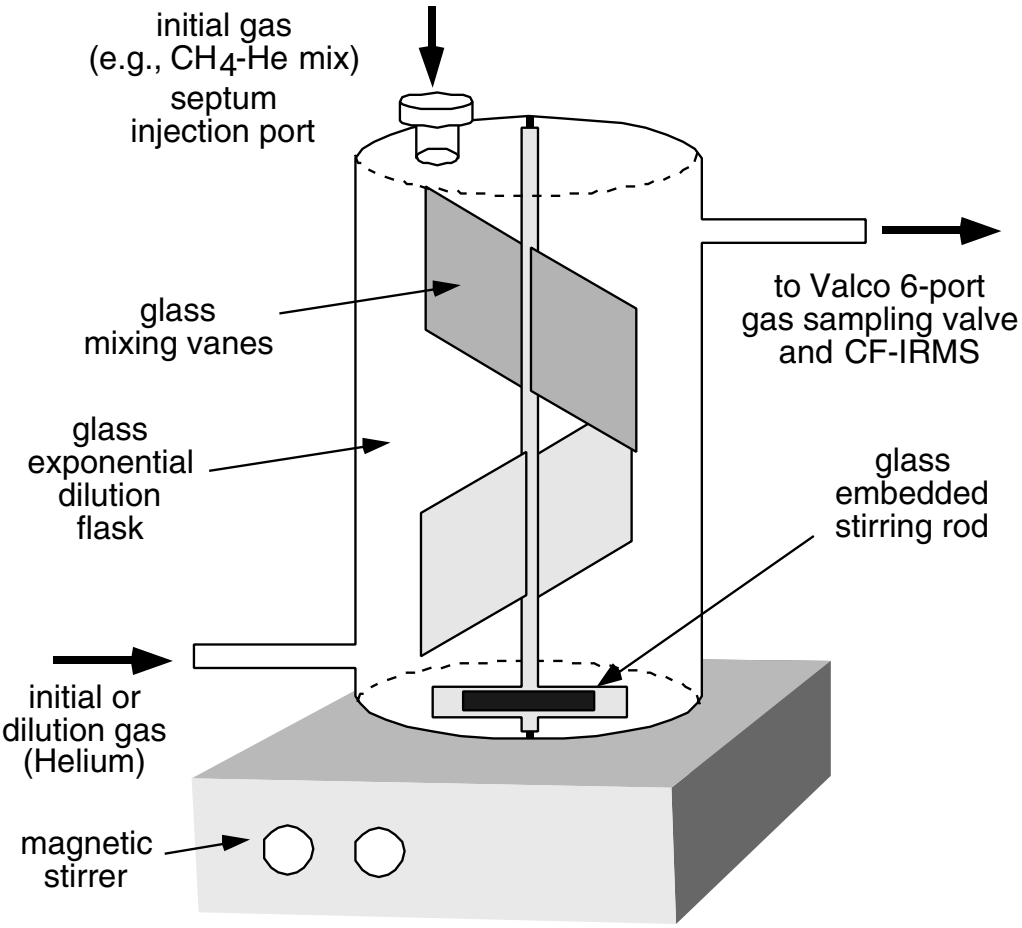

Fig. 4. Exponential dilution flask experimental set-up.

where f/v is the ratio of the helium flow rate to the volume of the flask. Fig. 5a shows the time-concentration curves for four experiments up to 185 min. Mass 44 peak areas for times shorter than 60 min are not reported because the CH_4 was still too concentrated for the IRMS (> 8V). The four runs, at 2 different micro-combustion tube temperatures (850°C and 900°C) are similar and show no systematic offsets. Values for f/v were consistently between 0.0405 and 0.0437, with r^2 values of 0.9999 to 0.9829. For reference the position of 80 picomoles of carbon is indicated on the plot. Fig. 5b is a scale expansion of Fig. 5a. By the end of the experiment, the amount of methane injected produced a peak area of 0.034 Vsec, or the equivalent of < 5 picomoles. Shown on the plot is the 80 picomole point that corresponds to the amount of carbon in methane in a 1 ml air sample, assuming a tropo-spheric mixing ratio of 1.8 ppmv.

The corresponding time series of the methane carbon isotope ratios is shown in Fig. 6. From 60 to 125 minutes, the carbon isotope ratios vary about a mean $\delta^{13}CH_4$ of -16.9‰ and fall within the 95% confidence interval calculated from all data and drawn on the plot (Panel b). Fig. 6 shows that at longer times, i.e. >125 min., the error in the carbon isotope ratios gradually increases, departing from the mean value. The peak area at 125 min. (see Fig. 5) is approximately 0.36 Vsec or ca. 42 picomoles C. for most purposes, this sets the lower measurement limit for $\delta^{13}CH_4$ determinations in a He mix. As discussed below, the precise $\delta^{13}CH_4$ measurements in real atmospheric samples is more challenging, i.e., CH_4 in air rather than CH_4 in He.

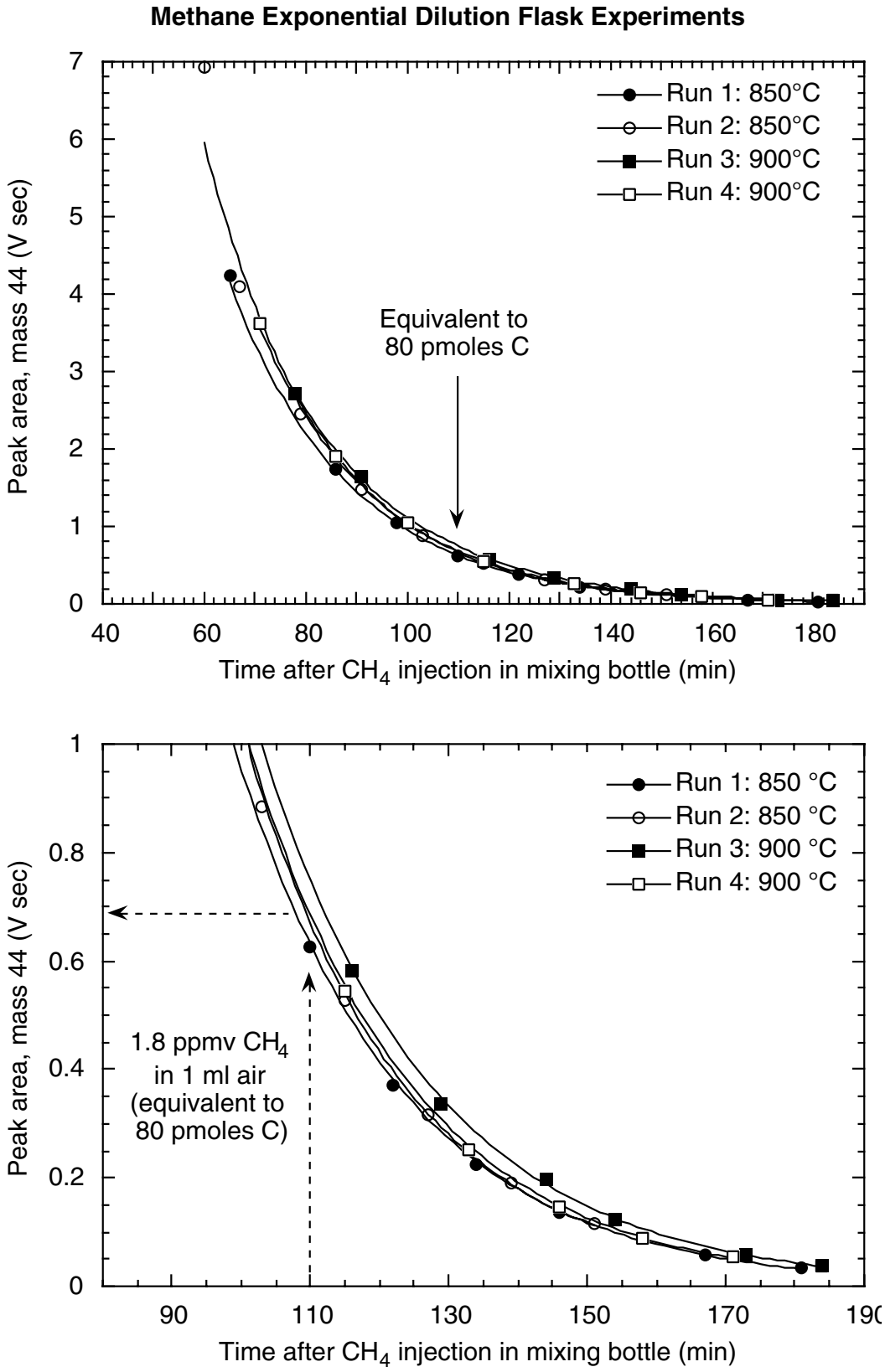

Fig. 5. Time series of methane concentration (peak area, mass 44 ion) in exponential dilution flask experiment.

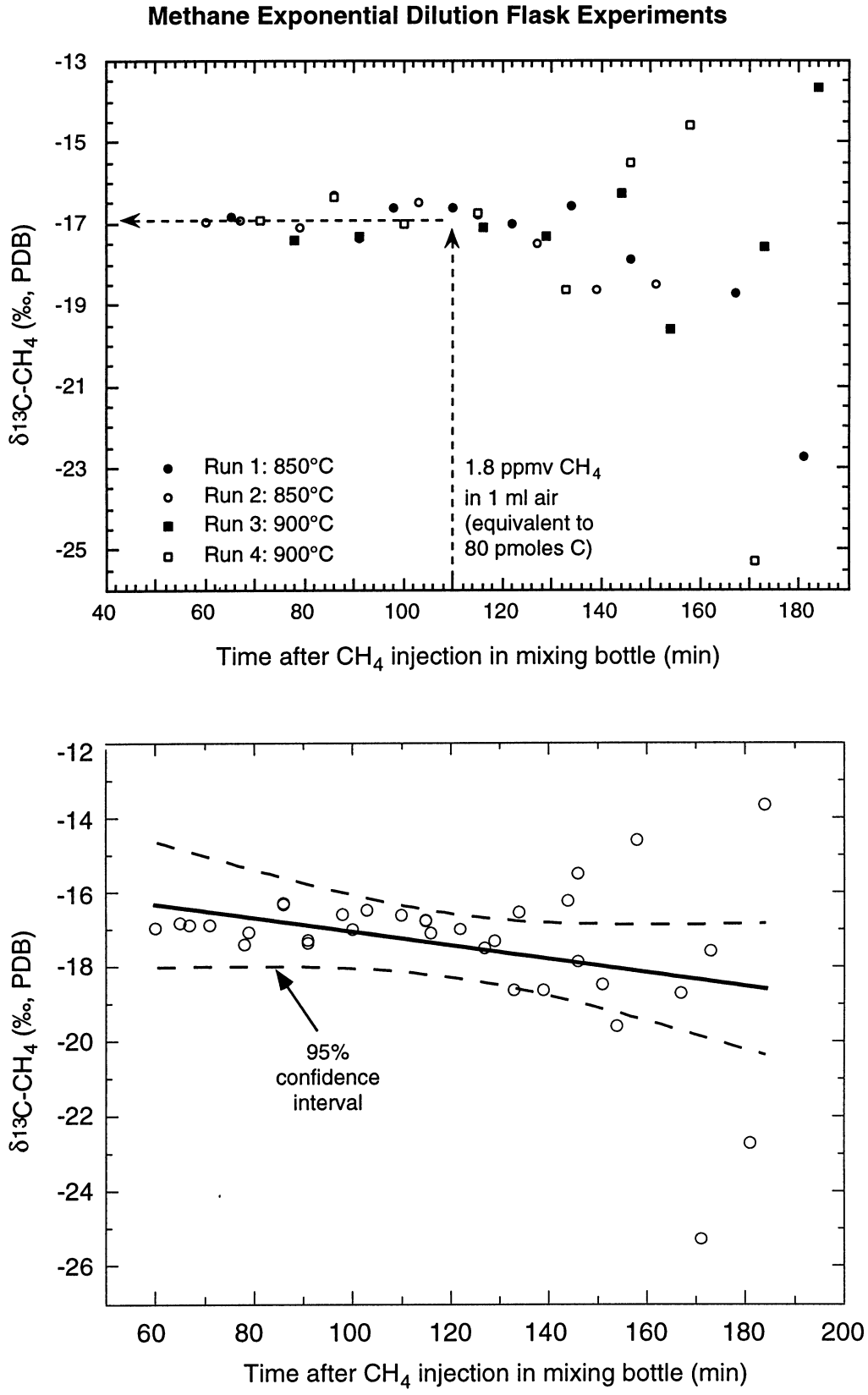

Fig. 6. Time series of methane carbon isotope ratio ($^{13}C/^{12}C$) in exponential dilution flask experiment.

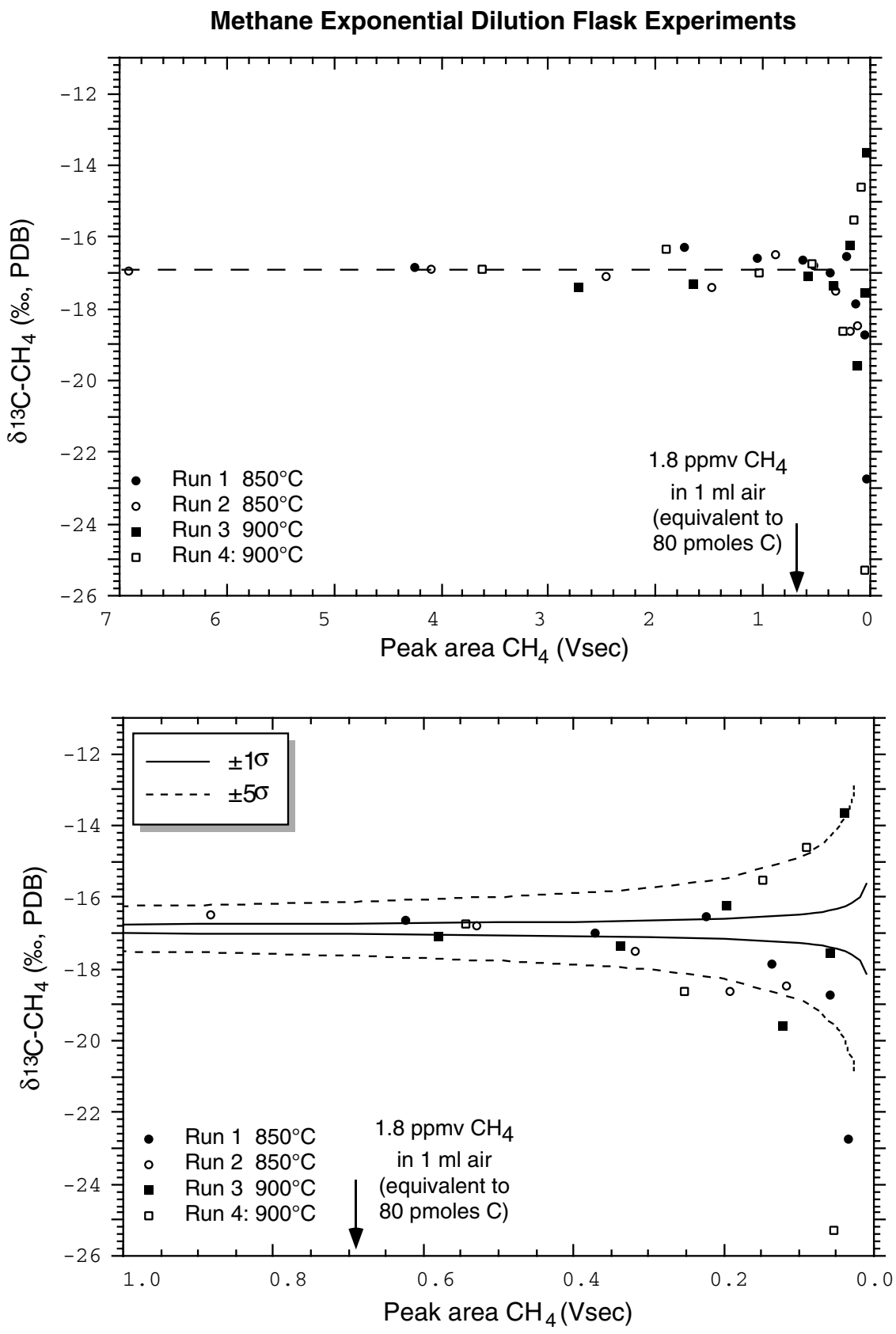

Fig. 7. Methane carbon isotope ratio ($^{13}C/^{12}C$) vs. peak area (mass 44) in exponential dilution flask experiment.

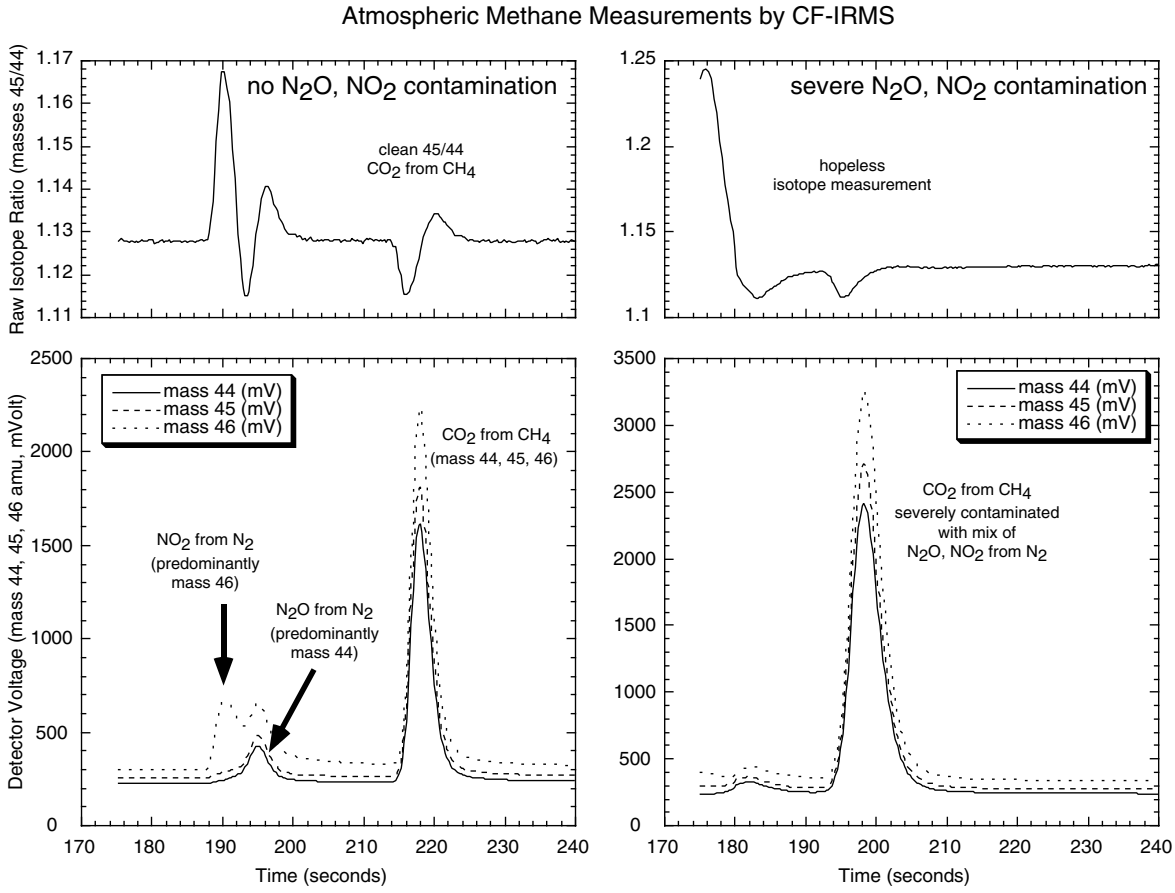

Fig. 8. Demonstration of the effect of air N_2 isobaric contamination on GC partitioning and stable carbon isotope measurements on atmospheric samples.

The relationship between peak area and $\delta^{13}CH_4$ from the dilution experiments is shown in Fig. 7a. The mean $\delta^{13}CH_4$ value of –16.9‰ is drawn as a dashed line. In Fig. 7b, the expanded scale, the shot noise shown in Fig. 3 is superimposed. At higher concentrations the variances from the mean $\delta^{13}CH_4$ approach the expected performance limited by ±1σ shot noise. However, at lower concentrations (< 0.5 Vsec) the error in $\delta^{13}CH_4$ is larger; around the ±5σ shot noise limit.

3.3. Influence of nitrogen on $\delta^{13}CH_4$ measurements of atmospheric CH_4

As discussed above, there is sufficient carbon in 80 picomoles of methane (ca. 1.8 nl CH_4 in 1 ml air) for an $\delta^{13}CH_4$ measurement by CF-IRMS. However, the nitrogen (N_2) in air presents a serious complication. If the large amounts of N_2 in the air sample are co-injected into the GC and subsequently oxidized in the microcombustion tube, then several things occur. The first is that the partitioning capability of the GC column is degraded, such that N_2 and CH_4 are poorly separated. As shown in Fig. 8b, this loss of column performance due to overloading with N_2 shortens the retention time of methane from ca. 220 sec. to 200 sec. Secondly, a small proportion of the N_2 leaving the micro-combustion tube is oxidized to NO_2 (predominantly mass 46) and N_2O (predominantly mass 44), which isobarically contaminates the CO_2 masses 44, 45 and 46 that are derived from the CH_4 combustion. Thus it is critical that the N_2 in air be removed such that it does not overlap or tail into the CH_4 peak. We perform this by cryo-prefocussing the air sample on a sample loop to effectively remove O_2 and N_2 from the gas prior to inletting in the GC (Fig. 1).

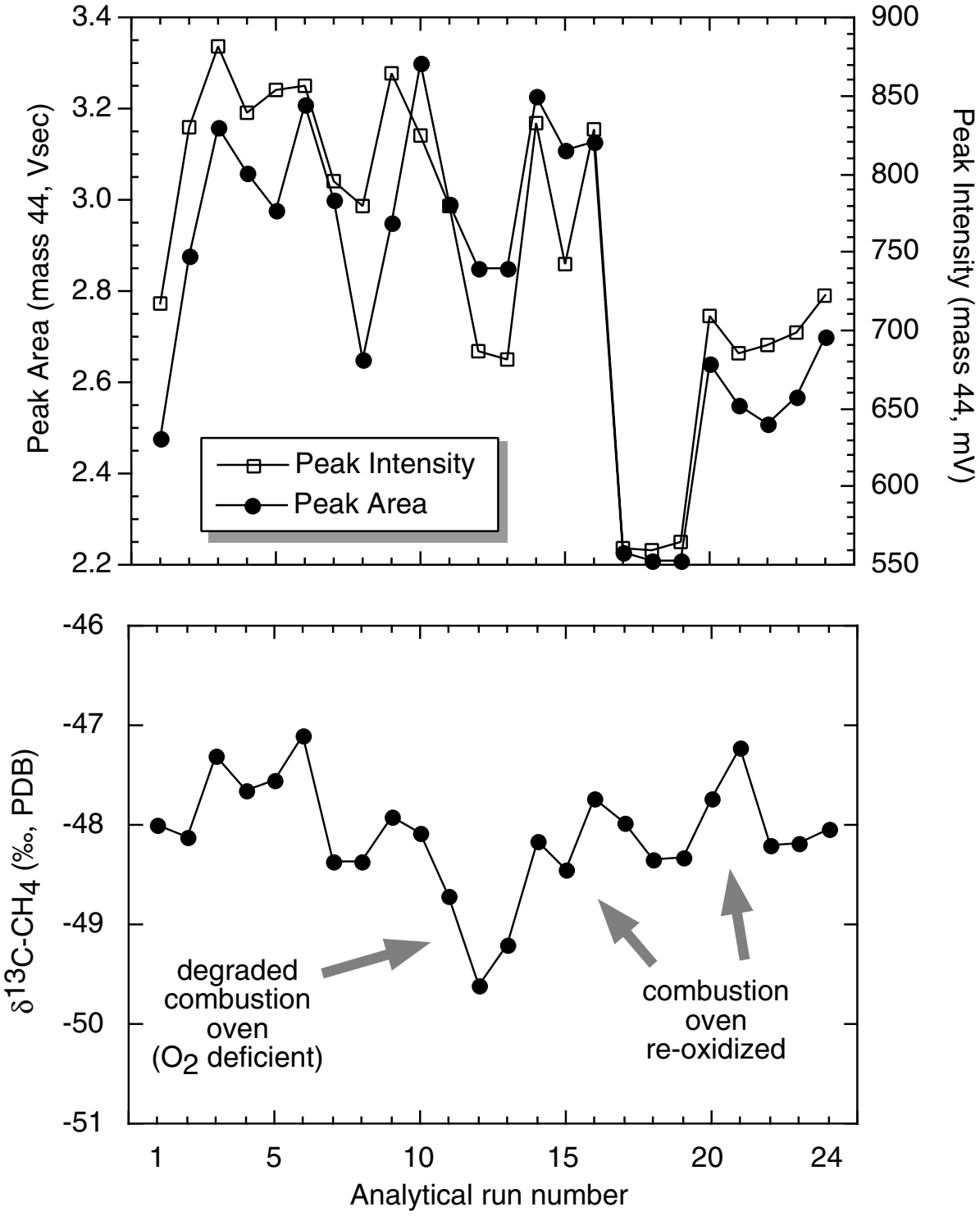

Fig. 9. Effects of microcombustion oven performance on peak area (mass 44 and carbon isotope ratio of atmospheric methane.

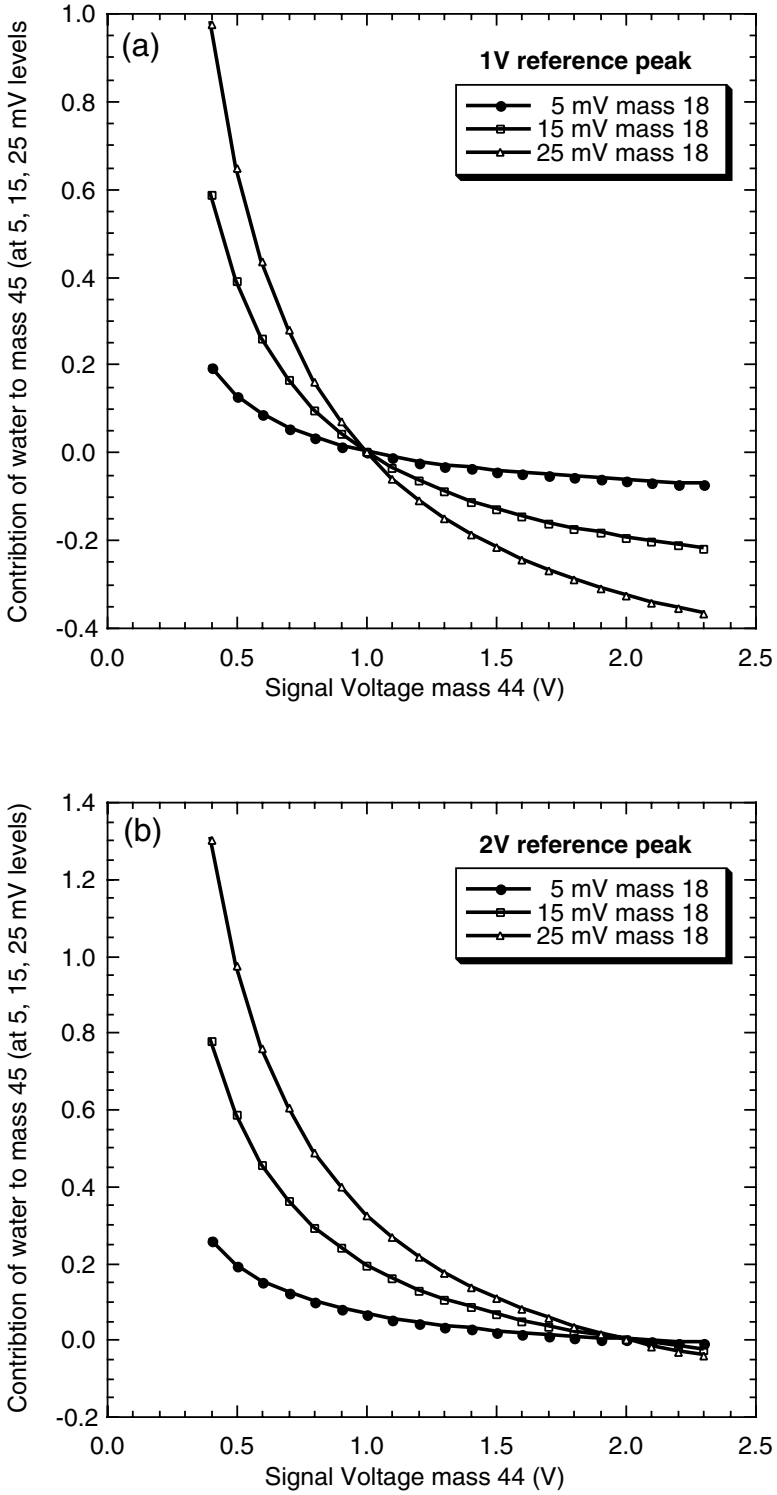

Fig. 10. Influence of water in the IRMS source on carbon isotope ratio. Panel (a) is the isotope shift on a sample with varying intensity at a fixed water standard content (mass 18 5, 15 and 25 mV) and reference gas intensity (1V on mass 44). Panel (b) is the same except the reference gas intensity is 2V (mass 44).

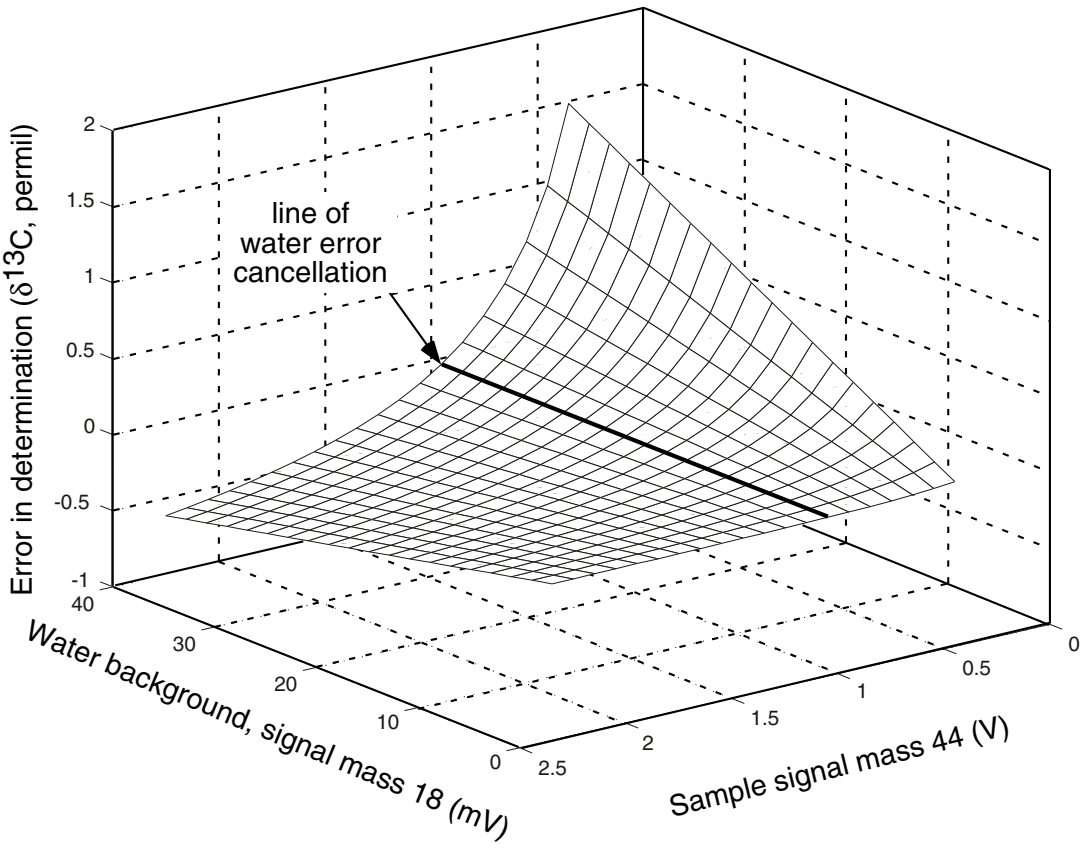

Fig. 11. 3D plot showing the influence of water in the IRMS source on carbon isotope ratio. In comparison with Fig. 10, the surface is created by allowing sample intensity and reference gas (1V mass 44) water content to vary continuously.

Fig. 8 is an example of a $\delta^{13}CH_4$ measurement of atmospheric methane in air by CF-IRMS. In Fig. 8a most of the air N_2 has been removed. The peaks of masses 44, 45, and 46 associated with the methane are cleanly partitioned with baseline separation from the preceding nitrogen oxides. The 45/44 trace in panel a clearly demonstrate the potential for contamination by nitrogen oxides if they overlap with those of the CO_2 from CH_4 combustion. Fig. 8b shows the effects of the nitrogen oxide contamination on the 45/44 of the methane-CO_2. Unlike normal GC data integration procedures, it is not possible to deconvolute a contaminated peak. This is discussed in detail below.

3.4. Influence of microcombustion tube performance on $\delta^{13}CH_4$ measurements

The conversion of organic compounds to CO_2 for 45/44 isotope measurements by CF-IRMS typically utilizes a microcombustion tube in an oven heated to temperatures ranging from 850°C to 1100°C. These capillary tubes are filled with strands of twisted metal wire, such as Cu, Pt and Ni. These wires serve two purposes, namely (1) providing a source of oxygen to oxidize the carbon-bearing compounds to CO_2 and (2) acting as a catalyst and reaction substrate to ensure quantitative combustion. The temperature of the tube is important because of O_2 streaming. At a too low temperature there is insufficient O_2 and incomplete oxidation results. At too high temperatures, the O_2 streams off the metal oxide too quickly and is depleted before required, again leading to incomplete oxidation. Because of this streaming, the choice of metal oxides in the tube dictates the operating temperatures of the microcombustion tube. Fig. 9 shows the effect of O_2 depletion in the micro-combustion tube on $\delta^{13}CH_4$ measurements by CF-IRMS. As O_2 depletes the carbon isotope ratio of methane becomes ^{13}C depleted. Re-oxidation of the oven returns the combustion performance.

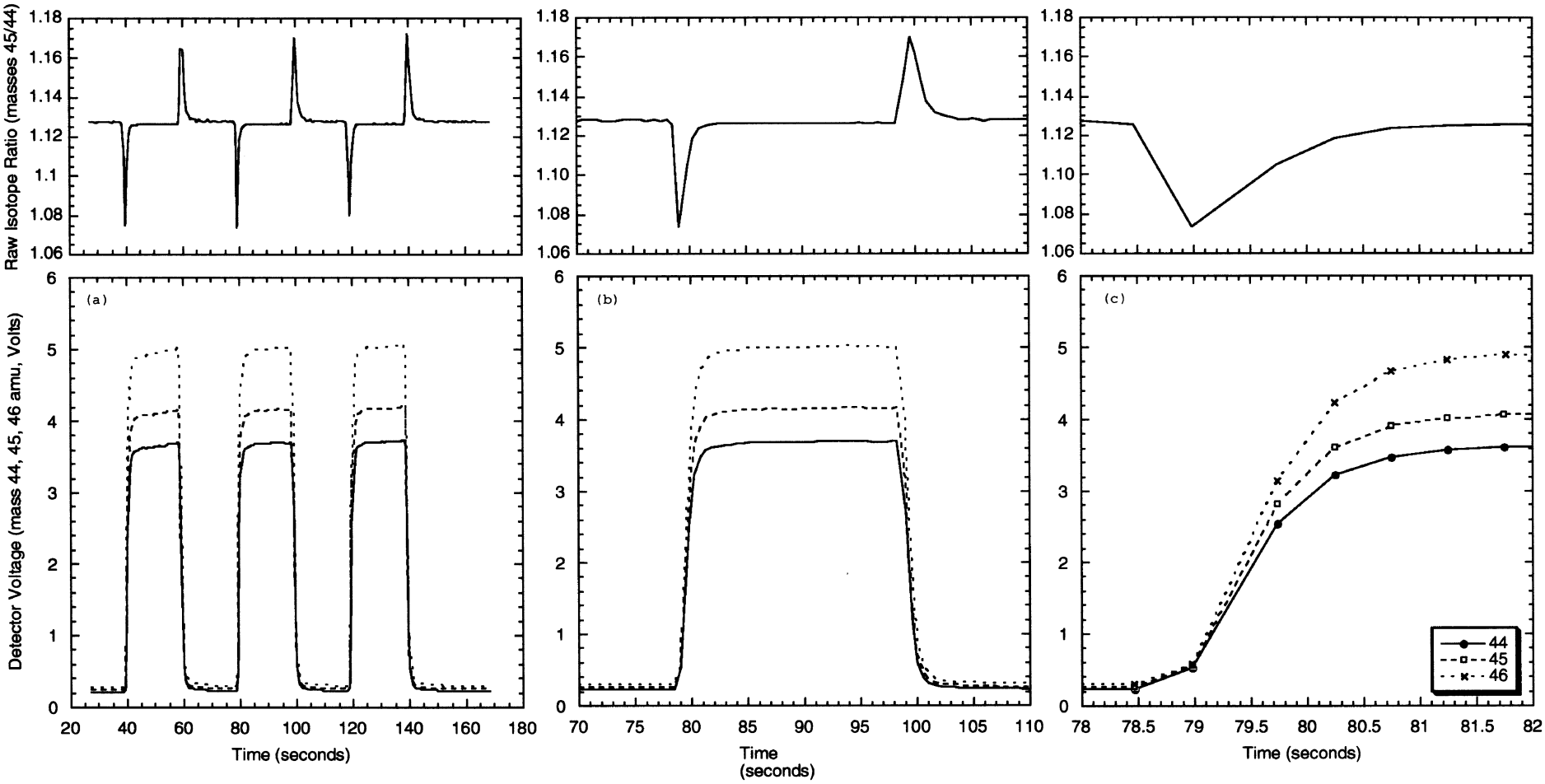

Fig. 12. Example using reference gas injection (see Fig. 2) of CO_2 showing 3D integration requirement of CF-IRMS. Isotope effects operating on the gases can partition them such that the peaks are not homogeneous with respect to masses 44, 45 and 46 for CO_2.

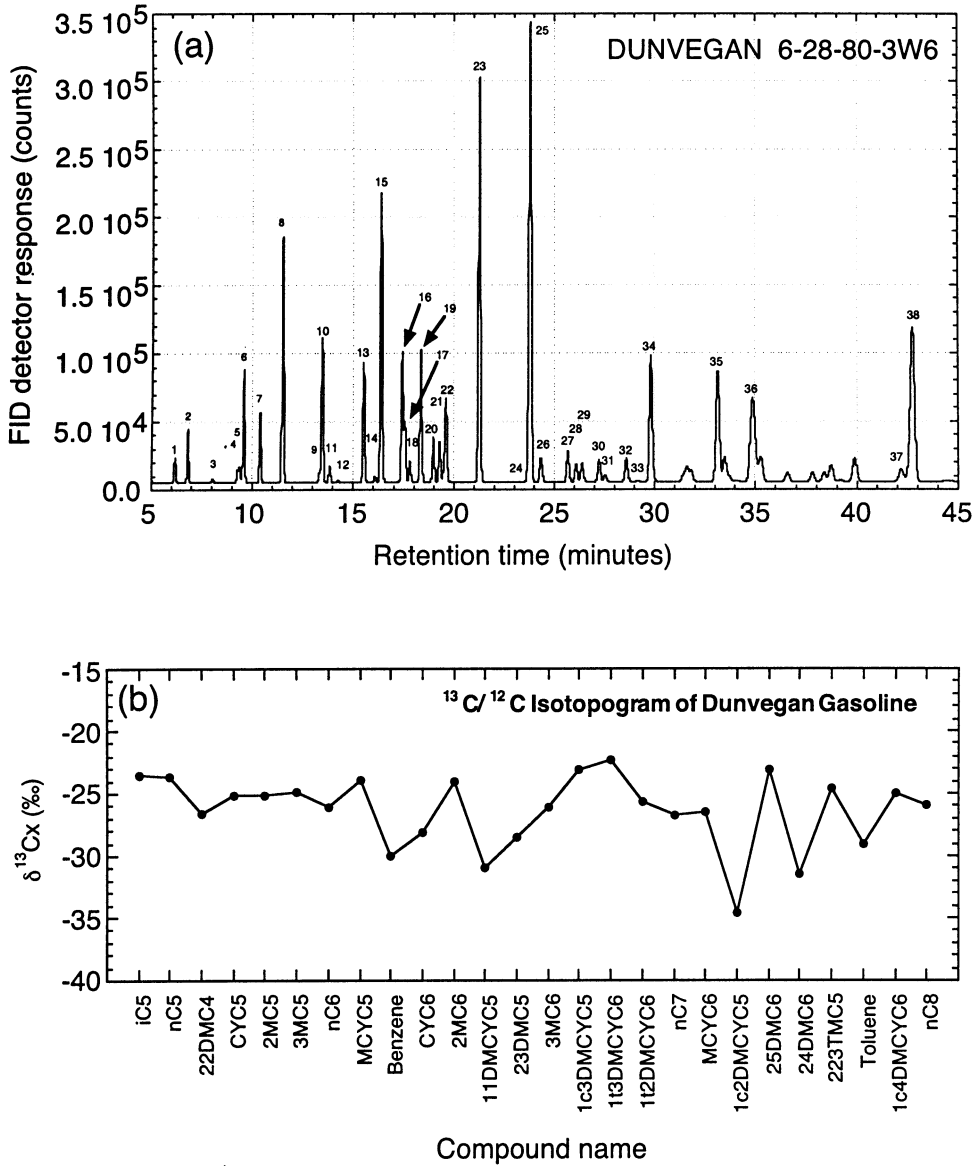

Fig. 13. Example of CF-IRMS measurement of individual hydrocarbons in the gasoline range fraction of a Albertan oil. Panel (a) shows the GC-FID trace of the sample (see Table II for peak ssignments). Panel (b) is the corresponding $\delta^{13}C_x$ "Isotopogram" for the sample.

B. Dias (USGS, pers. comm.) has produced a modification to the CF-IRMS system that introduces a constant "leak" of He/O_2 into the gas stream between the GC column and the microcombustion tube. This steady, low level stream of O_2 (50 mV mass 16) ensures that sufficient O_2 pressure is available in the microcombustion tube, especially for longer GC runs with high O_2 demand. The leak also obviates the need to re-oxidize the combustion tube, thereby saving considerable amounts of time.

Other improvements include the use of a capillary column as the microcombustion oven [7, 8]. In this configuration, the capillary extends from the backflush/O_2 make-up connection through to the water trap. This single tube design eliminates several connections that are notorious for leaks. However, our experience with the capillary tube approach is that the 0.32 mm fused silica tube becomes very brittle with time and is easily subject to breakage. Recently, we have moved to a silica-lined stainless steel combustion tube. This has proven to have the advantages of the fused silica capillary column combined with the robustness and ease of sealing of a steel column.

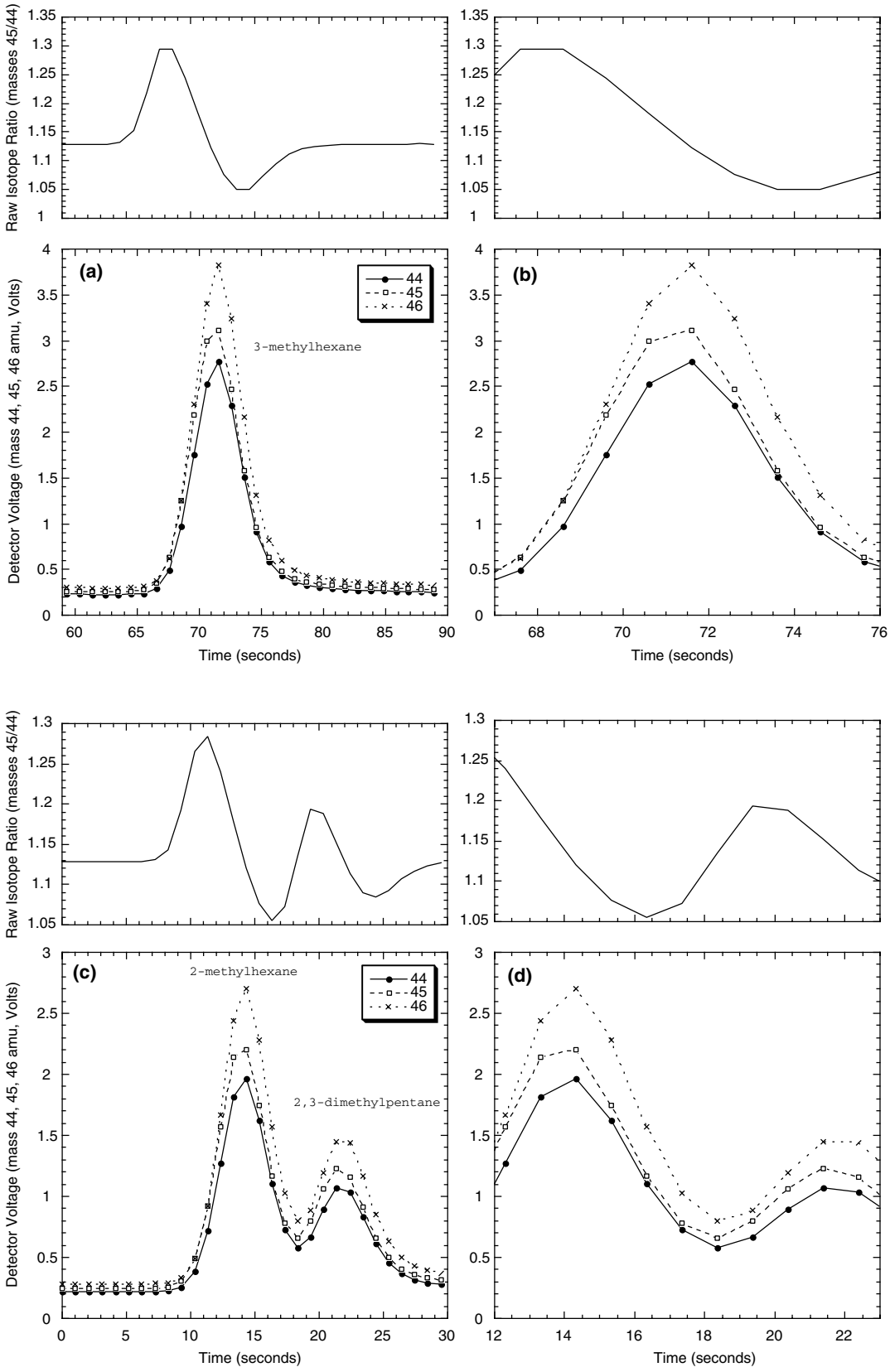

Fig. 14. Example demonstrating the importance of compound (peak) separation on carbone isotope ratio. The compounds are indicated in Fig. 13 (peaks 16, 17 and 19). Panel (a) shows a well-separated compound (baseline separation). Panel (b) is a scale expansion of (a). Panels (c) and (d) are an example of two poorly-separated peaks. The resultant carbon isotope ratios in (c, d) are highly unreliable.

3.5. Effects of water on CF-IRMS measurements

Water in the source of the IRMS can produce systematic errors during stable carbon isotope measurements, if not maintained at a constant, low level. By transferring hydrogen to CO_2 in the ion source via the reaction

$$CO_2^+ + H_2O \rightarrow HCO_2^+ + OH \quad (7)$$

and similar reactions, water causes changes in the 45/44 and the 46/44 ion currents, e.g., $H^{12}C^{16}O_2^+$ = mass 45 and $H^{13}C^{16}O_2^+$ = mass 46 [9, 3]. The consequences of these changes are an over-estimation of ^{13}C and a similar over-compensation in the ^{17}O correction.

A quantification of water induced errors in the determination of carbon isotopes was made by Leckrone and Hayes [10]. They derived a general relationship between the observed error in δ and the ratio of water to CO_2. This shows that the effects on the observed ion current ratios are such that the systematic errors on the true isotope ratios can be either positive or negative. The sign and magnitude of the offset error depends on the differential in the ion currents between the sample and the reference peaks.
For example, if the sample and reference peaks are identical and the water background is the same for both, then the water-based errors are identical and cancel out. However, if for example the area of the sample peak is smaller than the reference peak, i.e., the mass 44 ion currents of the two are different, then the resulting systematic error in the determination of the sample value will be positive, even though the water background remained the same for both. This contamination is manifested as a heavier bias or apparent mass 45 and 46 contributions to the CO_2. Conversely, a sample peak that is greater than the reference peak will produce a negative error with a bias in $\delta^{13}C$ towards a ^{12}C-enriched value.

Fig. 10 illustrates the calculated errors to the 45/44 ratio of the sample in δ-notation as a function of the sample peak size (mass 44, V) and level of water background. Curves are plotted in Fig. 10 for three water background levels of 5, 15 and 25 mV measured on mass 18 ($^1H_2{}^{16}O$). The reference peak level is fixed at mass 44 = 1V (Fig. 10a) and mass 44 = 2V (Fig. 10b), respectively. All of the curves intersect where the sample and reference peak intensities are the same. At this intersection point, the water-induced error on $\delta^{13}C$ is balanced and hence zeros. These plots demonstrate the negative and positive errors in $\delta^{13}C$ depending on the sizes of the sample relative to the reference peaks. At lower water contributions, e.g., 5V vs. 15V mass 18, the error becomes less significant. Fig. 11 shows the continuous 3D surface describing the $\delta^{13}C$ error as a function of changing signal intensities of water (mass 18) and the sample (mass 44), using a reference signal intensities of 1V (mass 44). Again, at the points in the plot where sample and reference intensities are matched, the water errors cancel as indicated by the bold line.

Thus, matching the intensities of the reference peak to the sample peak can be used to minimize the influence of the water-based error.

There are additional ways to control water-induced errors. For example, it is possible to reduce the systematic errors by changing the potential of the extraction lens and thereby shorten the residence time of the molecules in the ion source. A shorter residence time will lead to a lower number of collisions between water and CO_2 molecules. Hence, there will be less HCO_2^+ formed and subsequently a reduced error. Unfortunately, lowering the extraction plate potential will also decrease the sensitivity of the instrument, making this approach less optimal.

The best way to avoid the influence of water background without compromising the sensitivity of the instrument is to use an efficient water trap before entering the source of the IRMS. Currently we use a trap constructed with a 60–100 cm long Nafion™ tube inserted into 1/16-inch glass lined steel tubing with a 1/16-inch Swagelok™ stainless steel T-piece in either end. Approximately 30–40 cm of the

downstream end the trap is held at -20°C in an ethylene glycol/dry ice slurry. This configuration is sufficient to keep water background at a level where the systematic error should be reduced to less than 0.1 per mil.

3.5. Influence of isotope effects on reference gas measurements by CF-IRMS

Isotope effects including diffusion velocity and vapour pressure differences are operative during the online preparation stages. To illustrate this, Fig. 12 shows the introduction of three CO_2 reference gas pulses via the reference gas injector. Panel (a) is the time series of intensities for each of the 44, 45 and 46 masses of the three pulses. As the CO_2 capillary is lowered and raised in the mixing tube (Fig. 2) the ion currents for the three masses rise and fall. Comparison with the corresponding raw 45/44 ratio in Fig. 2 demonstrates that $^{12}CO_2$ arrives marginally earlier than $^{13}CO_2$. Once the reference gas is turned off, the $^{13}CO_2$ ion current persists longer, giving rise to the distinctive 45/44 ratio swing.

Panels (b) and (c) in Fig. 12 are time scale expansions to zoom in on the middle CO_2 reference pulse and the leading edge of this pulse, respectively. In this case the time shift between masses 44, 45, and 46 are not readily seen merely by looking at the intensity records, but obvious in the corresponding45/44 ratio. This example points out the need for extreme care in making isotope ratio measurements by CF-IRMS. Essentially, a $\delta^{13}C$ determination requires careful 3D integration, i.e., contemporaneous integration of three peaks (e.g., masses 44, 45, and 46). In the case of the reference gas, the 45/44 reading can be taken on the plateau region in the middle of the pulse. This avoids the front and tail problems.

3.6. Influence of isotope effects on sample gas measurements by CF-IRMS

Unfortunately, using a plateau region in a peak is not an option for the sample gas measurements, due to limited sample quantities and hence peak width. Furthermore, as gases such as CO_2 or CH_4 travel along the GC column, isotope effects discriminate between isotope species. In these cases, $^{12}CO_2$ and $^{12}CH_4$ elute from the column measurably faster than $^{13}CO_2$ and $^{13}CH_4$. Thus a compound peak is isotopically heterogeneous upon elution from the column. This isotope time lag is observed in the raw 45/44 trace of CH_4 in panel (a) of Fig. 8. This means that the $\delta^{13}CH_4$ at any point across the CH_4 peak is different, depending on the mix of mass 44, 45 and 46 contributions.

This factor is one of the greatest challenges in the use of CF-IRMS. Failure to completely and reliably integrate the three ion peaks in the case of CO_2, results in large errors. Certainly the individual baseline corrections required for each mass 44, 45 and 46 peak in this 3D integration dramatically complicate the measurements.

The isotopic heterogeneity across a peak means that as a general rule baseline separations are needed between the individual compound peaks eluting off the GC columns. The carbon isotope ratios of co-eluting or shoulder peaks are difficult, if not impossible, to determine by deconvolution of the three separate masses.

Fig. 13a shows FID gas chromatogram of the gasoline range fraction of a Dunvegan oil from the Western Canada Sedimentary Basin [11]. The chromatogram illustrates the poor separation between the compounds 2-methylhexane (compound peak 16) and 2,3-dimethylpentane (compound peak 17).
In contrast 3-methylhexane (compound peak 19) is well separated from the surrounding peaks. In order to create the “isotopogram” in Fig. 13b, good separations are essential.

Fig. 14 illustrates the importance of baseline separation. Panel (a) and the scale expansion panel (b) show a cleanly resolved 3-methylhexane peak with a good 45/44 trace. Panels (c) and (d) (Fig. 14) show the two overlapping 2-methylhexane and 2,3-dimethylpentane peaks. The resultant 45/44 trace

indicates the uncertain mixture of the two and the potential for large errors in the carbon isotope estimates.

4. CONCLUSIONS

Continuous Flow-Isotope Ratio Mass Spectrometry (CF-IRMS) is an exciting analytical development that permits isotope measurements on individual compounds in complex mixtures. This opens up tremendous opportunities to understand isotope characteristics and systematics at a much more refined level than available through bulk measurements. Although pioneered by geochemists, application of CF-IRMS is now extending rapidly to other fields.

Significant differences in the inlet configuration between the conventional dual inlet and CF-IRMS mean that new challenges must be met to make reliable measurements. In particular internal and external standards are critical to test and calibrate the instruments, but also to allow the interlaboratory exchange of information.

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CF-TECHNIQUES APPLIED TO INORGANIC COMPOUNDS

TAKING THE ATMOSPHERE'S PULSE: THE APPLICATION OF GC-IRMS TO STABLE ISOTOPES IN ATMOSPHERIC TRACE GASES

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Abstract. Since the industrial revolution, the abundance of many atmospheric trace gases has changed significantly. This is of concern because many of these trace species play a fundamental role in determining physical and chemical properties of the atmosphere important for maintaining life on earth. The impacts of the changes have been studied by a combination of analytical and theoretical modelling techniques. Stable isotope measurements made by conventional dual inlet IRMS for example, have provided valuable constraints on the budgets and removal mechanisms of key atmospheric trace gases. Unfortunately, in most cases, the application of these methods has been limited, because large air samples and cumbersome off line processing techniques are required to pre-concentrate enough gas for analysis. GC-IRMS offers a very attractive alternative because it combines on line processing with air sample size requirements typically 1000 times less than used in conventional techniques. In this article we focus on the requirements imposed on GC-IRMS by some of the current applications in atmospheric trace gas research. In addition, we examine some of the analytical and calibration aspects of the method applied to this kind of work. We finish with a summary of some of the comparative advantages and disadvantages of the GC-IRMS technique and some suggestions for future research using the method applied to specific atmospheric trace gases.

1. INTRODUCTION

The most abundant gases in the dry atmosphere are nitrogen, oxygen and noble gases. Their relative mixing ratios are remarkably constant throughout the atmosphere and they are not very reactive. In addition to the major gases, there is another class of gases, known as "trace gases". These are usually characterised by high reactivity and variability and make up less than 0.05% of the atmosphere's volume ranging from less than a few parts in 10^{15} to a few parts in 10^{4}. Despite this tiny fraction however, trace gases play a pivotal role in the radiative, physical and chemical properties of the atmosphere and provide many of the conditions essential for sustaining life as we know it.

Since the industrial revolution, significant variations in the abundance of trace gases, have been caused by various agricultural and industrial activities. These changes have led to major problems in both world wide and regional atmospheric chemistry including ozone depletion, photochemical smog and acid rain formation as well as implications for global climate change. Determining the effects of changing atmospheric trace gas concentrations and forecasting future perturbations to the properties of the atmosphere are amongst the most important environmental challenges facing humankind today.

How are such global problems addressed? First of all the magnitude of the problems have to be determined. This involves making high precision temporal and spatial mixing ratio measurements of the trace gases believed to be responsible for changing the properties of the atmosphere. For example the first high precision measurements of atmospheric CO_2, the principal anthropogenic "greenhouse gas", were begun in the late 1950's on a mountaintop in Hawaii [1]. Since that time many cooperative international atmospheric trace gas measurement programmes have been initiated because no single country can provide the resources or logistics required to provide the data needed. The data is currently used in mathematical models predicting the future state of the atmosphere based on assumed trace gas "emission" scenarios of trace gases. Based on this kind of work the very successful Montreal protocol aimed at limiting emissions of the CFCs believed to be involved in stratospheric ozone depletion, was ratified in 1987 and has since been signed by 169 countries.

More recently mixing ratio measurements of trace gases such as CO_2, CH_4 and CO have been complemented by determinations of their stable isotopic composition. These data have provided valuable additional constraints on the budgets and removal mechanisms of key atmospheric trace gases for example [2–4]. Most of these results have been provided by isotope ratio mass spectrometry (IRMS) operating in the traditional dual-inlet mode. Very recent developments, however, indicate that the coupled techniques of gas chromatography and IRMS (GC-IRMS) may prove to be a valuable technique in atmospheric trace gas work. Here we focus on the requirements imposed on GC-IRMS by some of the current applications in atmospheric trace gas research. In addition, we examine some of the analytical and calibration aspects of the method applied to this kind of work. We finish with a summary of some of the comparative advantages and disadvantages of the GC-IRMS technique and some suggestions for future research using the method applied to specific trace gases.

2. ISOTOPE RATIO MASS SPECTROMETRY

2.1. Requirements for IRMS in atmospheric trace gas research

The analysis of stable isotopes in atmospheric trace gases places high demands on IRMS for two main reasons:

(1) As mentioned in section 1, atmospheric trace gas concentrations are very low. Consequently non-fractionating enrichment and extraction techniques, for example cryo-focussing, are required to enrich the gases to levels that can be measured by traditional dual inlet IRMS. This in turn places demands on air sampling techniques because large air samples require specialised collection methods involving the use of clean compressors and pumps and are difficult to transport, store and manage.

(2) To provide meaningful data for use in mathematical models describing the implications of changes in atmospheric trace gases, high precision measurements are required. For example to be able to use $\delta^{13}C$ measurements to resolve seasonal cycles and small latitudinal changes in background atmospheric methane, external analytical precisions (1 sigma) of at least 0.05‰ are needed [5]. For atmospheric CO_2 analyses, the requirements are even more stringent, 0.01‰ and 0.02‰ for $\delta^{13}C$ and $\delta^{18}O$ respectively. Such requirements for high precision place extreme demands on the best IRMS equipment commercially available. Also, as mentioned above,
the situation is exacerbated further by the fact that atmospheric mixing ratios are low.

2.2. Use of traditional dual-inlet IRMS in atmospheric trace gas research

In dual-inlet IRMS, variable volume reservoirs are used to introduce sample and reference gases at closely matched flow rates through capillaries into an electron impact ion source. During analysis, the inlet system is repeatedly switched between sample and reference gases, and very small differences in isotope ratios, better than 1 part in 10^5, can be precisely determined. In order to maintain precision and minimise non-linearity, constant pressure and thus uniform flow rates through each capillary must be maintained on each side of the IRMS dual-inlet system. This requires the presence of a relatively large amount of sample and reference gas in the reservoirs. Most of this is not used in the analysis but serves only to maintain the pressure required to keep inlet flows constant and in the "viscous" or turbulent linear flow region of the capillaries.

One of the most effective uses of this technique has been the determination of $\delta^{13}C$ and $\delta^{18}O$ in atmospheric CO_2. For example Francey et al. [6] demonstrated that, with very careful calibration and techniques, small seasonal cycles and long term secular changes in $\delta^{13}C$ in atmospheric CO_2 can be used to estimate changes in oceanic and terrestrial carbon uptake. This work shows that $\delta^{13}C$ in atmospheric CO_2 is becoming more depleted due to fossil fuel CO_2 emissions at an average rate of about $0.03‰.yr^{-1}$ over the period 1982 to 1993. Over this period average atmospheric CO_2 mixing

ratios were about 350 micromole.mole^{-1} and about 50 mls of a 2 L air sample was used in the analysis. This is the smallest amount of air used in a dual inlet IRMS analysis of the stable isotopes in atmospheric CO_2 however. Other programmes involved with similar measurements use far more air, for example 3 L at Scripps Institution of Oceanography (C.D. Keeling, personal communication) $\delta^{13}C$ in atmospheric methane has also been analysed by dual inlet IRMS. Because atmospheric methane mixing ratios are low, typically 1.7 micromole.mole^{-1}, elaborate offline pre-concentration systems are required to quantitatively extract the methane and convert it to CO_2 (without isotopic fractionation!) [7]. Despite these difficulties however, high precision $\delta^{13}C$ in methane data (1 sigma = 0.02‰) have been used to determine changes in source strengths of atmospheric methane [5, 8]. To perform these measurements large air samples are required, typically 50 L.

Stable isotopes in atmospheric carbon monoxide have also been determined using techniques developed by Brenninkmeijer et al. [9]. Because carbon monoxide is a short lived relatively reactive species, this work has had a major influence on atmospheric chemistry. Atmospheric carbon monoxide mixing ratios are very low ranging from about 50 nanomole.mole^{-1} in the southern hemisphere, to about 200 nanomole.mole^{-1} in the background northern hemisphere. Air sample sizes for the stable isotope measurement range from about 300 to 1000 L.

2.3. Capabilities of standard GC-IRMS in trace gas research

Dual-inlet IRMS for trace gas analysis has the large disadvantage that, prior to analysis, the gas of interest must be quantitatively extracted from a large volume of air, the size of which increases as the gas mixing ratio decreases. Clearly in this approach there are potential problems caused both by incomplete recovery of the gas of interest as well as contamination by impurities. In the case of atmospheric CO_2 for example, where cryogenic separation techniques are typically used, atmospheric N_2O is co-extracted and introduced into the IRMS as a contaminant for which a correction has to be made. These problems can potentially be solved by on line contaminant separation before analysis by IRMS. Sano et al [10] first reported "on line" separation using a GC coupled to a mass spectrometer to monitor aspirin and its metabolites. This was followed by the pioneering work of Matthews and Hayes [11] where a system designed for biological work yielded sufficient precision to detect methane isotope variability in natural biological systems where the signal was relatively large. Nevertheless, these systems suffered a significant loss of precision when handling the transient signals derived when processing gases at low abundances. This situation was improved however, in the first reported attempts to measure atmospheric methane [12] and non methane hydrocarbons [13] using GC-IRMS where sample volumes used were 3 to 4 orders of magnitude smaller than those required for dual-inlet IRMS. These systems used on line pre-concentration techniques to increase the signal in the GC-IRMS and were significantly more advanced than initial methods but still offered relatively poor precision compared to dual-inlet IRMS for atmospheric trace gases.

GC-IRMS clearly offers huge advantages for atmospheric trace gas research because of the very low sample volumes used and the potentially contaminant free on line preparation of sample gases. However to our knowledge, at the time of writing, there are no published reports of GC-IRMS techniques capable of providing analyses with the precision required for many of the problems involved in trace gas research.

However we do know of several unpublished accounts of successful high precision analyses of atmospheric methane, carbon monoxide and CO_2. At NIWA, New Zealand, for example, we have a very successful prototype providing simultaneous analyses of $\delta^{13}C$, $\delta^{18}O$ and mixing ratios in atmospheric CO_2 to precision's 0.02‰, 0.04‰ and 0.4ppm respectively. The performance of this system is currently being tested as a joint project between NIWA and three other laboratories where dual inlet techniques are used in very successful long term projects designed to make high precision measurements of stable isotopes in atmospheric CO_2. Details of the NIWA technique will soon be published and it is likely that information on techniques from other laboratories will soon become available. This makes the GC-IRMS conference organized by the IAEA a timely event.

2.4. Factors limiting the performance of GC-IRMS for high precision determinations of stable isotopes in atmospheric trace gases

Current work at NIWA shows that many factors affect the precision of stable isotope ratio determinations by GC-IRMS. Among the most significant are water entrained from laboratory air through system leaks in either the gas transfer line and/or the open split system is used to switch GC effluent into the IRMS. Laboratory air is relatively "wet" and the protonation of CO_2 in the ion source contributes to loss of precision and accuracy through the formation of HCO_2^+ ions, mass 45 [14]. Other factors are laboratory temperature variations which can affect many parts of a GC-IRMS system for example gas regulators on sample cylinders, temperature sensitivity of resistors in electro-magnet power supplies and effects on open split performance.

The fundamental performance of a GC-IRMS system can be evaluated from considerations of the "shot -noise limit". This is based on ion collection statistics and refers to the precision that would be obtained if the ion beam was the only significant noise source [15]. In the case of CO_2 analysis performed on a Finnigan MAT 252 IRMS, simplified expressions from Merritt and Hayes [16] for the shot noise limited precision (σ_s) expressed as functions of the integrated m/z signal area (^{44}A Volt.s) are

$$\sigma_s^2 = 0.00446(1/^{44}A_{reference} + 1/^{44}A_{sample})$$

where the integrated ion currents of the reference and sample are not equal and

$$\sigma_s^2 = 0.00892/^{44}A$$

when the currents are equal.

Clearly the shot noise precision limit is a function of the size of the signal which is proportional to the amount of sample in the ion source. In all IRMS systems however, there is a limit to the amount of sample that can be in the source at any one time. In the case of CO_2 for a Finnigan MAT 252 IRMS the limit is about 3–4 nanomole. More CO_2 than this causes the detection system to "saturate". Using the formulae above, signals from samples of this size yield a shot-noise limited precision of 0.02‰ for $\delta^{13}C$ in CO_2. For only 150 picomole of CO_2 in the ion source the precision deteriorates to about 0.1‰.

3. CALIBRATION REQUIREMENTS

As mentioned in section 1, the problems involved in atmospheric trace gas research tend to be global in nature. No single laboratory can provide all the isotopic and mixing ratio data required to investigate the implications of the increasing global atmospheric burden of trace gases. For this reason many collaborative projects have been initiated between laboratories in different countries where data on various aspects of global atmospheric chemistry are required. Data from the laboratories is shared and pooled, but before it can be used strict inter-calibration protocols must be agreed to between the participants.

In the case of isotopic measurements for atmospheric trace gases, the most extensive and successful laboratory inter-comparisons have been carried out for CO_2 and the IAEA has played a major role in these.(reference) However, no similar strategies exist for other atmospheric trace gases like methane, non methane hydrocarbons and carbon monoxide.

The natural abundances of ^{13}C in atmospheric trace gases are very different. For example CO_2, methane and carbon monoxide have $\delta^{13}C$ values of about –8‰, –47‰ and –28‰ V-PDB respectively. Hence, because of the non linearity and memory effects inherent in IRMS systems, no single primary standard can be used for all species.

The IAEA has distributed a ^{13}C light barium carbonate reference inter-comparison material known as IAEA-CO-9 [17] with IAEA published values of –47.119±0.149‰ for $\delta^{13}C$. In principle this material

should be ideal for the calibration of working reference gases used by laboratories to make measurements of $\delta^{13}C$ in atmospheric methane. However the stated precision, based on results of determinations made by 10 different laboratories, is far too large to be useful for research into the natural variations of atmospheric methane. In addition the most recent (May 1999) determinations of this material versus NBS-19 by NIWA indicate a $\delta^{13}C$ value of –47.195‰ V-PDB which is close to the original value of –47.23‰ assigned to the material by Carl Brenninkmeijer (personal communication) who first prepared the material. The mean value, n = 20, obtained for the material at NIWA over a 3.5 year period is $\delta^{13}C = -47.181 \pm 0.011$‰ V-PDB.

Experience from dual-inlet IRMS stable isotopic determinations of atmospheric CO_2 has shown that real air samples are a very effective method for inter-comparing results from different laboratories [18]. Because whole air is used rather than pure CO_2, entire laboratory extraction systems as well as the IRMS procedures can be tested [19]. The same situation will apply for other atmospheric trace gases but to our knowledge no such reference or inter-comparison gases in air have ever been formally prepared or circulated amongst laboratories making these measurements.

With the likely wide spread use of GC-IRMS in atmospheric trace gas research, the IAEA could play a role in setting up protocols for the supply and use of inter-comparison materials and reference gases. These protocols could be based on existing procedures developed for dual inlet IRMS analytical systems used for CO_2 for example.

4. CONCLUSIONS

In this article we have shown that GC-IRMS is likely to have a major impact on the stable isotopic measurement techniques used in atmospheric trace gas research. GC-IRMS has the considerable advantage that sample sizes up to 1000 times smaller than those required for dual inlet IRMS can be analysed. This will allow multiple analyses of samples as a means to improve precision. In addition bulky, off line potentially contaminant-producing extraction systems can be replaced by small on line separation systems. A major disadvantage however is that the currently published GC-IRMS systems lack the precision required to solve many of the problems in atmospheric trace gas research. This situation is likely to change in the near future however as new systems currently under development in several laboratories become available.

Calibration and inter-comparison is a critical factor limiting cooperative research into several important trace gas species, for example atmospheric methane. The IAEA has played a valuable role in the establishment of protocols for work on atmospheric carbon dioxide. A similar role could be played by the IAEA in setting up calibration and inter-comparison procedures for GC-IRMS techniques for other trace gases.

Because GC-IRMS uses quite different analytical principles for the extraction and measurement of trace gas isotopic compositions, inter-comparisons with existing dual-inlet techniques for species like atmospheric CO_2, CH_4 and CO will be important. As is the case in other areas of analytical science, measurements made by techniques based on different principles often provide valuable quality control information.

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ISOTOPE RATIO ANALYSIS ON WATER: A CRITICAL LOOK AT DEVELOPMENTS

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Abstract. In this opinion paper a short overview is given of the methods by which the stable isotope ratios of water are determined, ranging from the classical equilibrium technique for $\delta^{18}O$ and the Uranium-reduction technique for $\delta^{2}H$ to the recent innovative continuous flow work. Then the extensive IAEA "GNIP" database is used to show that the overall intercomparison quality of water isotope measurements "in the field" is still not satisfactory at all, and has not really improved in the last decade. Some suggestions are made as to what are the main causes for this situation, and what can be done to improve the situation.

1. OVERVIEW OF TECHNIQUES

There is no doubt that isotope ratios of water are very important tools in a multitude of applications. The ratios concerned are those of the stable isotopes ^{18}O ($\delta^{18}O$), ^{2}H ($\delta^{2}H$) and also ^{17}O ($\delta^{17}O$), and the radioactive tritium (^{3}H). The latter isotope is actually the reason why IAEA got involved in the (stable) isotope ratio scientific community in the first place. Nuclear bomb tests in the early sixties increased the tritium content of precipitation by a factor of 1000. Driven by public health concerns, the IAEA started a monitoring network for isotopes in precipitation (originally only for tritium) that gradually extended into the present-day GNIP-network (Global Network for Isotopes in Precipitation [1]). Furthermore, the IAEA got involved in reference material development and maintenance as well.

Measurements of the stable isotope ratios in water are traditionally rather cumbersome, especially for $\delta^{2}H$. The traditional method for $\delta^{2}H$ isotope ratio determination is based on total reduction of H_2O to hydrogen gas, using a hot uranium surface [2].

The method offers a reasonable precision (if well-performed) of 1–2‰, and the quantity of water needed is small (typically 10 µl). Disadvantages are that it is a slow and laborious method, and very hard to automatize. The total preparation usually suffers from memory effects, caused both by the uranium surface and the glass surfaces present. Finally, uranium is very poisonous and radioactive, giving rise to many necessary precautions and regulations. Because of this last disadvantage, researchers have attempted to use other metals, such as Zn, Mn and Cr, with varying success. Especially Zn has been attempted by many laboratories, and results seemed to depend heavily on the quality of the Zn, but such that it is still disputed exactly what qualities do matter.

For $\delta^{18}O$ on the other hand, Epstein and Mayada designed the so-called equilibrium method [3], in which water is brought into contact with an amount of CO_2 gas. Through the carbonic acid reaction:

$$H_2{}^{18}O + CO_2 <=> H_2CO_3 <=> H_2{}^{16}O + C^{16}O^{18}O \quad (1)$$

isotopic equilibrium is formed. This does not mean, however, that in equilibrium the $\delta^{18}O$ of the water and the CO_2 are identical, but rather that there is a temperature-dependent difference between them. This implies that the method requires well-stabilized temperature conditions. If this is done carefully the CO_2 resembles the original water but for a constant factor, and thus this CO_2 can be used for the $\delta^{18}O$ measurement.

If performed well, precisions exceeding 0.05‰ can be obtained. The typical amount of water needed is 1 ml, which is only a problem in a selected class of applications. Preparation machinery for batches of water samples using this equilibrium technique are commercially available. One disadvantage, although this is generally not thought to be problematic, is that $\delta^{17}O$ cannot be measured using this

preparation technique. In this case the ^{17}O isotopic equilibrium will also be reached, but $\delta^{17}O$ cannot be measured in the CO_2 gas due to the mass overlap of $^{12}C^{17}O^{16}O$ with the much more abundant $^{13}C^{16}O_2$.

In the light of the above, it is logical that especially for δ^2H there is a continuous search for new techniques that overcome some or all of the disadvantages of the uranium system.

Horita and Gat [4], and Coplen et al. [5] reported about the successful implementation of the catalytic equilibrium technique for hydrogen:

$$\overset{(Pt)}{H_2 + HDO <=> HD + H_2O} \quad (2)$$

This method resembles the oxygen equilibrium method very much. In fact, both equilibria can be accomplished sequentially with the same amount of water, and in the same preparation set-up (and automatically!). The typical amount of water needed is, again, about 1 ml. A big disadvantage of the method is the extreme fractionation that exists between the δ^2H of the hydrogen in the water and that of the hydrogen gas: in equilibrium this fractionation amounts to about -700‰. This fractionation is extremely temperature dependent, so a very good (and reproducible) temperature stabilization is necessary. The second disadvantage is that the hydrogen gas coming from this equilibrium contains almost a factor of 4 less ^2H, and thus the (already low) signal for mass 3 is further decreased. This gives rise to noise problems, but much more critical is the increase (by a factor of 4) of the relative importance of the H^{3+} contribution for which a correction must be made. Despite these disadvantages reported precisions are rather good: <1‰.

An alternative for the ^{18}O equilibrium method, that enables the $\delta^{17}O$ measurement as well, is proposed by Meijer and Li [6]. They produce oxygen gas from water by electrolysis. This oxygen can then be used in the IRMS for $\delta^{17}O$ and $\delta^{18}O$ measurements. About 1 ml of water is necessary, the precision is comparable to the equilibrium method, and automation is relatively easy. Disadvantages are as follows: the extra preparation step of electrolyte admixture, the regular replacement of one of the electrodes, and above all the frequent, cell-specific calibration that is necessary. The primary advantage, the additional measurement of $\delta^{17}O$, is of use to specific branches of research.

A major change in preparation and measurement came about by the introduction of the continuous flow IRMS systems. These machines can perform measurements with orders of magnitude less sample, although at the cost of precision. Several groups have used these new instruments for the miniaturization of existing (HD) preparation techniques, using U [7], Zn [8] or Cr [9].

However, the most innovative step came when on-line pyrolysis was introduced in the CF-IRMS preparation line [10]:

$$H_2O + C \rightarrow H_2 + CO \quad (3)$$

Preceding this important step, the problem of HD (mass 3) measurement in the presence of an overwhelming abundance of He had to be solved. Tobias et al [11] used a palladium filter, which was permeable for hydrogen, but not for helium. Prosser and Scrimgeour [12] used a specially designed analyzer in the IRMS (with a larger dispersion). Hilkert et al. [13] also adapted the IRMS, but now with retardation filtering preventing the “tail” of He to enter the mass 3 cup.

The method is still relatively new. It allows very small sample sizes (a few µl), easy automation, and measurement of δ^2H and $\delta^{18}O$ on a single sample of water. At this stage there is not yet enough experience with (longer term) precision, eventual fractionation effects during the pyrolysis reaction, influences of traces of CO_2 formed, etc. First results look promising.

Still, principal problems remain. In all techniques mentioned, there are different reaction pathways leading to the δ^2H and $\delta^{18}O$ measurements. Even with pyrolysis, two gases are formed on which these measurements are sequentially being done. This can lead to different drifts, calibration problems, incomplete reactions for one of the isotopes, different scale contraction problems [14], etc.

2. INTERLABORATORY COMPARISONS

In spite of accuracy claims (which are sometimes exaggerated, unfortunately), the situation "in the field" is not at all satisfactory at the moment. Recently the IAEA organized a new and extensive ring test in which ~ 80 laboratories co-operated (ones that are participating in the GNIP network), in which all labs had to determine the δ^2H and $\delta^{18}O$ of 4 different waters [15]. The labs were instructed to report these waters on the VSMOW-SLAP scale, so calibrated to VSMOW, and normalized using the assigned values to SLAP.

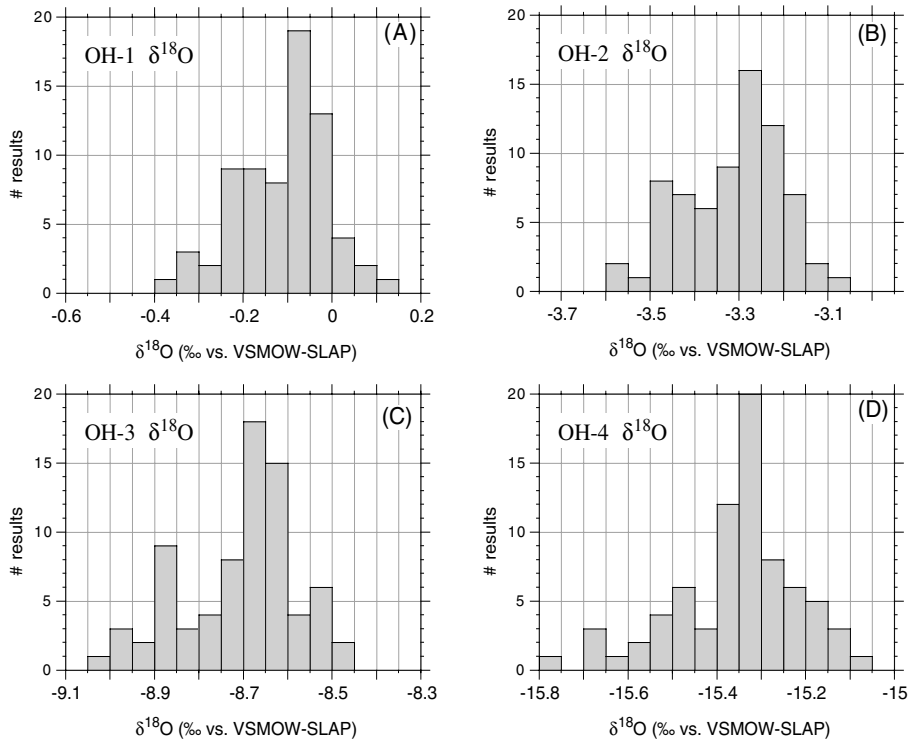

Fig. 1. Histograms showing the results of the ring test organised by the IAEA among ≈ 80 laboratories for the $\delta^{18}O$ analysis of four waters, OH-1–OH-4 (A-D). Apparent "outliers" have been removed (12 per plot for $\delta^{18}O$). The typical interlaboratory spread (2σ) is ±0.25‰, and specially for OH-3 and OH-4, the histograms show a deviation from a random Gaussian distribution, such that deviations of ± 0.4 ‰ from the average value are still not rare.

Fig.1 a-d shows the results of this ring test for $\delta^{18}O$, Fig. 2 a-d for δ^2H. Before making these plots, apparent "outliers" have been removed (about 10 for each plot)). As can be seen, the typical interlaboratory spread (2σ) is ±0.25‰ for $\delta^{18}O$, and ±3‰ for δ^2H. These results in fact give an alarming message about the use of the GNIP database, in which many laboratories participate. It means that on combining records from different laboratories, offsets in the range mentioned above can be expected. Furthermore, the spread in Figs. 1 and 2 can also be interpreted such that it is a measure for the calibration accuracy of all the GNIP records over time. If a laboratory was in this ring test on the "low" side of the averages, there is no guarantee at all that this has always been the case.

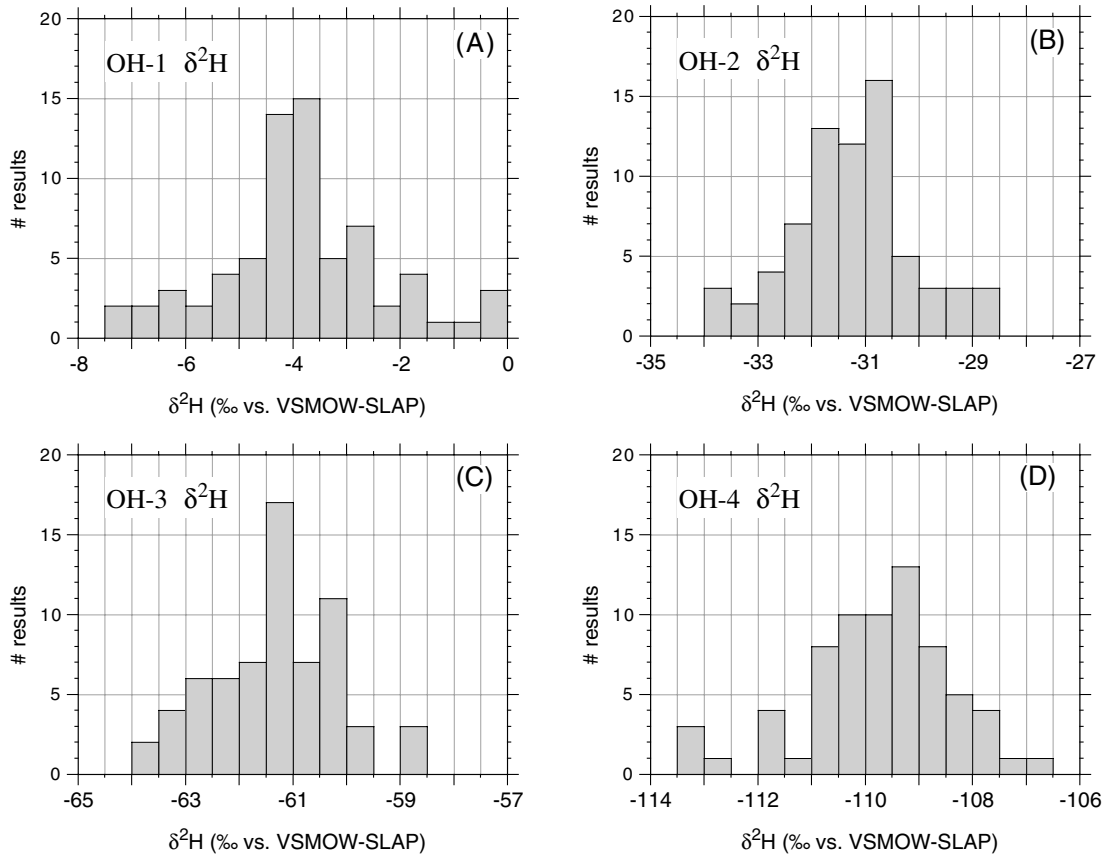

Fig. 2. As shown in Fig. 1, but for δ^2H. (8 outliers per plot have been removed). The typical interlaboratory spread (2σ) is ±3‰ for δ^2H. Also for δ^2H, deviations from a random Gaussian distribution can be observed. For a extended discussion of the ring test results, see [15].

What is clear though, is that if a laboratory performs too low on one of the waters for one of the isotopes, that it generally does so for the other waters for this isotope as well. However, there is no correlation between the isotopes. (For a complete report on this ring test, in which a considerable part of the spread is attributed to infrequent or not well-performed calibration and normalization procedures, see [15].)

The situation gets especially problematic for the calculation of the so-called deuterium excess:

$$d = \delta^2H - 8 \cdot \delta^{18}O \quad (4)$$

Since errors in δ^2H and $\delta^{18}O$ generally do not correlate, the error in d is still larger. Fig. 3 a-d shows the deuterium excess deviations for the four waters from the ring test. The spread has increased to almost ±4‰ (2σ), again interpretable both as being the interlaboratory spread, and the time-variability of the calibration of one record over the years.

If we plot an example from the GNIP database, the comparison of deuterium excess for the GNIP stations Valentia (Irish west coast) and Groningen (Netherlands), in Fig. 4 (both the smoothed trends over the years, and the average seasonal cycle), we have to keep the spread of Fig. 3 in mind while we interpret the findings. The measurements have been performed by two different labs, and thus a calibration offset of for example 5‰ would be possible. This means that the "obvious" result of the comparison of Valentia and Groningen, namely that the d-excess at the Irish west coast is lower than at the coast of the continent, cannot be deduced with high confidence from the GNIP database!

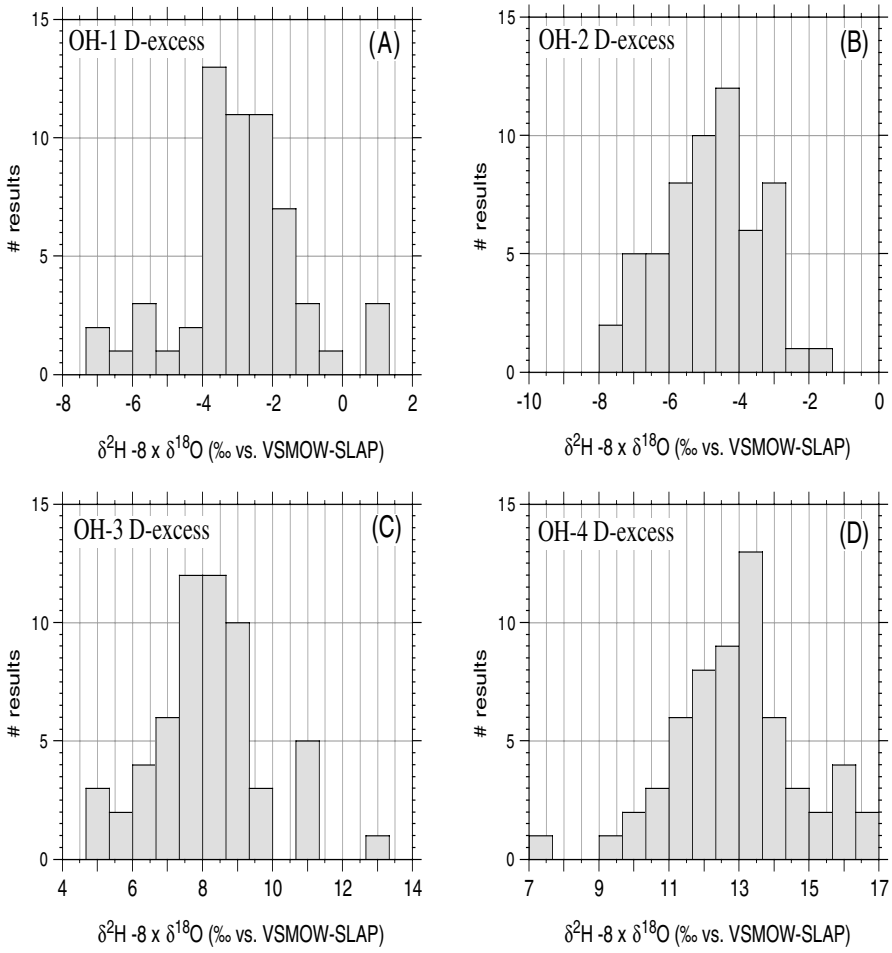

Fig. 3. Histograms for the deuterium excess d, computed from the $\delta^{18}O$ and $\delta^{2}H$ results in figs. 1 and 2. Since the deviations of specific labs for $\delta^{18}O$ and $\delta^{2}H$ are generally not correlated, the spread of d is larger than for $\delta^{2}H$ (or 8 x $\delta^{18}O$) itself. Results of these histograms should be kept in mind when using data from the GNIP database.

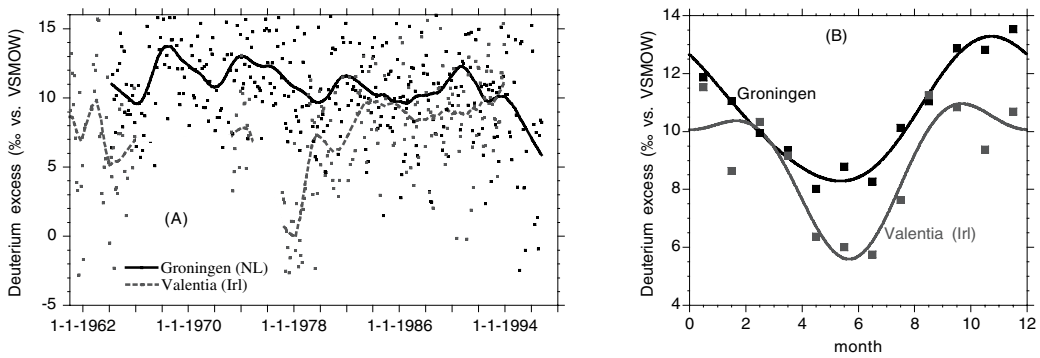

Fig. 4. Example of use of the GNIP database [1]: the deuterium excess of the GNIP stations Groningen (black lines) and Valentia (Ireland, gray lines). A) Smoothed long-year trend of d for the two stations. B) Average seasonal cycle for the two stations over the period 1981–1994. If compared to Fig. 3, one must conclude that the difference between Valentia and Groningen is still in the range of inter-laboratory spread (although the persistance of this difference over 3 decades increases its likelihood).

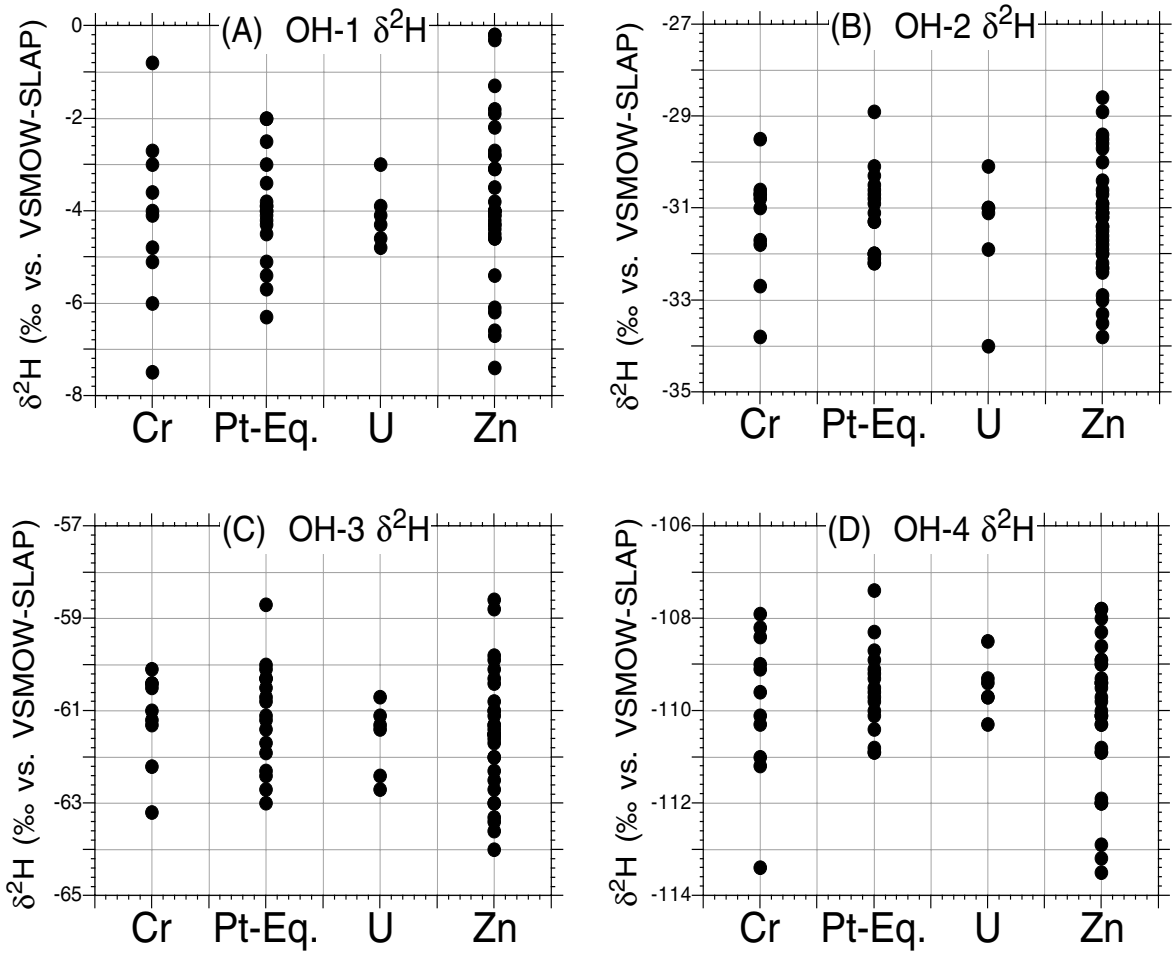

Fig. 5. The δ^2H results for the four waters of the IAEA ring test (cf Fig. 2), now with the used techniques as parameter (Cr is reduction using Cr, Pt-Eq. catalytic equilibrium, U reduction using Uranium, and Zn using Zinc, the method labelling is rather crude, since no further split-up between off-line preparation-dual inlet and on-line continuous flow has been made) No method seems to be performing better than another (only Zn seems to be worse).

More, interesting information can be extracted from the ring test results. Fig. 5 a–d shows the δ^2H results for the four waters *versus* the technique used (Cr is reduction using Cr, Pt-Eq. catalytic equilibrium, U reduction using Uranium, and Zn using Zinc). Although the method labelling is rather crude (no further split-up between off-line preparation- dual inlet and on-line continuous flow), the overriding message is that no superior method exists (only Zn seems to be worse).

In my opinion, the majority of problems are caused by scale contraction problems, i.e. the calibrated (but not yet normalized) values that various laboratories measure for SLAP show a very wide range. Fig. 6a shows the results for SLAP from the ring test performed by the IAEA during which SLAP has been assigned its values [16]. Values for SLAP vary between –395 and –441‰. Fig. 6b shows the results for $\delta^{18}O$ for SLAP from the same ring test; values vary between –54.0 and –56.2‰. The situation has not really improved in the mean time: the "Isogeochem" ring test performed by Brand and Coplen [17] in which, as a side-result, SLAP measurements were collected, still shows a similar spread (Fig 6c).

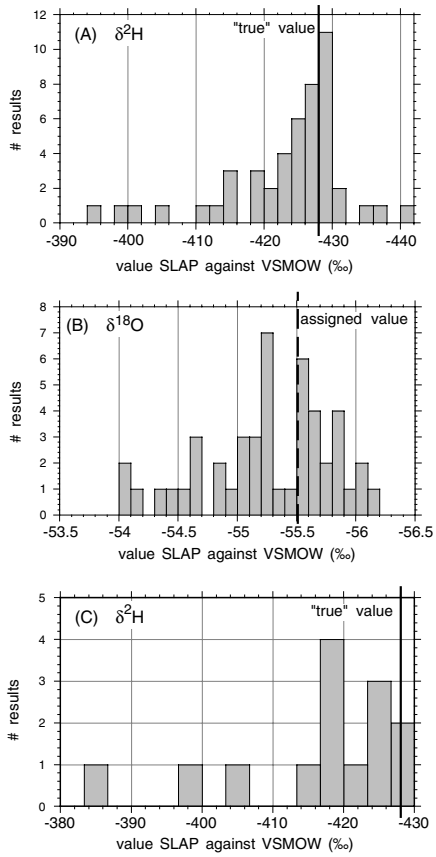

Fig. 6. The results for SLAP from the ring test performed by the IAEA during which SLAP has been assigned its values [16]. (A) ?^{2}H values for SLAP vary between -395 and -441‰. (B) ?^{18}O values for SLAP vary between -54.0 and -56.2 ‰. (C) ?^{2}H values for SLAP, being a side-result from the pure hydrogen ring test performed by [17]. The spread in values has not decreased compared to the 1984 ringtest (A) fifteen years earlier.

3. CONCLUDING REMARKS

Laboratories likely have serious problems if they are finding unnormalized δ^{2}H values for SLAP that deviate from the assigned values than by more than ~10‰. This information should not be ignored or "fixed" by just "normalizing" it away, as there is no guarantee that such vastly deviating values will stay constant, nor can it be assumed that the correction is linear. For δ^{18}O one should even be more strict, and put the limit of acceptable values at ~ 0.5‰.

Even more, laboratories should do their utmost to find unnormalized values for SLAP already as close to the assigned values as possible. This will make (1) correction factors much smaller and less significant, (2) temporal variations in the correction factors less important, and (3) the question whether the correction is linear less relevant. Therefore I plead for always explicitly publishing the unnormalised values for SLAP as well, both for δ^{2}H and δ^{18}O.

As is illustrated and discussed above the intercomparison between laboratories for water isotopes is still unsatisfactory. There are no indications that the new methods discussed above will get rid of, or even reduce these problems. I attribute the majority of the problems (if we leave sloppiness out as a possible cause!) to the fact that δ^{2}H and δ^{18}O are prepared as two different substances. The direct measurement of δ^{2}H and δ^{18}O on water itself has the possibility of eliminating these problems and, in principle, produce more reliable δ^{2}H and δ^{18}O, and especially deuterium excess results. Another contribution to this TECDOC [18] describes the first steps towards this goal.

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ON-LINE STABLE ISOTOPE MEASUREMENTS DURING PLANT AND SOIL GAS EXCHANGE

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Abstract. Recent techniques for on-line stable isotope measurements during plant and soil exchange of CO_2 and/or water vapor are briefly reviewed. For CO_2, these techniques provide means for on-line measurements of isotopic discrimination during CO_2 exchange by leaves in the laboratory and in the field, of isotopic discrimination during soil respiration and during soil-atmosphere CO_2 exchange, and of isotopic discrimination in O_2 during plant respiration. For water vapor, these techniques provide means to measure oxygen isotopic composition of water vapor during leaf transpiration and for the analysis of sub microliter condensed water vapor samples. Most of these techniques involve on-line sampling of CO_2 and water vapor from a dynamic, intact soil or plant system. In the laboratory, these systems also allow on-line isotopic analysis by continuous-flow isotope ratio mass spectrometry. The information obtained with these on-line techniques is becoming increasingly valuable, and often critical, for ecophysiologial research and in the study of biosphere-atmosphere interactions.

1. INTRODUCTION

The use of stable isotopes in environmental sciences is rapidly increasing. This is because stable isotopes provide powerful tracers of sources and sinks of elements in the biosphere-atmosphere system, as well as useful indicators of processes underlying biospheric response to change. In both cases the isotopic labeling by natural processes of gases such as CO_2, O_2 and H_2O exchanged between plants, soil and the atmosphere is often involved. The process-based mechanisms of such isotopic labeling must be studied in detail for quantitative use of the isotope approach at any scale, from the organism through ecosystem to the global scales. This, in turn, has motivated a great deal of research and development in the stable isotope methodology. One of the major changes in stable isotope analyses in recent years has been the advancement of a suite of on-line methods.

Notably, the term on-line technique has been adopted for two distinct operations. The first refers to on-line isotopic analysis. In this case, the traditional dual inlet of the mass spectrometer, using a sample and reference reservoirs, is replaced with a single inlet that introduces a continuous flow of a carrier gas (usually He). A pulse of sample or of reference gas is injected into the carrier stream. The sample line is first purified through a combination of traps and GC column to separate the gas of interest (e.g. CO_2). The reference gas is usually introduced after the purification step and the gas stream is then introduced to the source of the isotope ratio mass spectrometer (IRMS). The isotopic analysis is carried out by integration of the peak areas of the relevant mass beams (as opposed to beam heights in the dual inlet mode). The main advantages of this operation is in greatly increasing sample throughput and dramatically reducing the required sample size.

The second on-line operation refers to an on-line sampling of the gas of interest for isotopic analysis. Such experimental systems are often mentioned in the ecophysiological literature and are distinct from the on-line analysis in that they are not necessarily connected on-line to the IRMS. The main idea here is that sampling on-line allows measuring the isotopic discrimination of interest in a non-destructive manner in active biological systems. For example, leaf discrimination against $C^{18}O^{16}O$ or $^{13}CO_2$ can be measured repeatedly and in response to changes in environmental conditions by sampling the air passing above an active leaf [1]. From the change in the isotopic composition of the air entering and exiting a chamber in which a leaf (or a patch of soil, etc.) is enclosed, it is possible to accurately infer the isotopie effect associated with the leaf acitivity. The two aspects of the on-line methodology, on-line sampling and on-line analysis are equally important in the context discussed here. While on-line analysis of material collected in a destructive way (say, dried plant material), will make the analysis more efficient, but on-line sampling of the gas exchanged by active leaves in a real

time fashion will provide information that cannot be obtained otherwise. A combined operation is often required since on-line sampling produces small gas samples. Sampling and analysis of atmospheric gas sample such as CO_2 and O_2 in on-line technique takes a 2–5 minutes and requires small air samples of a few μL (considering differences in ambient concentrations, such as between CO_2 and O_2). Analysis of water vapor samples requires equilibration with CO_2 over several hours. But equilibrating water samples with ambient CO_2 in the air samples greatly simplifies the analysis. Alternatively a small water sample can be pyrolize to CO and analyze on-line.

On-line methods such as described here allow us to make investigations of almost undisturbed, in-vivo, systems and to observe short term, dynamic responses to changes in environmental conditions. This is in sharp contrast to original geochemical methods in which samples were stored in containers and analyzed separately from the experimental system and after long delays, in a specialized stable isotope laboratories.

2. ISOTOPIC DISCRIMINATION DURING CO_2 EXCHANGE OF LEAVES: LABORATORY MEASUREMENTS

2.1. Introduction

The ^{18}O of atmospheric CO_2 is influenced by CO_2 exchange with the land surface. In leaves and soil, CO_2 readily dissolve in water via the bicarbonate system and oxygen isotopic exchange occurs. Since in natural system there are always trace amounts of CO_2 and large quantities of water, the water impose its isotopic composition on that of CO_2. Because the isotopic composition of water in leaves and soil is unique, measurements of the ^{18}O in atmospheric CO_2 can provide information on specific fluxes of CO_2 exchanged with the land surface. The quantitative aspects of the ^{18}O exchange between leaf water and CO_2 diffusing in and out of leaves has been studied in laboratory experiments at the single leaf level. In this case, a leaf attached to the plant is sealed within a cuvette with an air stream flowing through it. By sampling and analyzing both the air going into and exiting the cuvette, the interactions of the leaf with the atmosphere inside the cuvette can be investigated.

2.2. Gas exchange system

The gas exchange system is described in Fig. 1 [2]. Synthetic air was mixed from N_2, CO_2 and O_2 cylinders using mass flow controllers, MFC (MKS Instruments, USA), and humidified via bubbling a variable portion of the air stream through water at room temperature ($\delta^{18}O = -4.5‰$, hence vapor ~ −14.5 ‰), acidified with two drops of 80% H_3PO_4. The airflow was split into reference and analysis air streams, the latter flow, range 800–1500 ml min^{-1}, passing to a Parkinson 'conifer pod' leaf cuvette (PLC3C, ADC Scientific, UK) and measured via another MFC. Illumination was from a 250 W projector lamp (GE, USA), passing through 3 cm depth of water to reduce infra-red radiation. Incident radiation on the leaf was controlled by shading with a predetermined number of 'miracloth' filters. Absolute CO_2 and H_2O concentration in reference and analysis streams were monitored alternately via an infra-red gas analyzer, Li-6262 (LI-Cor, USA).

2.3. Isotopic measurement of CO_2

The outflow of the leaf chamber, min 700 cm^3 min^{-1}, after passing through the IRGA, was split and 100 cm^3 min^{-1} was pumped first through a Nafion® dryer, and then a sample loop (0.85 ml) fitted onto a six port, two position valve (Valco Instruments Co, USA — see Fig. 1). CO_2 was trapped at liquid nitrogen temperatures for 30 s. After warming to room temperature, the sample was swept with helium carrier gas (120 mL min^{-1}; Gordon Gas and Chemicals, Israel) through a magnesium perchlorate drying trap and a 2 m. packed column of molecular sieve 5A, 80/100 mesh (Alltech, USA). The large peaks of N_2 and O_2 which eluted first from the column were diluted via a gas diluter (Micromass, UK), followed by the non-diluted sample CO_2. The gas was introduced into the source of

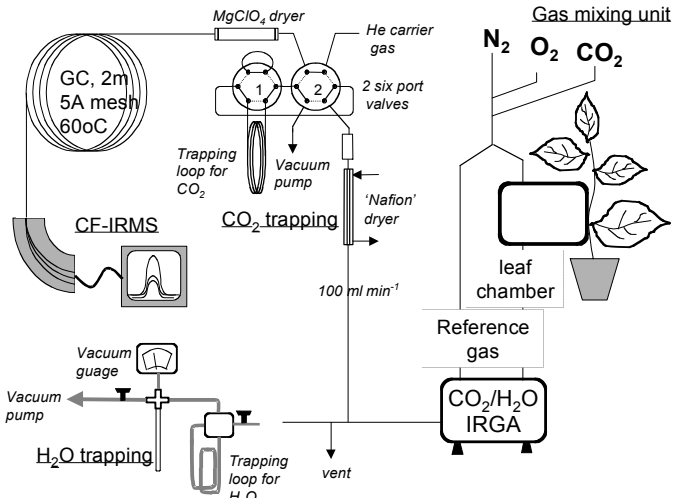

Fig. 1. On-line sampling and analysis system for gases exchanged between leaves and atmosphere.

an OPTIMA mass spectrometer (Micromass, UK) via an open split. $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios were measured from the integrated peak areas of masses 44, 45 and 46, normalized against a 30 second CO_2 reference pulse injected prior to each sample. Sample size was standardized by adjusting the cryogenic trapping time according to the CO_2 concentration in the outflow from the leaf chamber. N_2O was assumed to be constant in air (310 ppb) and absent from 'synthetic' air, thus δ values corrected accordingly [3] and expressed in the small delta notation vs. VPDB (for ^{13}C) and VSMOW (for ^{18}O), derived from calibration of the reference gas with CO_2 of known isotopic composition. Precision for repeated sampling of CO_2 was 0.06‰ ($\delta^{13}C$) and 0.07‰ ($\delta^{18}O$).

2.4. Isotopic measurement of water vapor: Equilibrium with ambient CO_2

The remaining airflow from the leaf chamber was passed at positive pressure to a 1/4 inch i.d. stainless steel vacuum line (pressure < 1×10^{-3} torr), where CO_2 and water vapor were trapped from the air stream (3 min. at 500 mL min^{-1}) in a coil cooled with liquid nitrogen. After trapping, the line was evacuated and the trap was heated with a flame, distilling both CO_2 and H_2O into a Pyrex side-arm immersed in liquid N_2. After quantitative transfer the Pyrex tube was flame sealed. The sample was left for CO_2-H_2O equilibrium at constant temperature (29°C, Labline Instruments Inc, USA) for 72 hrs. The CO_2 was then dried in a vacuum line with an ethanol trap at –70°C, before isotopic analysis on a MAT 250 dual inlet mass spectrometer (Finnigan-MAT, Germany). $\delta^{18}O$ of water vapor was calculated from that of the CO_2, according to Scrimgeour et al. [4], correcting for the amount CO_2 and H_2O (calculated from the concentration, flow rate and time of trapping) and $\delta^{18}O$ of the pre-equilibration CO_2, taken from the corresponding measurement on the continuous flow system. Precision of $\delta^{13}C$ CO_2 and $\delta^{18}O$ water vapor was 0.04‰ and 0.11‰.

2.5. Experimental procedure

Light responses (in which the rate of photosynthesis is gradually modified by changing the light photosynthetic photon flux density PPFD) were conducted from high to low PPFD, in 21% O_2. Collections of CO_2 for isotopic analyses were carried out for 3 minutes, while water vapor was trapped continuously (i.e. two samples of CO_2 and one of water were analyzed per light level). Photosynthesis measurements were averaged for the collection period. At the end of the experiment, the portion of leaf inside the cuvette was excised and placed in a 15ml vacutainer (Becton Dickinson, USA), for extraction of leaf water. The complete light response analysis (ca. 10 determinations) was

done with CO_2 relatively depleted in ^{13}C and ^{18}O ($\delta^{13}C$ = –30 ‰ and $\delta^{18}O$ = +10‰) to maximize the precision of measurement, or ambient air pumped through a 50 L external buffering volume ($\delta^{13}C$ = –8‰ and $\delta^{18}O$ = +41‰).

2.6. Isotopic calculations

Isotope ratios were expressed in the delta notation, $\delta‰ = 1000 \bullet (R_a/R_s - 1)$, where R_a and R_s are the rare/common stable isotope ratios for the sample and standard respectively (standards are VPDB, $^{13}C/^{12}C = 0.01118$, and VSMOW, $^{18}O/^{16}O = 0.0020052$ respectively). Instantaneous discrimination, Δ, for ^{13}C and ^{18}O was determined from [1],

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1000 + \delta_o - \xi(\delta_o - \delta_e)}.1000 \quad (1)$$

where $\xi = c_e/(c_e - c_o)$, c_e, c_o and δ_e, δ_o referring to the CO_2 concentration (corrected to the same humidity) and isotopic composition of air entering and leaving the cuvette, respectively. Additionally, $\Delta^{13}C$ was described simply in terms of the enzymatic and diffusive fractionation model [5] as $\Delta_i = a + (b' - a)c_i/c_a$, where c_i and c_a refer to CO_2 concentration in the sub-stomatal cavity and atmosphere respectively, a is the fractionation during diffusion in air (4.4‰) and b' is the fractionation during carboxylations (29‰). The additional reduction in CO_2 concentration from c_i to the chloroplast, c_c, was estimated from the difference between the simple model and the measured discrimination, $\Delta_i - \Delta_{obs}$, [1] as

$$\Delta_i - \Delta_{obs} = \frac{(b' - a_i)}{g_i} . \frac{A}{p_a} \quad (2)$$

where Δ_{obs} is the discrimination measured in equation 1, g_i refers to the total internal conductance, a_i is the combined fractionation (+1.8‰) during dissolution (+1.1‰) and diffusion through the liquid phase (+0.7‰). Internal conductance was derived from the gradient of the Δ_I–Δ_{obs} response vs. A/c_a, which avoids introducing error due to the uncertainty of b' and photorespiratory fractionation [6], [7], [8].

Discrimination against ^{18}O in CO_2 , $\Delta^{18}O$is estimated in a similar way [9]:

$$\Delta^{18}O = \frac{\overline{a} + \varepsilon\Delta_{ea}}{1 - \varepsilon\Delta_{ea}/1000} \quad (3)$$

where $\Delta_{ea} = 1000 \cdot [(\delta_e/1000 + 1)/(\delta_a/1000 + 1) - 1]$, $\varepsilon = c_c/(c_a - c_c)$; δ_a, δ_e represent the $\delta^{18}O$ of CO_2 in the overlying air and in full isotopic equilibrium with water in the chloroplast, and c_a, c_c the respective CO_2 concentrations (see Fig. 1); is the weighted-mean diffusional fractionation through boundary layer, 5.8‰, stomata, 8.8‰, and aqueous leaf media, 0.8‰. The oxygen isotopic composition of CO_2 in equilibrium with chloroplast water, δ_c, was determined by solving equation 3* for δ_c, so that

$$\delta_c = 1000.\left(\frac{c_a - c_c}{c_c}\left(\frac{\Delta^{18}O}{\Delta^{18}O/1000 + 1} - \overline{a}\right) + 1\right).\delta_a \quad (4)$$

Thus, δ_c was estimated from equation 3 using direct measurements of discrimination and the appropriate estimates of CO_2 concentrations.

The $\delta^{18}O$ of transpired water vapour, δ_t, was calculated as

$$\delta_t = \xi_L(\delta_{out} - \delta_{in}) + \delta_{\text{in}} \quad (5)$$

where $\xi_L = e_o/(e_o-e_e)$, e_e, e_o and δ_e, δ_o referring to the H_2O vapor pressure and isotopic composition of air entering and leaving the cuvette, respectively [10]. Following their example, δ_t was then substituted into the Craig and Gordon model [11] of surface water enrichment (as modifed by Flanagan et al. [12]) to predict the $\delta^{18}O$ at the sites of evaporation, δ_e. This method has two benefits over using the source water value based on the assumption of isotopic steady state, because a) water arriving at the leaf may differ from source water applied to the roots (due to evaporation from the soil or from unsuberised stems) and b) it does not restrict estimation of δ_e to isotopic steady state (ISS) conditions.

3. ISOTOPIC DISCRIMINATION DURING CO_2 EXCHANGE OF LEAVES: FIELD MEASUREMENTS

3.1. Introduction

Although laboratory conditions offer the best conditions to investigate leaf scale processes, these studies must eventually be extended to field conditions. Only under these conditions different plant species can be investigated in their natural habitat and under natural conditions. At present, IRMS is not a transportable, field instrument and samples must be collected in the field and transported to the lab. However, on-line field sampling systems have been used in the field in several studies such as [9], [13]. In these cases, the gas exchange and gas sampling systems is similar to that described above but simplified and made portable. The gas samples are purified in the field and sample of, say, CO_2 ready for analysis are transported to the lab. The term "on-line" applies in this case to the experimental and sampling system only and not to the analytical system.

3.2. Gas-exchange measurements and plant sampling

Rates of photosynthesis and transpiration, stomatal conductance, as well as micro-meteorological conditions (relative humidity, photon flux density and air and leaf temperatures), were measured using a portable leaf gas exchange system (ADC-3 with PLC-3 leaf cuvette, ADC, Hoddesdon, Herts, England) and calculated according to [14]. Boundary layer conductance in the leaf cuvette was estimated to be about 2 mol m^{-2} s^{-1}. Leaf area was determined from traces made for each leaf in the field. Subsequent to each gas exchange measurement, a similar, nearby leaf and a woody (non-green) stem sample were sealed, separately, in test tubes (Vacutainers, Rutherford, NJ, USA). Notably, in several cases, sampling was repeated twice for the same plant to evaluate within-plant variation. These tests, and previous experience indicated that a careful sampling strategy keeps such variations to less than 1‰. Atmospheric moisture samples were simultaneously collected by flowing air (250 ml min^{-1}) through a cryogenic trap (–80ºC) at a field site exposed to turbulent air away from nearby canopy.

The above mentioned leaf cuvette was used for measurements of leaf discrimination against $^{13}CO_2$ and $C^{18}O^{16}O$ by attaching it to a 1/4" stainless steel vacuum line (better than 1.0×10^{-3} torr) used for cryogenic drying (–80°C) and trapping of CO_2 at liquid nitrogen temperature (Fig. 2). CO_2 and water vapor in the air flow (ca. 250 ml min^{-1}) entering and exiting the cuvette was measured with an infra-red gas analyzer (LiCor-6262, Lincoln, NE, USA). CO_2 samples were trapped for 2 min, after which the vacuum line was evacuated and the CO_2 transferred and sealed in a glass ampoule. After each measurement, the leaf was cut and sealed in a test tube. Ambient environmental conditions at the time of the measurements were also recorded to allow estimation of leaf water $\delta^{18}O$ values.

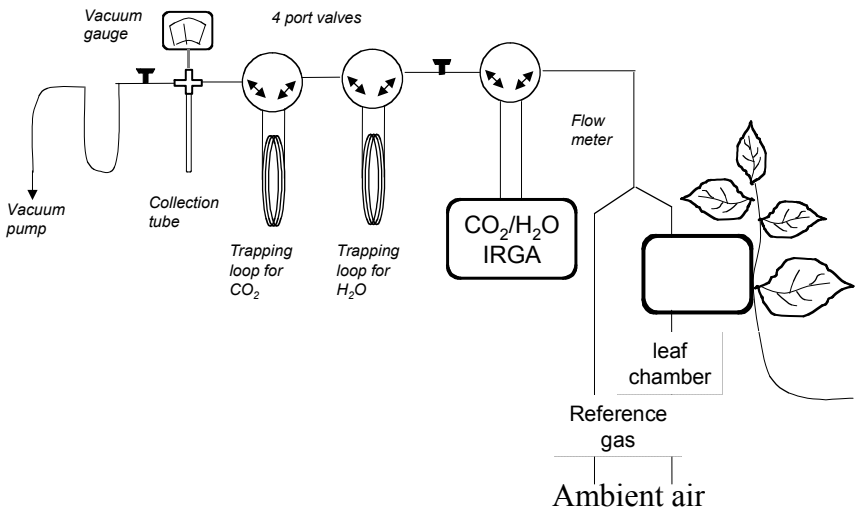

Fig. 2. On-line sampling system for the field sampling of gases exchangeed between leaves and atmosphere. An attached leaf is enclosed in a leaf cuvette and an air stream is samples before and after passing above the leaf. Water vapor and CO_2 concentrations are measured by an infra-red-gas analyzer, water is traped at –8°C and CO_2 at –170°C. Samples are cryogenically transferred to a collection tube for the mass spectrometric analysis.

3.3. Isotopic analysis

Leaf and stem water was extracted by vacuum distillation at 60°C in the lab. $\delta^{18}O$ values of the water samples were determined by equilibration with CO_2 at 25°C over night followed by cryogenic separation of CO_2 for the mass spectrometric analysis (Finnigan MAT-250). Precision for the water analysis was ±0.1‰. Samples of CO_2 from the on-line discrimination measurements were directly analyzed on the mass spectrometer with an external precision of ±0.05‰ for $\delta^{13}C$ and ±0.1‰ for $\delta^{18}O$. Estimating $\delta^{18}O$ values of chloroplast water and other calculations are similar to those described in the previous section.

4. ISOTOPIC DISCRIMINATION DURING SOIL CO_2 EXCHANGE

4.1. Introduction

Soil CO_2 exchange carries a unique isotopic signature and influence the isotopic composition of atmospheric CO_2 at the local, regional and global scales. Using ^{13}C, soil respiration can trace the source of the CO_2 and help distinguish between root respiration and organic matter decomposition, as well as in estimating raters of its turnover in the soil. Using ^{18}O in CO_2, soil respired CO_2 provides another useful link between the land biosphere and the atmospheric ^{18}O budget. In this case, the principal process in the soil is similar to that in leaves, namely the isotopic exchange of oxygen between water and CO_2. However, other factors such as the rate of exchange, competition between diffusion and exchange, atmospheric invasion into soils and spatial and temporal variations in the $\delta^{18}O$ of soil water require addressing the soil system as an independent experimental system. Here again, both on-line sampling and on-line analysis were carried out in the lab., but on-line sampling methods were also adapted to field work. Both systems are briefly described below.

4.2. Direct soil CO_2 measurements

A newly designed system using a gas-chromatograph-isotope-ratio-mass-spectrometer (GC-IRMS) was built for sampling small quantities of soil air at 1 cm vertical resolution from near the soil surface

for direct, on-line, isotopic analysis [15], (Fig. 3). 200 μL aliquots of soil air were sucked by vacuum from different soil depths within the collar described above through a fused silica capillary (0.32 mm i.d., 0.45 mm o.d.) and a stainless steel sample loop fitted to a two position, six-port Valco valve (Fig. 4). After switching the valve position, the air sample trapped within the sample loop was carried with a He carrier gas through a Nafion membrane drying trap (0.4 mm I.D. x 15 cm), and onto a capillary GC column (0.32 mm × 25 m, J&W GS-Q at 35°C). The column effectively separated CO_2 from air and N_2O without any cryo-focusing. The GC eluant passed through an open-split and changeover valve into the mass-spectrometer (Micromass Optima). The changeover valve allowed the air peaks to be pumped to waste and the remainder of the sample to be directed to the ion source of the mass spectrometer. The CO_2 peak eluted within two minutes of the six-port valve switch with a peak width of 5 s (FWHM), and peak height between 1.5 and 15 nA, depending on sample loop size and sampling depth. Peaks for the different isotopic masses (44, 45, 46) were integrated and their ratios normalized to a CO_2 standard injected one minute after each sample from the bellows of the dual inlet of the mass spectrometer.

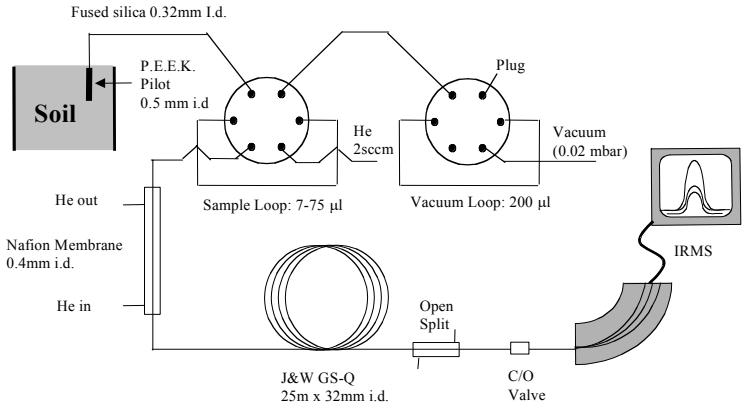

Fig. 3. On-line sampling and analysis system for soil CO_2.

Insertion of the sampling capillary into the soil must insure that the capillary did not become clogged with soil and that room air did not mix with soil air. A plastic pilot tube (PEEK, 1/16" o.d. × 0.5 mm i.d.) was first inserted to the appropriate depth and immediately plugged with a dummy sampling capillary. The dummy capillary was also used to clear any soil present in the pilot tube. The snug fit of the fused silica inside the PEEK tubing prevented room air from mixing down to the sampling depth. Small soil fractures around the outside of the PEEK pilot, resulting from its insertion, were immediately pressed down by hand. After the fused silica sampling capillary had been flushed with dry He, the dummy capillary was removed and quickly replaced by the sampling capillary. Soil air profiles were always measured from the top down to avoid mixing of deeper soil air into the sampling region as the capillaries were withdrawn. The 200 μL air samples sucked out of the soil corresponded to a volume of soil with a radius of 4 mm, assuming a soil air fraction, θ_a, of 0.25 and isotropic removal of soil air. 200 μl of air was sufficient to flush our largest sample loop (75 μl) and sampling capillary more than twice. Suction of 200 μL air was achieved by connecting the vent of the Valco valve to a second valve containing a 200 μL vacuum loop that could be, according to the valve position, connected to the vacuum system of the mass spectrometer (Fig. 3). After evacuation, the 200 μL loop was switched on-line to the sampling system described above, filling up with soil air while flushing and filling the sample loop on the first valve.

Precision of the ^{18}O analysis of repeated sampling of tank air (360 ppm) was better than 0.1‰. However, soil CO_2 mole fractions often varied from atmospheric levels near the surface to more than 10,000 ppm 8 cm below the surface. It was therefore critical to establish any correlation between the isotopic ratio and the sample size. This "linearity" was checked by measuring isotopic ratios of standard tank air with sample loops ranging in volume from 7 to 75 μL. Any possible correlation over this range was smaller than the measured precision. Our approach of using increasingly smaller sample loops with increasing depth allowed us to measure $\delta^{18}O$ in soil CO_2 with mole fractions varying by a factor of more than twenty with no apparent non-linearity. Although this approach is somewhat limited in its dynamic range, experiments with on-line dilution methods exhibited poor reproducibility.

Drying sample air prior to analysis is a prerequisite for obtaining accurate results [16, 17], especially since soil moisture can exceed 30%. Water vapor extracted from the soil may re-condense in the sampling or measurement apparatus and exchange oxygen atoms with the sampled CO_2, modifying the original $\delta^{18}O$ value of the sampled CO_2. The installation of the Nafion drying tube immediately downstream of the sample loop ensured measurement of dry air while retaining excellent peak shapes. The small sample size insured that disturbance of soil air as a result of sampling was negligible, and the five minute analysis time minimized potential changes in environmental parameters during the course of an experiment.

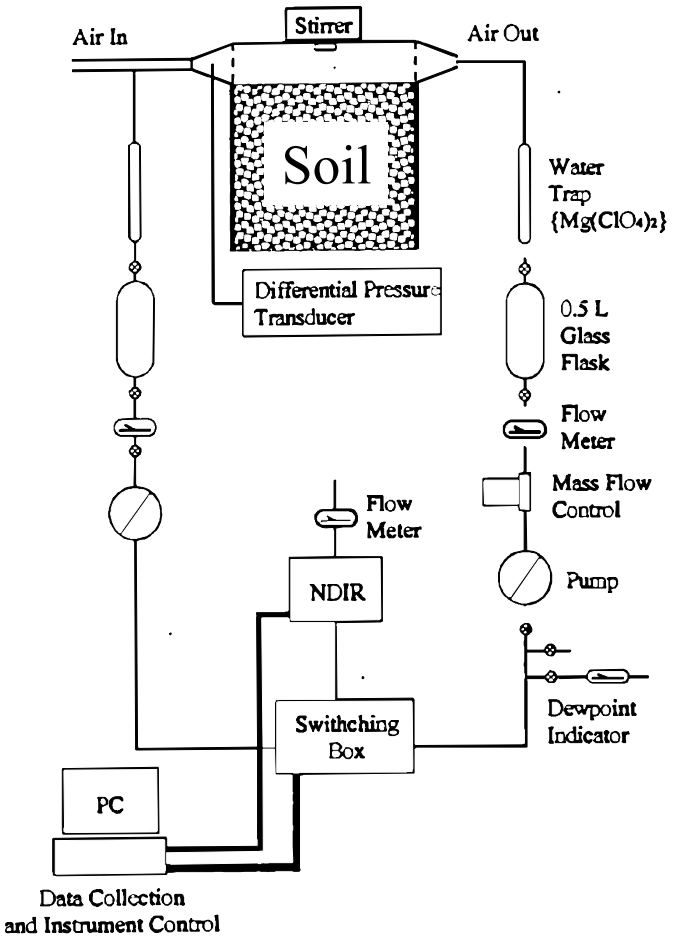

Fig. 4. On-line sampling system for the isotopic analysis of soil respired CO_2.

4.3. Dynamic chamber measurements

An open-bottomed flow-through chamber [15]; Fig. 4 based on the design of [18] was placed into a groove on top of the steel collar and sealed with putty (Apiezon Q). Both the collar and the chamber were leak-tested using an infra-red gas analyzer (IRGA, Li-Cor 6251) and pure CO_2. Before sealing the chamber, we placed a magnetic stir-bar on the ceiling of the chamber with a standard lab magnetic stirrer placed on top of the chamber. Using the stirrer at the minimum speed prevented the development of laminar flow inside the chamber. The chamber intake air was pumped (KNF diaphragm pump) from a 120 L plastic reservoir located inside the laboratory but was fed by a separate pump with outside air. Air exiting the chamber passed through a 8" × 0.5" I.D. $Mg(ClO_4)_2$ drying trap and a 0.5 l glass flask (J. Young stopcocks with Teflon o-rings.) The airflow was then split with a constant flow of 100 ml min^{-1} into an IRGA via a switching box. Flow was regulated with a mass flow controller (Edwards 825) with a range of 0–4 L min^{-1}. The intake air was sampled through a tee in the intake hose immediately prior to the chamber into a parallel drying and sampling system, except that flow was controlled by a needle valve and monitored by a ball flow meter. The switching box alternated the intake and outlet streams that passed on to the IRGA at a computer controlled frequency of 0.0083 Hz. Data from the IRGA was recorded only after the appropriate flushing time between cycles. During experiments, the system was allowed to reach steady state, indicated by a constant difference between the CO_2 mole fractions of the intake and outlet flows. After steady state was reached and mean CO_2 mole fractions were recorded, the stopcocks of the 0.5 L flasks were closed, and samples of the intake and outlet air were taken for isotopic analysis.

The pressure difference between the chamber and atmosphere was continuously monitored by a differential pressure transducer (Edwards 1018) fitted to the wall of the chamber. At all times, measured pressure differences were smaller than the 0.2 Pa needed to avoid significant advective contributions to CO_2 diffusion in and out of the soil [18]. Dewpoints of the dried air streams were measured to insure that the flask air samples were dry enough to eliminate the possibility of isotopic exchange between CO_2 and liquid water on the walls of the flasks or the tubing [17]. Dewpoints were kept below −15°C by changing the $Mg(ClO_4)_2$ drying traps as needed.

5. ISOTOPIC DISCRIMINATION IN O_2 DURING LEAF RESPIRATION

5.1. Introduction

The first measurements of ^{18}O discrimination in atmospheric O_2 were made in 1956 when Dole [19] reported several single point measurements of respiratory discrimination by a variety of organisms ranging from bacteria to human beings. Thirty years later stable isotope methodology was applied to the terminal oxidases of the mitochondrial electron chain and a substantial difference was found in the discrimination of the alternative and cytochrome oxidases [20]. This difference forms the basis of a new technique which can be used to estimate steady state partitioning of electron flow between the two mitochondrial pathways. This method has since been developed to enable rapid on-line measurements of the dynamics in oxygen isotope discrimination during respiration [21, 22].

At least two samples are necessary to calculate discrimination, a reference sample (with $^{18}O/^{16}O$ ratio R_O) and a subsequent sample with isotopic ratio R taken after a substantial proportion of the available O_2 has been consumed in respiration. For statistical purposes several such comparisons are made over a broad range of O_2 concentrations. D is calculated as the slope of a linear regression of $\ln(R/R_O)\bullet 1000$ plotted against -ln f (where f is the fraction of oxygen remaining in the reaction vessel). Two types of "environments" have been used. First, liquid-phase environment in which marine organisms, cells in suspension or isolated mitochondria can be analyzed [23–25], and second, air-phase environment in which whole tissues of higher plants can be analyzed [22]. Note that in all cases it is necessary to follow both the changes in isotopic composition and in the partial pressure of oxygen in the system's atmosphere (head-space). The elemental analysis can be accomplished with a gas chromatograph, corrected for the co-elution of oxygen and argon. Alternatively, both the isotopic

composition and the elemental concentration can be determined from the mass spectrometric analysis. As shown here, the change in oxygen partial pressure over time can also be used to estimate the rate of respiration. Care must be taken, hoever, to account for the unique conditions in the chamber due to decrease in barometric pressure with sampling and increase in CO_2 partial pressure.

5.2. On-line ^{18}O measurements during leaf respiration

Respiring tissue is enclosed in a leak tight vessel from which samples can be withdrawn (Fig. 5). In the liquid phase system, samples of the solution is withdrawn and the air is separated by sparging [23] or gravimetrically [24] and dried. In the air-phase system a sample of the "head-space" is withdrawn and dried. In both cases isotopic analysis of the O_2 in the air is accomplished by direct measurement in a mass spectrometer [21, 22, 24] or by first converting the O_2 to CO_2 on hot graphite [23]. An accurate determination of the O_2 concentration is also required for the calculation of D. A complication arises because O_2 pressure in the closed system is reduced both by respiration and by the gas sampling. To account for this effect, the amount of oxygen in the samples is normalized to that of a conservative gas in the air, such as nitrogen (which is not consumed in respiration). Determinations of the two gases can be achieved by separating them in a GC [21, 22] or in the mass spectrometric analysis of the air mixture [24], Fig. 5. In both cases, CO_2 is removed from the air prior to analysis. Argon does not interfere with the mass spectrometric analysis, and can be corrected for in the GC determination. Recently, both an air-phase and liquid-phase systems have been developed which are connected on-line to a MS. These systems significantly increase the speed of overall analysis, minimize contamination and reduce sample size [21]. Further, it now seems that the combined isotopic and elemental oxygen analysis on the MS allows also estimation of the rate of respiration [26]. The operation of a gas-phase system [26] is briefly described below.

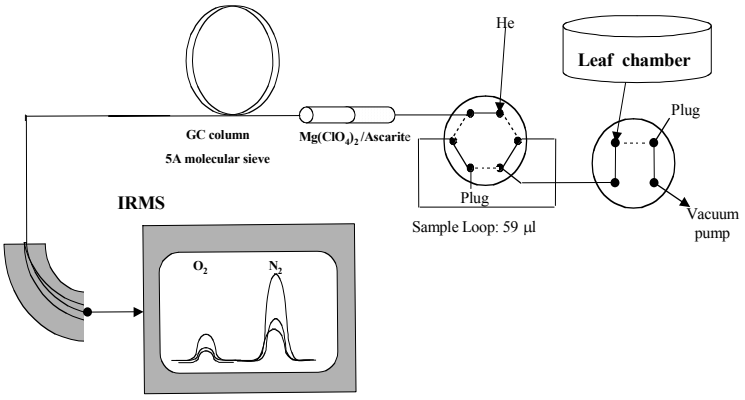

Fig. 5. On line sampling and analysis system for O_2 isotopic composition during respiration.

To measure *D* of plant materials we used freshly cut tissue samples (leaves, stems, and whole seedlings) from the selected plants. Samples were sealed in chamber (Hansatech Instruments Ltd, England) (Fig. 5). The chamber was vented and the tissue sample allowed to equilibrate with ambient air. The inlet vent was then closed and the tissue was allowed to respire in the dark. Samples of the head space were withdrawn at regular time intervals (5–20 minutes depending on rate of respiration) through a capillary (5.2 µL) into a pre-evacuated loop (59 µL) on one of two six-port, two positions Valco valve (Valco Instruments Co. Inc.). The valve was then switched to a carrier helium flow at flow rate of ~75ml/min. Carbon dioxide and water vapor were removed from the He and sample stream by using Ascarite and $Mg(ClO_4)_2$ traps, respectively. Separation of N_2 and O_2+Ar gases was done by 5A Molecular sieve GC column (Elemental Microanalysis Limited) kept at 40°C. The GC

peaks were transferred to the MS via a 1/16" stainless steel capillary. The MS detected and O_2 and N_2 by a peak jump procedure (magnet jump using Hall probe). Ratios of masses 32/28 (O_2/N_2) were used to correct oxygen uptake in respiration for sampling effects, and of 34/32 for estimating changes in ^{18}O. External precision was estimated by repeated measurements of $\delta^{18}O$ of lab air sampled through the system, yielding a precision of ±0.2‰.

5.3. Calculating ^{18}O discrimination in respiration

To calculate the discrimination factor two measurements are required for each sampling time the concentration of O_2 in the reaction vessel and the $^{18}O/^{16}O$ ratio of the sample. Oxygen must be separated from the other gases in air (N_2, CO_2, H_2O and Ar) using traps and gas chromatography (GC) before the isotopic ratio can be measured by mass spectrometry (MS). In Guy's system respiration occurred in a liquid phase reaction vessel from which samples were removed at regular intervals via a syringe. Oxygen was then purged from these samples using He, separated from the other gases and converted to CO_2, from which the isotopic ratio of oxygen could be determined [23, 27]. This method is both technically demanding and time consuming. A similar method for liquid phase determinations has been used by [24] to measure discrimination in various marine organisms. This method is simpler because the O_2 isotopic ratio is determined directly thus eliminating the steps associated with conversion of O_2 to CO_2. An on-line system developed at Duke University [21] allows direct measurements of respiratory oxygen isotope discrimination in the gas phase. Samples are removed directly from the gas phase surrounding the tissue and fed into a GC/MS system which provides measurements of both the concentration and the isotopic ratio of oxygen in the chamber. The on-line system is considerably faster than the previous method enabling measurements of discrimination in less than an hour. It also dispenses with the sample preparation steps which are the main source of contamination or error associated with the vacuum line system [27]. The volume requirement is reduced a 100-fold and some of the diffusional problems encountered previously are overcome in the gas phase system. Although many of the limitations to the stable isotope method identified by [27] have been overcome by the on-line system, the equipment remains expensive.

Traditionally, discrimination (D) is determined by comparing the isotope ratios (R, e.g. $R=^{18}O/^{16}O$) of the substrate (Rs) and of the product Rp:

$$D= (1-Rp/Rs)1000 \quad (6)$$

Note that $D = (1-\alpha)1000$; and $\Delta = (D/1000)/(1-D/1000)$, where α and Δ are alternative ways for defining isotopic discrimination [28, 29]. Obviously, in respiration isotopic analysis of the product (H_2O) is difficult. This problem can be overcome by considering that in a close system changes in Rs can be related to D by a Rayliegh type equation:

$$D=[(\ln R/R_0)/(-\ln f)]1000, \quad (7)$$

where R is the isotope ratio of the substrate at sampling time, R_0 is its initial isotope ratio of the substrate and f is the fraction of unconsumed substrate. In practice, D is obtained from the slope of the linear regression line of $\ln R/ R_0$ against -lnf of a series (ca. 5) of samples. In this approach, although only the substrate is analyzed, it is important to accurately determine its concentration, f, (O_2 concentration when respiration is considered).

The partitioning of electrons to the alternative pathway in the absence of inhibitors (P) was calculated as outlined by [27]:

$$P = [(D_n - D_c)/ (D_a - D_c)] \cdot 100 \quad (8)$$

where D_n is the net uninhibited discrimination on a linear scale between D_a (discrimination by the alternative oxidase) and D_c (discrimination by the Cyt oxidase). The flux of electrons through the alternative pathway was calculated by multiplying the total respiratory rate in the absence of inhibitors by the partitioning factor, *P*.

5.4. Calculation of respiration rates

Rates of respiration were estimated directly from the MS measurements (Fig. 5). Assuming atmospheric composition of 20.95% O_2, 78.08% N_2, 0.93% Ar plus trace gases, we denoted:

$$\theta_{air} = P_O/P_N \tag{9}$$

where P_O and P_N are the partial pressures of oxygen and nitrogen respectively and during measurements θ_{air} at time zero ($\theta_{air(0)}$) is equal to θ_{atm} in the open atmosphere (0.26, standard conditions). However, due to different instrument sensitivity to N_2 and O_2, $\theta_{air(0)}$ as measured by the MS is slightly different from θ_{atm} and must be corrected:

$$\theta_{air} = k \cdot O_t/N_t \tag{10}$$

where O_t and N_t are the measured peak areas at time t of oxygen and nitrogen. The sensitivity correction factor, *k*, was found to be a constant over variable conditions in continuous measurements performed in a closed and empty chamber.

We follow the decrease in O_2 concentration due to respiration only, and remove the affect of sampling by first considering the change in the non reacting nitrogen:

$$P_N = 0.78N_t/N_0 \tag{11}$$

We then estimated P_O at any time step due to sampling changes only (P_O^S) by assuming this change to be similar to that in N_2:

$$P_O^S = P_N \cdot \theta_{atm} \tag{12}$$

The O_2 partial pressure at a time step is then given by:

$$P_O = P_N \cdot \theta_{air} = (0.78N_t/N_0)\ \theta_{air} \tag{13}$$

and due to respiration only (P_O^R) by subtraction:

$$P_O^R = P_O - P_O^S = P_N \cdot \theta_{air} - P_N \cdot \theta_{atm} = P_N(\theta_{air} - \theta_{atm}) \tag{14}$$

values of P_O^R were converted to mole units with the ideal gas equation where V, the volume of the chamber ($5.34*10^{-3}$L) was corrected for the volume of the tissue sample by assumed for convenience it is mostly water and using fresh weight. Finally, the respiration rate was expressed on the leaf dry weight basis and the time of the relevant time interval (μmol O_2 min^{-1} g^{-1}). Precision for respiration rates was estimated from the calculated "rates" obtained in an empty chamber (0.001 μmol O_2 min^{-1}).

Notably, respiration rates often decreased during the time of the measurement. Several reasons were suggested as possible cause for a decrease in dark respiration in chamber measurements. The CO_2 concentration buildup in the closed chamber during the measurement might inhibit the respiration, especially via the Cty pathway [31, 33]. Since elevated CO_2 inhibit the cytochrome respiration,

the discrimination against ^{18}O could increase during the measurement. In our experiments, even when respiration rates decrease substantially, the *D* values were relatively constant during the time course of the measurements. Moreover, adding CO_2 absorber (Soda lime, Fisher Scientific) to the chamber did not affect the decrease in the respiration rate or the *D* value.

The dark respiration may also decrease due to changes in the carbohydrates status in the leaves. In our measurements, dark respiration decreased immediately upon darkening which might be consistent with carbohydrates status. It is also possible that the sampling and the resulting decrease in pressure in the chamber caused decrease in respiration. The range of respiration rate measured for alfalfa sprouts (control plant) at the beginning of the measurements was 0.11–0.13 (μmol O_2 $min^{-1}g^{-1}$), consistent with the respiration rate reported by the accepted method [23]. Therefore, the respiration rates, which were determined at the beginning of each sampling set, were considered reliable.

6. ON-LINE MEASUREMENTS OF ^{18}O IN WATER VAPOR: PYROLYSIS OF μL SIZE WATER SAMPLES

6.1. Introduction

Methodology for isotopic analysis of large water samples is well documented [30]. Recent attempts to apply stable isotopes to the study of evapotranspiration at the canopy scale required, however, modifications associated primarily with the analysis of small quantities of atmospheric moisture. Although for hydrogen isotopic analysis the conventional methods using Uranium [32] or Zinc [34] were designed for small water samples of a few μL, oxygen isotopic analysis was usually carried out on samples of a few mL (but see section on small sample equilibration above). The availability of an efficient method for the ^{18}O analysis of μL size water vapor sample has been a major limiting factor in the incorporation of stable isotopes to eco-hydrology studies. Here I briefly describe an on-line pyrolysis-IRMS method for the ^{18}O analysis of very small water samples that has been successfully used in field studies of atmospheric moisture.

6.2. Air moisture sampling

A general method was recently employed by [35] for simple short term sampling of ambient air moisture at different locations within and above a canopy. In this approach, air is sucked, at different levels, by a small diaphragm pump through low-adsorption plastic tubes (e.g. Teflon, Bev-a-Line) and a small, low cost cryogenic trap at –80°C, at a rate of about 250 ml min^{-1} for about 30 min. Pump and traps are located on the ground down wind of the sampling site and the tubings are flushed with sample air before the actual trapping. After sampling, traps are sealed and transported to the lab for analysis. Small, simple and inexpensive glass traps were used both for the moisture trapping and sample storage.

The trap design was based on that of [36] used for trapping reduced sulfur compounds and consisted of a 6 mm o.d. Pyrex tube looped six times over a central arm (Fig. 6). The trap had two bulb type configurations with the first to prevent clogging by ice and was positioned at the top of the cold portion of the trap on the intake side; the second was at the bottom of the trap, where most of the moisture accumulated. In addition, Pyrex wool was fitted a few cm below the outlet of the trap to prevent the escape of flakes. The trap had two simple tube endings and were connected on-line with conventional connectors (e.g. ultra-torr Cajon fittings). After sampling, traps were disconnected and sealed with plastic fittings (e.g. Swagelock).

Large water samples could be decanted off the trap, while small samples were quantitatively distilled under vacuum at 60°C and collected by freezing into a short 6 mm tube on a vacuum line. After thawing, 20~30 μL aliquots were stored in 50 μL uncoated Pyrex capillaries (Monoject Scientific, USA) that were flame sealed at both ends (Blazer, Piezo micro torch, Japan). These samples could be stored for extended periods and were convenient for shipping when necessary.

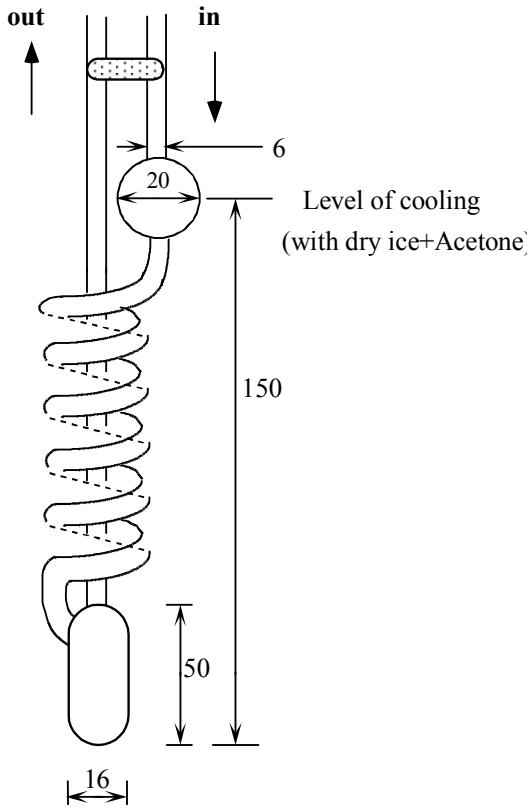

Fig. 6. A water trap for collecting small air moisture samples (b) used in the field setup. The trap is made of Pyrex tube and the dimensions shown are in mm.

6.3. ^{18}O analysis of μL water samples

The conventional water-CO_2 equilibrium approach can be used for small water samples [37], see section on equilibration above), but is labor intensive and requires corrections for equilibrium and evaporation isotope effects in the equilibration vessels. Small water samples can also be analyzed by the Guanidine Hydrochloride method in which the oxygen of water is quantitatively converted to CO_2 for the isotopic analysis [38]. This method, and a few others [30, 39] have not been widely applied, probably because of their complexity and the relatively low sample output. Here we describe a simple and rapid technique for the analysis of 0.2~2 μL water samples carried out on-line with an isotope ratio mass spectrometer. This method is based on the original approach of [40].

An elemental analyzer (e.g. EA1108, Carlo Erba Instruments, Inc., Italy) is used for the pyrolysis (1090°C) of 0.1–2 μL of water samples (the same method is used also for pyrolizing about 1 mg of organic matter) on a nickelised carbon (Elemental Microanalysis Limited, Devon, UK) column to quantitatively produce carbon monoxide. The CO sample was separated from other gases (primarily N_2) and focused on a packed GC column (9 mm, molecular sieve 5A, 80/100 mesh, 70°C) and was carried, on-line, by the He carrier gas (120 mL min^{-1}) through an open split into the isotope ratio mass spectrometer through an on-line port (e.g. Optima, Micromass, UK). The 30/28 mass ratio of the sample and a reference CO gas (injected between samples from the reference-bellow of the mass spectrometer's dual inlet) were used for determination of the $\delta^{18}O$ values of the samples. Because mass 28 used for the analysis of CO is the same as that for N_2, care must be taken to avoid air leaks in the system. The pyrolysis is also sensitive to both blank and memory effects [40]. The major blank effect was due to oxygen interactions with glass (e.g. the reaction tube, glass chips and glass wool). This was minimized by replacing the conventional quartz reaction tube with one made of ceramic (e.g. Carlo Erba, Italy; Bolt Technical, Texas). Similarly, any quartz chips required for the column packing

were replaced with ceramic chips (prepared by sacrificing a reaction column), and quartz wool was replaced with silver wool for packing purposes. The memory effects due to interactions of oxygen with the carbon in the reaction tube, was minimized by a combination of doping the helium carrier stream with 5% CCl4 in heptane, reducing by half the quantity of the carbon reactant, as compared to conventional packing for oxygen analysis, and by "flushing" the system with a "blank" sample prior to sample analysis. Doping was achieved by placing a glass capsule (ca 10 mm long, 5 mm o.d. with a 1 mm hole on its side) filled to about half with the doping solution inside ca. 200 mm long 13 mm o.d. glass tube that was fitted on the carrier gas line. A packed reaction column used in the above analysis was 46 cm long and filled, in going from bottom to top, with 3 cm quartz wool (positioned outside the furnace), 18 cm ceramic chips, 1 cm silver wool, 2 cm Nickelised Carbon (substituted in some cases by spectrographic graphite). The top of the carbon reagent was positioned at the center of the furnace. The speed of the analysis was about 4 min. per sample.

Handling of water samples was critical for the analysis, and two approaches were used. The first employed the standard multi-sample carousel of the EA. In this case ca. 1 μl water sample was placed with a gas-tight syringe (Hamilton Co., Reno, Nevada, USA) in a silver capsule and dropped directly into the first sample position with no waiting time for the EA analysis. Silver capsules were made hydrophobic by annealing at 400^{o}C and slow cooling under vacuum. This allowed the placement of a single ca. 1 μl droplet onto the bottom of the capsule and sealing the capsule without "smearing" the water sample. Such arrangement avoided evaporative loss for the time needed for the transfer and initiation of the pyrolysis (tested as a weight drift<0.01 mg per minute, compared with about 10 sec for sample handling). The second approach employed an injector provided by the manufacturer of the analyzer (Carlo Erba Inst., Italy) that replaces the standard multi-sample carousel. This injector allowed conventional GC mode operation by injection of the small water sample directly into the reactor through a septa. A gas tight syringe with a long hypodermic needle was used to facilitate the delivery of the sample to the center of the furnace. The first approach described above was generally adopted.

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CONTINUOUS FLOW IRMS APPLICATION TO CH_4, NMHCS, AND N_2O IN THE ATMOSPHERE AND THE OCEANS

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Abstract. The application of CF-IRMS to measurement of methane (CH4), non-methane hydrocarbons (NMHCS), and nitrous oxide (N_2O) is outlined and preliminary information on isotopic variations in the atmosphere and oceans is presented. Labelling of these compounds is expected to provide a robust method for tracing sources, sinks and controlling processes in the environment.

1. INTRODUCTION

Methane and N_2O are naturally occurring trace gases which have important roles in global warming and stratospheric ozone budget, while non-methane hydrocarbons (NMHCs) are controlling the atmospheric oxidation reactions which are major methane sinks as well. Isotope containing molecules (isotopomers) of these gases are considered to have information of their sources, sinks, and geochemical cycles. It was, however, difficult to determine their isotope ratios before the application of continuous flow IRMS, a most sensitive and precise analytical method at present.

Samples of 0.3 to 1 nmol are analyzed for several background atmosphere and for samples collected from terrestrial and marine environment along with latitudinal transect. Carbon 13 isotopic composition of methane and NMHCs has been found to have distinct information for the difference in their sources.

Information and data base for the global budget, sources and sinks strength are more limited for N_2O than methane and carbon dioxide. Nitrogen and oxygen isotope ratios of atmospheric N_2O are determined by tropospheric sources and stratospheric sinks, especially nitrification and denitrification in terrestrial and marine environments and UV photolysis. We have recently originated the isotopomer analysis of N_2O.

Isotopic analysis of these naturally occurring gases have been found to have potential importance to provide quantitative constraints for reducing uncertainties with their budget, source and sink processes, and relative importance of the area or sphere to act as sources and/or sinks. Wider circulation and interlaboratory calibration of the standards for these gases in pure and the background levels becomes very important. Isotopomer standards should also be taken into account very soon.

2. CARBON ISOTOPIC COMPOSITIONS OF CH_4, NMHCS AND METHYL CHLORIDE

Studies on the stable isotopic composition of atmospheric carbon species of greater than about ppmv mixing ratio, such as CO_2 and CH_4, have provided valuable information for understanding the relative strengths of the production and removal processes involved in atmospheric cycling [1–3].

Samples of 0.3 to 1 nmol are analyzed for several background atmosphere and for samples collected from terrestrial and marine environment along with latitudinal transect using continuous-flow GC/C/IRMS. The samples collected from Indian Ocean are analyzed for their ^{13}C revealing the importance of the air-sea interactions and the oxidation of CH_4 in the deeper water columns.

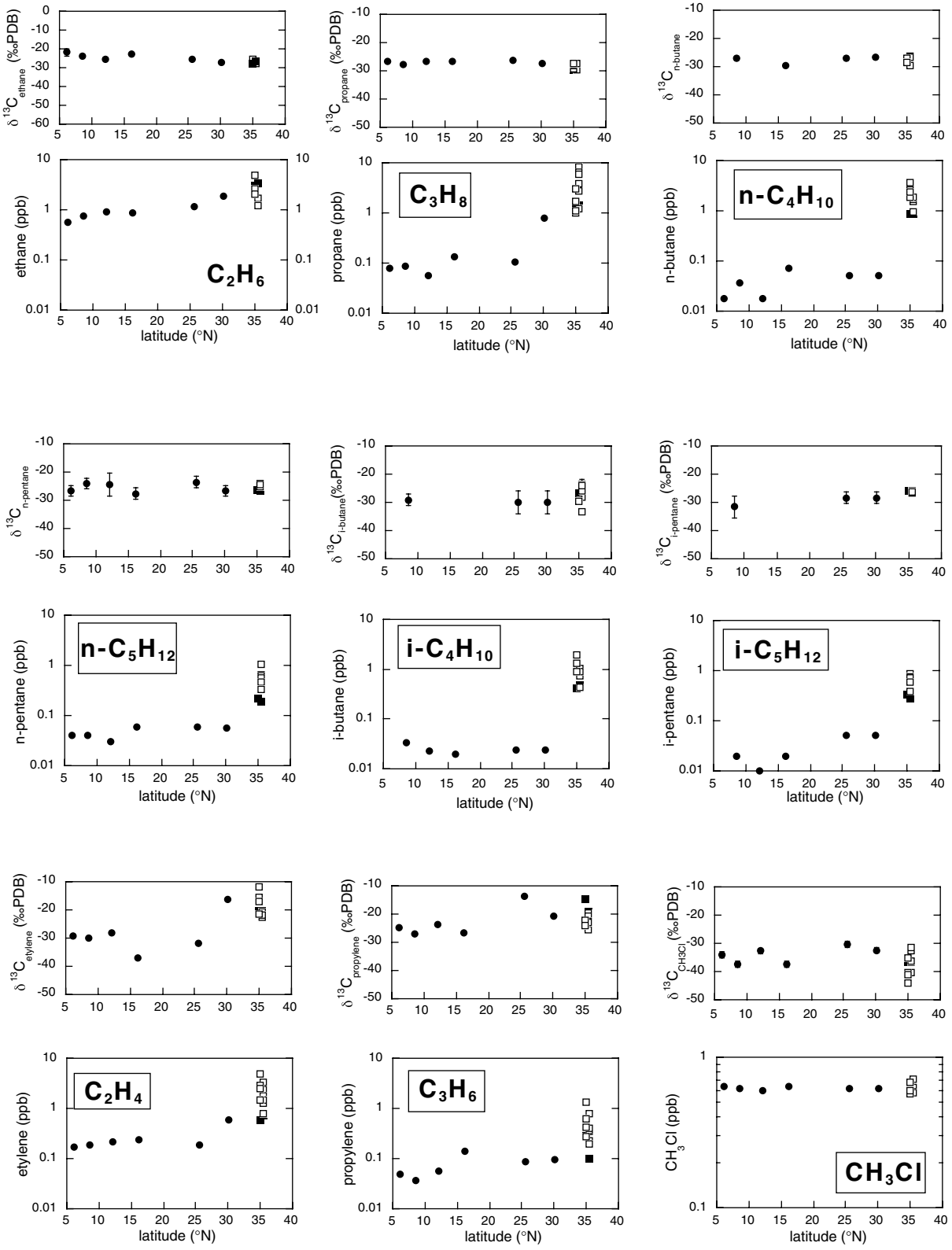

Fig. 1. Latitudinal variations of the measured mixing ratios and the carbon isotopic compositions of NMHCs and CH_3Cl in maritime (solid circles), coastal (solid squares), and urban atmospheres (open squares).

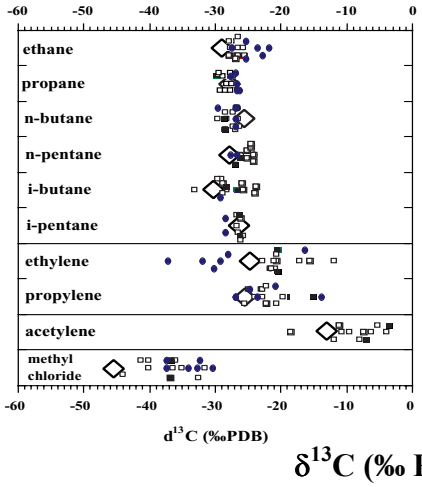

Fig. 2. Isotopic compositions of each component in samples (see Fig. 1 for the symbols), together with that of biomass burning plume (C-3 plant [5]) (large open diamonds). Isotopic data with more than 2 per mil error are excluded.

In the case of trace components such as light NMHCs and methyl chloride, however, data describing their isotopic compositions are scarce [4, 5], especially regarding those in maritime atmosphere. C_2–C_5 non-methane hydrocarbons (NMHCs) and methyl chloride in the remote maritime atmosphere are analyzed in regard to their variation of mixing ratio and $^{13}C/^{12}C$ ratio ($\delta^{13}C$), together with those in polluted urban and coastal atmospheres in Japan [6]. NMHCs show large atmospheric mixing ratio differences between urban (coastal) and maritime atmospheres probably due to emission from urban areas and degradation within the maritime atmosphere as shown in Fig. 1. Reflecting isotopic fractionation during the degradation within the maritime atmosphere, ethane shows large and systematic $\delta^{13}C$ variation between urban (around –27‰ Peedee belemnite(PDB)) and maritime atmospheres (up to –22‰ PDB). Except for ethane, however, alkanes show small isotopic variation around $\delta^{13}C = -27 \pm 2$‰ PDB (1σ) without systematic isotopic differences between urban and maritime atmospheres, suggesting both small $\delta^{13}C$ variation within major emission sources and also little isotopic fractionation during atmospheric degradation for alkanes other than ethane.

Alkenes show large $\delta^{13}C$ variation from –37 to –12‰ PDB for ethylene and from –27 to –14‰ PDB for propylene as shown in Fig. 2. Combination of both large $\delta^{13}C$ differences between major sources (especially between land and maritime sources) and large isotopic fractionation effect during atmospheric degradation can be suggested for alkenes. Methyl chloride also shows large isotopic variation from –44 to –30‰ PDB in spite of their similar atmospheric mixing ratios from 580 to 710 parts per trillion by volume (pptv), probably due to the contribution of highly ^{13}C-depleted, anthropogenic methyl chloride especially to urban atmospheres.

The general $\delta^{13}C$ pattern of NMHCs and methyl chloride in polluted urban city air agrees strongly with those of biomass (C-3 plant) burning plumes, suggesting that thermal breakdown of C-3 plant (or related organic matter) is one of the representative sources of these hydrocarbons in urban atmospheres. Further investigations of the isotopic signature of source materials as well as laboratory studies of isotopic fractionation processes resulting from atmospheric degradation will improve our understanding of the sources, sinks, and atmospheric distributions of NMHCs and methyl chloride.

3. DETERMINATION OF NITROGEN ISOTOPOMERS OF NITROUS OXIDE

Nitrous oxide (N_2O) is a trace gas in the atmosphere that plays an important role in the tropospheric greenhouse effect and stratospheric chemistry, which regulates the ozone layer. Although its tropospheric concentration is increasing by 0.2–0.3% per year [7], the global budget and/or cycle of N_2O has not been well understood because it has a variety of sources (both natural and anthropogenic)

and sinks. Nitrogen and oxygen isotope ratios of N_2O for several environments have been reported and they are considered to be useful parameters for examining the source–sink relationship.

Previous studies of isotopic characterization of N_2O have been based on the elements contained in the molecule, i.e. nitrogen and/or oxygen [6–19]. Additional information, however, would be obtained if the intra-molecular distribution of nitrogen isotopes could be determined, since the N_2O molecule has an asymmetric linear structure (N-N-O) and thus site preference of nitrogen isotopes in naturally occurring fractionation processes is expected. For example, Yung and Miller [20] estimated that $^{15}N^{14}N^{16}O$ and $^{14}N^{15}N^{16}O$ have different fractionation factors in the stratospheric photodissociation based on a theoretical calculation. Hence, determination of isotopomers of N_2O in the environment and those incorporated in processes simulated in the laboratory has the potential to reveal the contribution of sources and sinks of N_2O, either recognized or proposed ones.

A new method [21] for the determination of the nitrogen isotopomers (intra-molecular distribution of the nitrogen isotopes) of nitrous oxide has been developed in the present study as illustrated in Fig. 3. The precision of isotope ratio measurements at each specific site relative to the reference gas is better than 0.1‰ for pure N_2O samples introduced through a conventional dual-inlet system on a mass spectrometer equipped with a special ion collector system.

Although it is found that the observed isotope ratio is affected by rearrangement reactions in the ion source, a correction can be applied using an experimentally determined rearrangement fraction. Calibration of the standard N_2O for isotopomer measurements is performed by two procedures: (1) preparation of an N_2O standard by thermal decomposition of NH_4NO_3, (2) relative measurements with pure NO.

This new technique is expected to become a useful tool for studying mechanisms of production and consumption, and the global budget of N_2O. Applications of this technique to various environmental samples are now under investigation.

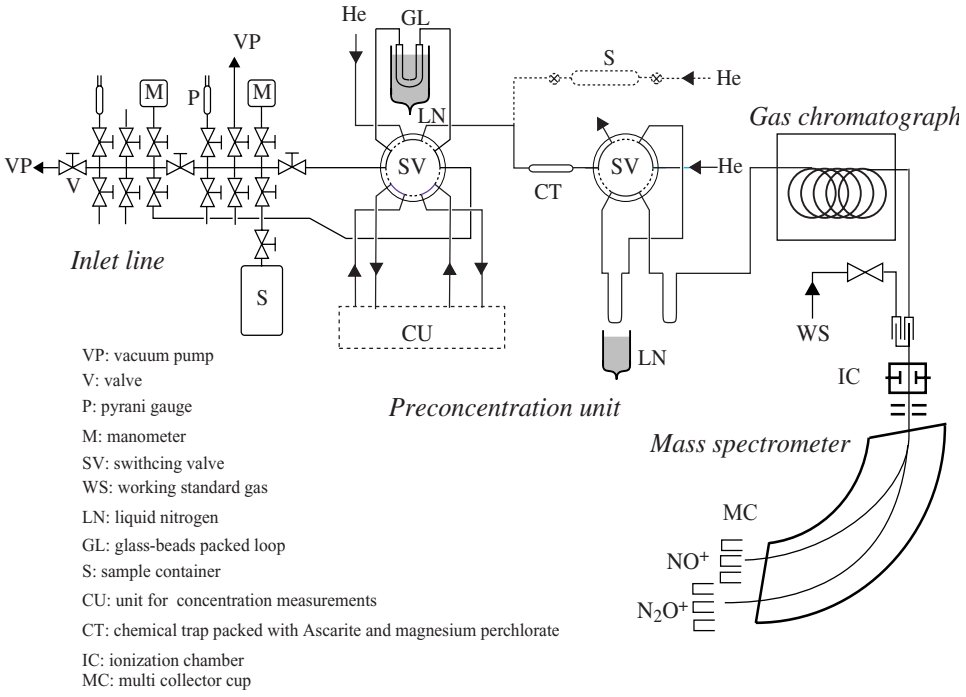

Fig. 3. Schematic diagram of the measurement of N_2O isotopomers on a mass spectrometer.

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ANNEX

TABLE I. LIST OF CANDIDATE REFERENCE MATERIALS
AS PROPOSED BY THE WORKING GROUPS

Compound	Isotope	Technique	Reference material	Precision required for RM
Cellulose	^{13}C, ^{18}O	CF-IRMS		<0.2‰
	^{2}H	CF-IRMS		<2‰
Thiourea	^{13}C, ^{15}N, ^{18}O, ^{34}S	CF-IRMS		<0.2‰
	^{2}H	CF-IRMS		<2‰
Methionine	^{13}C, ^{15}N, ^{18}O, ^{34}S	CF-IRMS		<0.2‰
	^{2}H	CF-IRMS		<2‰
N-Methyl anthranilic acid ester	^{13}C, ^{15}N, ^{18}O, ^{34}S	CF-IRMS		<0.2‰
	^{2}H	CF-IRMS		<2‰
Fatty acid methyl ester (C12, C18)	^{13}C, ^{18}O	CF-IRMS		<0.2‰
	^{2}H	CF-IRMS		<2‰
GROB-Test (mixture)	^{13}C, ^{18}O	GC-IRMS		<0.2‰
Enriched dilution series	^{13}C, ^{15}N, ^{18}O	CF-IRMS, optical techniques	H_2O, C-containing material (CO_2, carbonate, other), N-containing material	0.1‰ 1‰ of δ
	^{2}H	CF-IRMS, optical techniques		0.8‰ 1‰ of δ
CH_4	^{2}H	CF-IRMS, TD-LAS	Whole air samples	<0.5‰
	^{13}C	CF-IRMS, TD-LAS	Whole air samples	<0.05‰
CO	^{13}C, ^{18}O	CF-IRMS	Whole air samples	<0.1‰
N_2O	^{15}N (bulk), ^{15}N (center), ^{18}O	CF-IRMS, TD-LAS, FT-IR	Whole air samples	<0.05‰
H_2, CH_4, CO, N_2O	^{2}H, ^{13}C, ^{14}C, ^{15}N, ^{18}O	IrmMS	Trace gas concentrate cocktail	as above
CH_4, CO, N_2O, O_3 in stratosphere	^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{18}O	irmMS, TD-LAS, FT-IR	Enriched (pure) gases (^{2}H: 0‰, ^{13}C: 0‰, ^{15}N: 150‰, ^{18}O: 100 ‰)	as above
NMHC	^{13}C	CF-IRMS	?	?
CH_4	^{2}H	CF-IRMS, TD-LAS	Whole air samples	<0.5‰

WORKING GROUP REPORT A — ATMOSPHERE

Stable isotope techniques are being increasingly used to study problems in the atmosphere ranging from global background studies of the principal greenhouse gases, source and sink process studies, Kyoto protocol compliance issues, to air pollution work.

The present techniques are limited because in most cases very large air samples (for example >50 litre for 13C in methane) are needed due to very low concentrations of species of interest. Another complicating factor is that the range of delta value variations in nature are quite small so that high precision techniques are needed for background determinations in the atmosphere, the oceans, and in the terrestrial environments.

1. AIM OF THE REPORT

Up until recently almost all stable isotope measurements of species of interest in atmospheric studies have been made by conventional dual inlet IRMS. However there are now many new optical and IRMS techniques available for the measurement of isotope ratios in nature. The use of these techniques is not widespread and they have not been widely evaluated at present. One of the aims of this report will be to provide some recommendations for calibration and implementation of these new analytical techniques, e.g. CF-IRMS and some new optical techniques in and atmospheric studies.

2. PRESENT WORK

We estimate that there are 20 to 30 labs making stable isotope measurements in CO_2 world wide using conventional dual inlet IRMS. The IAEA and the WMO have already had considerable involvement with calibration protocols for this gas. However very recent work shows that CF-IRMS will meet the IAEA target precision for ^{13}C and ^{18}O and hence new protocols may be needed.
For atmospheric methane we estimate that there are about 10 laboratories world wide making ^{13}C determinations and about 5 making D determinations. There are no protocols for international inter calibration and inter comparison procedures.
For atmospheric N_2O there are for bulk ^{15}N and ^{18}O about 5 labs, ^{17}O determinations are being done by 2 labs, ^{15}N isotopomer determinations just started by 3 labs.

3. RANGE OF APPLICATIONS

(A) Direct applications to environmental problems

- Principal Greenhouse gases, CO_2, CH_4 and N_2O determinations for Kyoto protocol, atmospheric measurements in background air
- Marine environment/water column measurements
- Time series data from ground station network
- Firn air and ice core determinations
- Active trace gases
- Air pollution studies
- Budget studies

(B) Purely scientific studies

- Mass independent fractionation
- Mass dependent fractionation
- Reaction mechanisms and kinetics

4. RANGE OF SAMPLES

Categories

- Principal Greenhouse gases, CO_2, CH_4 and N_2O determinations for Kyoto protocol atmospheric measurements in background air
- Marine environment/water column measurements
- Time series data from ground station network
- Firn air and ice core determinations
- Active trace gases
- Air pollution studies
- Budget studies
- Tropospheric air/stratospheric air

5. RANGE OF ANALYTICAL EQUIPMENT

In atmospheric studies to date principally used dual inlet IRMS with some work done by FTIR and TD-LAS and small amount by continuous flow IRMS.
In the future likely to be much more use of optical techniques as well as widespread use of CF-IRMS. The new techniques have huge advantages for example they are fast, sample size can be up to 1000 times smaller, more selective than dual inlet IRMS and offer high resolution. The present disadvantages of the new techniques are that precision may not be sufficient for some atmospheric studies and that calibration techniques are not worked out

6. CALIBRATION

The only atmospheric species for which inter calibration and intercomparison protocols exist is CO_2 and these are all based on dual inlet methods. A very promising CF-IRMS technique has been developed for ^{13}C and ^{18}O determinations in atmospheric CO_2 and will require some adaptation to existing procedures used for calibrating conventional dual inlet IRMS procedures for atmospheric CO_2. However there are no similar protocols for other atmospheric species including methane and N_2O for any technique.

7. LOGISTICS

- Protocol for the intercomparison of CH_4, N_2O, O_3, CO, NMHC should be settled.
- Prepare standards of these gases in the isotope ratios of tropospheric and stratospheric end members referred to the primary IAEA standards.
- Identify the labs undertaking background level measurements and deliver these standards.
- Integrate and analyse the results performed by different methods and hardware, such as, dual-inlet IRMS, CF-IRMS, GC/C/IRMS, TDLAS, FTIR, and other new optical methods.

8. RECOMMENDATIONS

- Set up techniques to trace back working standards to primary IAEA calibration standards VSMOW, NBS19 and atmospheric nitrogen.
- Use whole air samples as intercomparison materials to exchange between labs making these measurements. This has the advantage that the same preparation and analysis path as unknown air samples is followed. With dual inlet IRMS this is difficult because samples are huge and require very expensive shipment of high pressure cylinders of air as dangerous goods. CF-IRMS will bring in the great advantage that much smaller air samples can be used. We recommend that low pressure air samples in glass flasks are used. These can be sent by regular parcel post very cheaply.

- For the studies in the stratospheric background, highly enriched standards are needed for CH_4, N_2O, O_3, and CO. We recommend to have standards for the enriched end member in D, ^{13}C, ^{15}N, and ^{18}O in the range of around 0 for δD, 0 for $\delta^{13}C$, 150 for $\delta^{15}N$, and 100 for $\delta^{18}O$ respectively.
- Another possibility to avoid the use of high-pressure cylinders would be the preparation of a synthetic ”trace gas concentrate” mixed into pure N_2 at relative concentration levels as well as isotopic compositions close to the environmental values of the different components (e. g., CH_4, CO, N_2O and H_2). The ”trace gas concentrate” could be shipped in amounts of only 1 liter STP to laboratories around the world where the initial concentrations would then be mixed by ”zero air” depleted by the relevant component(s). The reconstitution could be achieved either dynamically using mass flow controllers or statically by mixture into a large volume. The required gasses would be extracted from ambient air, wherever applicable. The isotopic and concentration integrity of the enriched material in the primary cylinder have to ascertained.
- For other species like NMHC, isoprene and terpenes the situation is more difficult and will require further research.

TABLE II. LIST OF ATMOSPHERIC SPECIES OF INTEREST
AND PRECISION REQUIRED TO MEET SCIENTIFIC REQUIREMENTS

Gas	Isotope	Measurement technique	Precision needed	Standard
CO_2	^{13}C	IrmMS		
	^{14}C	AMS		
	^{17}O	IrmMS		
	^{18}O	IrmMS		
CH_4	^{13}C	DI-irmMS, CF-irmMS, TD-LAS	<0.01‰	NBS19
	^{14}C	AMS	<1 pMC	Oxalic acid
	^{2}H	DI-irmMS, CfirmMS, TD-LAS	<0.5‰	VSMOW
N_2O	^{15}N (bulk)	DI-irmMS, CF-irmMS, TD-LAS, FT-IR	<0.05‰, better 0.02‰	air-N_2
	^{15}N (center)	DI-irmMS, CF-irmMS, , TD-LAS, FT-IR	<0.05‰, better 0.02‰	air-N_2
	^{17}O	DI-irmMS, , TD-LAS, FT-IR	<0.1‰	VSMOW
	^{18}O	DI-irmMS, CF-irmMS, , TD-LAS, FT-IR	<0.05‰, better 0.02‰	VSMOW
CO	^{13}C	DI-irmMS, CF-irmMS	<0.1‰	NBS19
	^{14}C	AMS	<1 pMC	oxalic acid
	^{17}O	DI-irmMS	<0.1‰	VSMOW
	^{18}O	DI-irmMS, CF-irmMS	<0.1‰	VSMOW
NMHC	^{13}C	CF-irmMS	<0.2‰	NBS19

WORKING GROUP REPORT B — BIOGEOCHEMISTRY, FOOD AND ECOLOGY

1. RANGE OF APPLICATIONS/TECHNIQUES

EA-Combustion-IRMS
EA-Pyrolysis-IRMS
GC-Pyrolysis-IRMS
GC-Combustion-IRMS
MDGC-Combustion-IRMS
MDGC-Pyrolysis-IRMS

2. ACCURACY AND PRECISION

There are large differences between the capabilities of the instrument technologies and the demands each application requires.
It is important to recognise that there is a distinction to be made between the analytical accuracy and precision of the IRMS. Precision obtained for isotope ratios of simple gas species (CO_2, H_2, N_2) introduced through dual inlet is generally better that achieved with continous flow techniques. For example, precisions of 0.02‰ are readily achieved for CO_2 gas generated off-line for dual inlet. In contrast, in GC-IRMS applications precisions are commonly of 0.3‰.
The situation becomes more complicated by the use of the derivatization reagents which makes the errors much larger for back calculations of original isotope compositions of compounds.

3. CALIBRATION NEEDS

There are two types of calibration needed for the above applications. One is in the use of **bulk material isotope standards** for continuous flow applications (EA-Combustion-IRMS, EA-Pyrolysis-IRMS) and the other is a unique molecular component for **compound specific isotope analyses (CSIA)**. Examples of these applications include GC-C-IRMS, GC-Pyrolysis-IRMS, MDGC-C-IRMS, MDGC-Pyrolysis-IRMS. These compounds specifically designed for CSIA could also be used for EA techniques.

4. BULK MATERIAL ISOTOPE STANDARDS

Although there exist organic standards for ^{13}C bulk material isotope analysis, there is no single reference material for both carbon and oxygen stable isotope abundances. Presently there is a standard of cellulose (IAEA-CH-3) in the intercalibration stage that holds promise for use on carbon, oxygen and hydrogen isotope determinations. However, the calibration of this cellulose still needs to be accomplished as a reference material versus the primary calibrated standards for these elements.
At present there is no appropriate organic standard material which contains nitrogen (or sulphur). Such a material could be developed, and could contain all five light isotope elements of interest to the above defined group of users. Examples of materials which fulfil this composition requirement could be thiourea or methionine. This material because of the known chemical formula will also serve as a CHNOS element abundance calibrant.

5. COMPOUND SPECIFIC STANDARDS

It will be impossible for the IAEA or any other Agency to provide CSIA standard compounds for all possible analytical schemes. Our working group suggest that the needs for secondary reference materials can be addressed through a limited number of calibrated materials that bracket a range of elemental compositions, analytical conditions and technologies. This could be achieved through between two to four selected compounds within an homologous series, example of which the N-methyl anthranilic acid esters. We suggest the methyl and a longer carbon chain (perhaps octyl) derivative would be appropriate. This compound contains carbon, oxygen, nitrogen and hydrogen and

could be used for assessing the multielement isotope determinations by a variety of techniques. Additionally, the series of compounds could be useful for testing chromatographic and IRMS interface performances, calibration biases as a result of molecular weight and software capabilities to eliminate background contributions from, for example, column bleed.

At the present time, and additional need exists in GC-Pyrolysis because of the co-elution of CO with N_2. There exists a need for calibration with a material which does not contain nitrogen as an alternative to the multielement standard recommended. Such a material could be two compounds such as, C_{12} and C_{18} fatty acids methyl ester.

For a new material of this nature, there will be a need for rigorous testing for chemical and isotope stability as a result of, for example, volatilization, oxidation, decomposition, etc. The compounds could be available as either a single component or in a mixture.

6. LOGISTICS

- Cellulose (IAEA-CH-3): continue IAEA interlaboratory calibrations against primary standards. For ^{18}O and maybe ^{13}C calibrations, coordinated activities should be made with the current on-going European project SUGAR 180. Such intercalibrations should also evaluate the results based on different methodologies that will include:
  - reagent materials (Ni-carbon, glassy-carbon; spectroscopic-carbon).
  - tube materials(quartz, ceramic, carbon).
- Thiourea, methionine, C_{12} and C_{18} fatty acids methyl esters and N-methyl anthranilic acid esters
  - Produce homogeneous and pure materials for these molecules
  - Preliminary tests for stabilility of the materials including storage and packaging should be planned.
  - The necessary amount is thought to be of the order of a few kg for each compound. These tasks can be attributed to selected laboratories having the necessary equipment and experience to carry out these tasks under the supervision of IAEA.
- Identify laboratories for comparison of EA-IRMS and off-line dual inlet analysis for testing multielement references materials against IAEA primary standards

7. OUTLOOK IN THE FUTURE

All primary standard materials must be certified according to the ISO 9000 guidelines.
For ^{18}O the VSMOW-SLAP and the VPDB scale have to be connected for organic and inorganic substances.
One has to keep in mind intramolecular isotopic composition for future applications and future technologies should also take in account sulphur compound standards.
Most relevant applications seem to be: food-webs and material fluxes in ecology, biochemical substances (amino acids, fatty acids, lipids), forensic application and the range of palaeoclimatic research as palaeoclimate, ancient food or bones, and finally food control (authenticity).
The most critical point seems to be the errors due to separation, interferences with the column material and for hydrogen the isotopic exchange.

8. RECOMMENDATIONS

- All primary standard materials must be certified according to the ISO 9000 guidelines. For ^{18}O the VSMOW-SLAP and the VPDB scale have to be connected for organic and inorganic substances.
- The cellulose standard (IAEA-CH-3) calibration for carbon, oxygen and hydrogen isotope determinations needs to be accomplished as a reference material for bulk analysis versus the primary calibrated standards.

- At present there is no appropriate organic standard material for bulk analysis which contains nitrogen. Such a material could be developed, and should contain all five light isotope elements, an example of which could be thiourea or methionine.
- For compound specific standards, there is a need for a calibrated material that contains carbon, oxygen, nitrogen and hydrogen. This standard could be used for assessing multielement isotope determinations by a variety of techniques. We suggest the methyl and a longer carbon chain (perhaps octyl) derivatives of the N-methyl anthranilic acid esters.
- An additional need exists in GC-Pyrolysis for calibration with a material which does not contain nitrogen as an alternative to the multielement standard recommended. Such a material could be two compounds, such as, C_{12} and C_{18} fatty acids methyl esters.
- A need for rigorous testing for chemical and isotope stability is required for all these new standards.
- The compound specific reference materials should be available as either a single component or in a mixture.

WORKING GROUP REPORT C — HYDROLOGY AND OCEANOGRAPHY

No detailed assessment was made on the situation in hydrology and oceanography. Since this is the most traditional field of application of stable isotope techniques, sufficient documentation exists on conventional analytical techniques, calibration strategies and interlaboratory comparisons. Recently in the field of on-line techniques for δ^2H analysis several new methods were proposed and implemented, facilitating the analytical procedures and increasing the sample throughput, but unfortunately not yet significantly increasing the precision of measurements.

RECOMMENDATIONS

- All laboratories handling water samples should follow a routine for calibration and normalisation on a daily basis. This calibration and normalisation should be based on 2 local water standards, that are distinctly different (preferably one similar to VSMOW, the other to SLAP). The local water standards should be checked for stability, and should be periodically calibrated against the primary standards VSMOW and SLAP.
- It should be realised that the value of the “light” local water standard w.r.t VSMOW (that is, calibrated, but not yet normalised) forms a valuable record, with information about stability of the IRMS system and the water preparation line.
- All laboratories co-operating in maintaining the IAEA GNIP network (Global Network of Isotopes in Precipitation) should recognise that careful and reliable calibration & normalisation is crucial for the quality and usefulness of the GNIP database. Only in that way the total GNIP network, consisting of measurements of tens of individual laboratories, can be merged successfully. To test the consistency, regular intercomparisons are necessary.

WORKING GROUP REPORT D — LABELLED COMPOUNDS

1. RESEARCH USING LABELLED COMPOUNDS

Medical, Biological and Agricultural Applications

2. DEFINITIONS, APPLICATIONS, SAMPLES

Applications of stable isotope ratio measurements using labelled compounds encompass the use of isotopic ratios for medical research, clinical medicine, drug synthesis, biological and agricultural studies. Samples often are highly enriched beyond natural abundance, where we take highly enriched to be roughly 2 or more times the natural value.

Samples to be analysed may be of two enrichment levels- first, the highly enriched labelled compound, and secondly the metabolic products- ranging from highly enriched to near natural values. Such samples may be derived from breath, blood, sweat, saliva, urine, faeces, hair or other tissue including plant tissue for crop research.

Deviations from normal natural abundance values for certain samples may also be important to measure, even in the absence of a labelled compound precursor as an indication of diet, geographical origin and health or disease status. However, in these cases the problems, calibrations etc. are similar to those in isotope geochemistry.

In addition, stable isotope studies may be research based or clinic based, where research involves laboratory studies including the use of animals and informed volunteers; and clinical use involves isotope ratio measurements of patient samples for the purpose of medical diagnostics or treatment.

Though many isotopes and compounds have been used, most common is the use of ^{13}C in drugs and foods for metabolic studies, and ^{18}O and D in doubly labelled water studies for energy expenditure. Next most important is ^{15}N for protein turnover and agricultural studies.

3. ANALYSIS

Due to the range of isotopic ratios to be analysed and the use of the results for both quality control, research and clinical decisions, two regimes for analysis are identified.

First is quality control and authentication of labelled material. For this purpose new procedures and techniques, discussed below, are required. As drugs are now uniformly subject to control for chemical purity and stability, similar tests are required for isotopic purity and stability. For drugs this may also involve verification of isotopic site selectivity in the compound.

Second is analysis of samples at and near natural abundance where analysis requirements are similar to those of environmental samples. Especially important is the analysis of changes in isotope ratio with time over minutes to several days.

4. EQUIPMENT

The equipment required for labelled compound work, as in other fields, depends on sample type, element studied and precision required. There are unique requirements, however, for equipment used in a clinical and field setting.

Standards are in place in most countries for medical devices which include analytical instruments used to evaluate patient samples. Thus far, few isotope ratio devices have been approved as medical instruments for routine clinical use and standards for such instruments have not yet been set. The ^{13}C

urea breath test for *Helicobacter-Pylori* infection diagnosis is the first stable isotope breath test approved for clinical use in the United States and EC. Instruments for clinical use must have procedures and safeguards in place to insure that results obtained are correct within a quoted precision and accuracy. As clinical instruments are often operated by semiskilled personnel, self testing and self calibration are important.

The requirements for field deployment are similar to those required for clinical use — ease of use, self diagnostic, robust and moderate cost.

5. ACCURACY, PRECISION AND STABILITY REQUIRED

Analysis of metabolised or diluted material often requires a differential analysis where time series differences in enrichment are the important parameters, rather than absolute values with respect to a reference material. For this, internal calibration protocols must be developed. Dependent on the analysis technique used, the individual samples are either directly compared to each other (dual inlet IRMS where one of the samples serves as "working gas", the other one as "sample", or in optical techniques), or are introduced into the measuring device sequentially (CF-IRMS).
For these "differential" measurements, for which only one isotope is of interest, requirements in terms of precision, accuracy and stability are rather relaxed compared to other fields of applications, and we believe can be easily achieved by several types of instrumentation on the market.

For moderately enriched samples, (i.e. in the same range as the natural variations for the specific isotope) typical individual measurement precision would be <0.1‰, and the stability of the apparatus should be such, that variations of the produced isotope ratios would remain within +/-0.1‰ borders during the time necessary to perform the full time series measurement.

A different item is the so-called scale variability: the way the measuring device reproduces a true 1‰ variation. IRMS devices are known to generally "contract" their scales towards the machine reference gas used or, to put it in other words, a true 1‰ difference is usually measured as somewhat less than 1‰. For optical instruments there are quite different mechanisms involved, and not much experience has been gained, but similar scale contractions (or expansions) cannot be ruled out.
Typical scale deviations in IRMS machines amount to up to 2% of signal range for CO_2, and somewhat more for H_2.

For moderately enriched samples, the scale variability uncertainties are therefore usually of no importance. Still, scale variability should be checked on a regular basis.

For highly enriched samples (at least 100–1000‰ enrichment) the scale variability is the most important source of error. However, even here, a +/-2% error in the measurement result usually does not present a problem. Furthermore, these errors can be avoided to a large extent using an "isotope dilution" series of enriched standards, as proposed below.

The so-called Doubly Labelled Water method presents a special case: the measurement result for this technique is the difference between two differential measurements: the ^{18}O and ^{2}H turnover in body water in the course of time after the administration of highly enriched doubly labelled water. Since the turnover times for these two isotopes are usually quite close, measurement errors in either one of them, most notably the scale variability, tend to get amplified in the final result. Therefore, in applying this technique, the regular check of the instrument (preferably on a daily basis) using an isotope dilution series of standards is a necessity.

Many of the calibration procedures described here are already common practice (or are expected to be) in a clinical setting. Requirements for instruments in such a setting are that they "check themselves". This means that procedures which adhere to good clinical practice as well as good laboratory practice must be installed to check precision, accuracy and stability.

Publications about new techniques should distinguish between precision, accuracy, as well as instrument stability. They should be adequately compared with existing state-of-the art equipment and checked against isotope reference materials that are back-traceable to primary calibration materials (IAEA). In addition they should clearly state the procedure followed for calibration (defining the scale zero) and normalization. In particular the results for the scale multiplication factor(s) should be presented. In practice this means, e.g. for the case of water, that VSMOW, SLAP, GISP and a number of local standards should be measured.

6. CALIBRATION MATERIALS

Special for the field of (bio-)medical applications is the use of (highly) enriched materials. This usually starts with the administration of a substance in which the abundant isotope has been nearly completely substituted by a rare isotope. The samples to be analysed are obviously less enriched, but can still show enrichment levels far beyond the natural range (e.g. samples for DLW analyses in small animals show enrichments of up to 2000‰ for $\delta^{18}O$ and 40000‰ for $\delta^{2}H$).

Due to scale variability problems described above, a set of reference materials for this range is necessary. However, because of this same scale variability, assigning values to such a set of RMs by an intercomparision exercise is not a fruitful procedure. Instead, it is possible, by successively diluting a 100% substituted material with a “normal abundance” material, to produce RMs that have gravimetrically determined delta-values with sufficient precision. Such a set must then be used on a daily basis for scale variability determinations and subsequent corrections.

At an international scale, the following materials should be made available in future: (1) making available a “dilution series” of RM materials for water (both ^{18}O and ^{2}H), a C-containing material (either pure CO_2 gas, carbonates or some other material), as well as a N-containing one. (2) publishing guidelines for the preparation of dilution series, along with the recommendation to uses these dilution series routinely. Since it is the IAEA policy to provide RMs only for calibration purposes of local working standards, laboratories should make their own local dilution series standards. For clinical use, it is to be expected that such dilution series will become available commercially. However, these too should be back-traceable to both the preparation guidelines and the RM dilution series of the IAEA.

The dilution series technique is also highly suitable for testing purposes: commercial suppliers offering “99.9% substituted” materials should be put to the test regularly. This can be done by preparing a dilution series based on the manufacturer’s product certificate, and by checking the resulting standards against another dilution series (preferably based on a “99.9% substituted material” which has already been checked using an independent method; but at least against a substituted material from another source).

7. OUTLOOK

Clinical samples, indeed the bulk of all medical samples, generally involve isotope ratios in either CO_2 or water, hence equipment specialised for these molecules are desirable. Optical instruments due to their molecular sensitivity may ultimately prove best for these applications. Medical research and clinical use of labelled compounds is under intensive development and is growing rapidly driven both by its success and the world wide decrease in the use of radioactive compounds for such work. Growth is limited by the cost of separated isotopes- especially ^{18}O, but also ^{13}C, and the analytical instrumentation limitations (primarily cost per analysis). As research moves to the clinical side, the expected growth will be further enhanced. On-line monitoring in real time during hospitalization and/or medical procedures may be useful, but requires development of suitable instruments. Developments in speed, accuracy and precision in the routine analysis of water and CO_2 samples for medical, drug, and labelled compound studies will influence, and be influenced by, similar developments in atmospheric and hydrology stable isotope ratio work.

8. PREPARATION OF REFERENCE MATERIALS & LOGISTICS

General requirements for the prepartion of a dilution series of a certain substance are:

(1) The availability of a material with the element under study fully substituted by the rare isotope,
(2) The availability of the same material with natural abundances,
(3) A way to make fully homogeneous mixtures of these materials. For gases and liquids, mixing does not produces a problem; for solids, however, the only method proven to work is the process of dissolution ñ mixing ñ recrystalisation. This process can be performed for large quantities industrially/commercially.

For a dilution series of water, requirements (2) and (3) are obviously met. Requirement (1) is unproblematic for deuterated water, for ^{18}O-water, however, the availability is extremely limited (it is also expensive), and ordering times can well be over a year.

For C-containing compounds, CO_2 gas would be suitable. Also some solids might be applicable, but attention should be paid to the above-stated requirements. Also, because all these molecules contain more than 1 C-atom, extra care should be taken with respect to which atom(s) exactly is/are substituted in the molecule by ^{13}C (and to how much a degree!), and whether the other C-atoms remain at natural abundance or they are affected to some extend by the chemical reactions used for the substitution. Obviously, the primary criterion for selection of a solid should be that it can be used as a standard in a wide variety of methods (EA-IRMS, GC-c-IRMS, off-line preparation for dual inlet IRMS and optical methods).

For N-containing compounds, the situation is very similar to the C-containing compounds mentioned above.

9. RECOMMENDATIONS REFERENCES AND STANDARDS

- The establishment of the following RMs is recommended: a dilution series of enriched reference materials for water (both ^{18}O and ^{2}H), a C-containing material (either pure CO_2 gas, carbonates or some other material), and N-containing material. These materials are to be used to calibrate a local dilution series which each laboratory will need to prepare individually, or to obtain it from a commercial supplier (as in the case of medical applications is to be foreseen). The local dilution series RM should be used for daily calibration of the instrument.
- Commercial suppliers offering isotopically substituted materials should be put to the test regularly, as the quality (isotopic signature) of such materials are critical for the derived local dilution series (as well as for medical diagnostics). This can be done by preparing a dilution series based on the manufacturer's product certificate, and by checking the resulting standards against a well-characterized dilution series.

10. INSTRUMENTS

During the past few years a variety of new techniques have appeared. Here we discuss in particular clinical applications of isotope measurements and laser spectrometry techniques. The requirements for clinical use are similar to those required for field deployment - ease of use, self diagnostic, robust and moderate cost.

The molecules in which the isotope ratios are determined are predominantly CO_2 and H_2O.

For such new techniques to be excepted by the isotope community they should be adequately compared with existing state-of-the art equipment and checked against isotope reference materials that are back-traceable to (IAEA) primary standards. Publications should distinguish between precision, accuracy, as well as instrument stability. In addition they should clearly state the procedure followed for calibration (defining the scale zero) and normalization (scale multiplication factor). In practice this means, e.g. for the case of water, that VSMOW, SLAP, GISP and a number of local standards should be measured.

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