On-line Quantitative Determination of ^{2}H/^{1}H Isotope Ratios in Organic and Water Samples Using an Elemental Analyser Coupled to an Isotope Ratio Mass Spectrometer

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A rapid continuous-flow technique for the quantitative determination of hydrogen isotope ratios in organic materials and water, at natural abundance levels, is described. Samples are pyrolysed in a helium stream at 1080 °C over an inert form of carbon. Hydrogen is separated from the other pyrolysis gases by gas chromatography and enters the ion source of the isotope ratio mass spectrometer (IRMS) via a crimp. The ^{2}H/^{1}H ratio of the gas was determined by the simultaneous integration of the m/z 2 (^{1}H^{1}H) and m/z 3 (^{2}H‰^{1}H) ion beams over time. δ^2H‰ values of organic and water samples were determined by comparison with organic and water standards, respectively. The precision of the on-line δ^2H‰ measurement is <3.3‰. The accuracy of the method was demonstrated by the analysis of a laboratory standard water (NTW), a European Community Bureau Reference material, N,N-tetramethylurea (TMU) and an International Atomic Energy Association polyethylene reference material (IAEA-CH-7). The measured values were NTW −39.4‰, TMU −144.1‰ and IAEA-CH-7 −100.7‰ (reference values are NTW −38.5‰, TMU −147.4‰ and IAEA-CH-7 −100.3‰). No apparent memory effect was observed when measuring samples at the natural abundance level. Calibration of organic measurements with water reference materials did not give satisfactory results, probably owing to differences in their respective pyrolysis pathways. The potential of the technique for food analysis was demonstrated by measuring the δ^2H‰ values of vanillin samples derived from natural and synthetic sources and by the analyses of hexamethylenetetramine synthesized from beet, cane and apple juice sugars. © Crown copyright 1998. Reproduced with the permission of the Controller of Her Majesty's Stationery Office.

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The measurement of stable hydrogen isotope ratios in organic molecules has been used widely in the authentication of foods and natural products. The possibility of differentiating between natural and synthetic flavours and aromas by hydrogen isotope ratio mass spectrometry and deuterium NMR (D-NMR) analysis has been demonstrated.[1–4] Site-specific D-NMR has also been used extensively in the detection of added beet sugar to a range of food commodities such as wine[5] and fruit juice.[6]

D-NMR studies have the advantage of providing site-specific ^{2}H/^{1}H ratios and this may yield more information about the botanical and geographical origin of a food. However, deuterium is a relatively insensitive nucleus for NMR studies, possessing a nuclear spin of 1 and a low natural abundance (~150 ppm). This means that long analysis times, of the order of 2 h per sample, are required to obtain satisfactory signal-to-noise ratios and measurement precision. Furthermore, food com-

ponent molecules must be purified and used in large quantities to ensure reasonable analysis times.

Isotope ratio mass spectrometry requires organic hydrogen to be quantitatively converted to hydrogen gas prior to analysis. Organic samples can be converted to hydrogen or water by pyrolysis and combustion, respectively. The techniques for preparing hydrogen gas from organic hydrogen have been extensively reviewed by Wong and Klein.[7] Generally, these off-line techniques for the preparation of hydrogen are experimentally difficult to perform, time consuming and to some extent operator dependent.[8–12]

Continuous flow isotope ratio mass spectrometric (CF-IRMS) techniques offer great advantages in terms of minimal sample preparation, smaller sample quantities and high throughput. This technique has been demonstrated for the 'light' isotopes ^{13}C,[13] ^{15}N,[14] ^{34}S[15] and, more recently, ^{18}O[16,17] and ^{2}H[18,19] in organics. However, the systems described in the literature for organic ^{2}H/^{1}H analysis use capillary gas chromatography (GC) as a means of sample introduction. The published systems have only investigated volatile model compounds and the GC capillary column is used essentially as a transfer line, thus reducing chromatographically induced misalignment of the H_2^+ and DH$^+$ peaks to a minimum. The introduction of a GC/IRMS

system capable of determining $^2H/^1H$ ratios of any chromatographically separated compound is some way off. In this paper we describe a robust on-line technique based on the pyrolytic conversion of organic materials directly to hydrogen and carbon monoxide or carbon using an elemental analyser coupled to a commercially available isotope ratio mass spectrometer capable of resolving $^4He^+$ from $^2H^1H^+$. The aim of this study was to develop a simple and rapid CF-IRMS method which could be applied to both extracted and purified food components and whole foods to determine their $^2H/^1H$ ratios.

EXPERIMENTAL

Materials

Reference materials with defined $\delta^2H‰$ values were obtained from the International Atomic Energy Association (IAEA, Vienna, Austria) and the European Community Bureau of Reference (BCR; supplied by the Joint Research Centre, Ispra, Italy). Laboratory organic standards were calibrated by deuterium NMR and the laboratory water standard was calibrated by the off-line zinc reduction method.[9]

Silver capsules for solid samples (3.5×5 mm) were obtained from EA Scientific (East Grinstead, UK), silver capsules for liquid samples (2×5 mm) were obtained from Thermoquest (Hemel Hempstead, UK) and glassy carbon (Sigradur G), 3150–4000 μm grit, was obtained from HTW (Thierhaupten, Germany).

Samples of authentic vanillin from known sources were kindly provided by Bush Boake Allen (London, UK). Samples of hexamethylenetetramine (HMT) were synthesized from authentic samples of apple juice, beet and cane sugars in our laboratory. The HMT molecule retains the $^2H/^1H$ isotope ratio from the C-6 carbon of glucose and the C-1 and C-6 carbons of fructose. Hydrogen from these sites on the parent sugar do not undergo exchange with the fruit juice water. The synthesis and measurement of HMT will be the subject of a future publication.

Apparatus

The system used for pyrolysis of the samples was similar to that developed by Werner et al.[16] A Fison's model 1108 CHN elemental analyser (EA) was connected to a

Europa Scientific Hydra IRMS via a crimp. Solid samples (vanillin and HMT, 2 mg $\cong$ 100 μg H) were wrapped in silver capsules. Liquid samples (water, TMU and NBS22; 2 μL $\cong$ 200 μg H) were dispensed into silver capsules using a 10 μl Hamilton syringe and sealed. The samples were pyrolysed at 1080 °C in a quartz reactor in the EA. The pyrolysis gases were swept by a flow of helium carrier gas (grade 5.6, flow rate 100 ml min^{-1}) through a 20 cm high bed of glassy carbon grit. In Werner's method, water vapour and carbon dioxide were removed by a chemical trap (0.2 m $\times$ 10 mm i.d.) containing magnesium perchlorate and Carbosorb, respectively. The chemical trap was left in situ during this analysis. The pyrolysis gases then passed through a GC column packed with molecular sieve 5A (1 m $\times$ 4 mm i.d.) heated to 60 °C, which separated H_2, N_2 and CO. A portion of the effluent (~ 0.05 ml min^{-1}) flowed via the crimp into the ion source of the IRMS. The ion source and analyser pressures were 3.9×10^{-6} and 3.2×10^{-7} mbar, respectively, under normal continuous-flow conditions. A 2 mg HMT sample typically gave a major beam (m/z 2) intensity of 10 nA. The m/z 2 ($^1H^1H$) and m/z 3 ($^2H^1H$) ion beams of H_2 gas entering the source were simultaneously integrated over time. The $^2H/^1H$ ratios were corrected by the Hydra software for the H_3^+ effect, by linear regression of a three-point data series of measured ratios versus m/z 2 ion beam intensity. An extensive discussion of the mass spectrometer's performance characteristics during continuous-flow ^2H measurement was presented by Prosser and Scrimgeour.[20]

RESULTS AND DISCUSSION

Organic samples

The IAEA mineral oil reference material NBS22 (assigned $\delta^2H‰$ value $-118.5 \pm 2.8‰$) was used to calibrate the measurement and determine the H_3^+ correction for the analysis of the BCR reference material (RM116), N,N-tetramethylurea (TMU) and the IAEA polyethylene reference material IAEA-CH-7 (Table 1). Good agreement was found between the measured value for TMU of $-144.1‰$ (s.d. 2.1‰, $n = 8$) and the reference value of $-147.4 \pm 1.9‰$. Similarly, the measured value for IAEA-CH-7 of $-100.7‰$ (s.d. 2.1‰, $n = 8$) agreed well with the reference value of

Table 1. $\delta^2H‰$ results versus VSMOW for organic reference materials obtained with the elemental analyser-IRMS continuous-flow technique and calibrated with the International Atomic Energy Association reference material NBS22

Reference material	Measured values	Mean ± s.d.	n	Reference value
BCR reference TMU	−142.6, −144.3, −141.4, −146.0, −146.8, −142.1, −144.3, −145.3	−144.0 ± 2.1	8	−147.4 ± 1.9
IAEA reference polyethylene foil (IAEA-CH-7)	−99.3, −98.4, −101.3, −99.9, −99.7, −101.5, −98.6, −106.5	−100.7 ± 2.6	8	−100.3 ± 2.0

Table 2. $\delta^2H\%_{00}$ results versus VSMOW for vanillin samples obtained with the elemental analyser-IRMS continuous-flow technique and calibrated with the International Atomic Energy Association reference material NBS22

Sample	$\delta^2H\%_{00}$	s.d.	n
Vanillin (natural)	−100.7	1.1	4
Vanillin (prepared from turmeric)	−114.7	2.1	4
Vanillin (synthetic)	−58.9	3.1	4
Vanillin (Sigma)	+50.1	3.3	4

$-100.3 \pm 2.0\%_{00}$. These data demonstrate the reliable performance of the on-line CF-IRMS system, relative to the VSMOW scale, for the analysis of organic reference materials.

The data presented in Table 1 have not been normalized against a VSMOW–SLAP linear calibration because it was found that the international water standards could not be used to calibrate the $\delta^2H\%_{00}$ measurement of organic samples. Substantial inaccuracies in $\delta^2H\%_{00}$ values of 71.6 and 65.2‰ were obtained for NBS22 and TMU, respectively. These inaccuracies probably result from differences in the pyrolysis pathways followed by water and organic samples. However, it is clear from the accuracy and precision of the reported organic analyses that if a suitable organic reference material is carefully chosen, then reliable $\delta^2H\%_{00}$ measurements may be performed. Any isotopic fractionation, that is specific to organic samples, therefore proceeds in a controlled and repeatable manner.

Vanillin samples were also measured against the NBS22 standard to assess the repeatability of measurements and to demonstrate the potential of the technique for flavour authenticity studies. The vanillin sample results, shown in Table 2, appear to be in agreement with earlier investigations by Culp and Noakes,[2] where the natural vanillin from vanilla beans possessed depleted $\delta^2H\%_{00}$ values relative to synthetic vanillin produced from sources such as guaiacol. The repeatability of the measurements of the four vanillin samples ranged from 1.1 to 3.3‰.
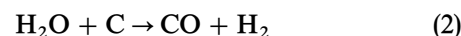
A laboratory HMT NMR standard was used to calibrate the measurement and determine the H_3^+ correction for the analysis of three HMT samples synthesized from hydrolysed apple, beet and cane sugars. All of the H atoms in HMT are equivalent in D-NMR analysis and therefore a good correlation is expected between these techniques, so long as the HMT does not contain

hydrogenous organic contaminants. Proton NMR studies in our laboratory have shown the HMT samples to be very pure. The D-NMR and CF-IRMS results are compared in Table 3. These results are reported conventionally as a ratio in ppm versus VSMOW. Their is good agreement between the D-NMR and CF-IRMS techniques (correlation coefficient, $r^2 = 0.9834$). Furthermore, the repeatability of the on-line IRMS technique ranges between 0.1 and 0.2 ppm compared with an average value of 1.5 ppm typically obtained by D-NMR for the HMT molecule.

The long-term repeatability of the CF-IRMS technique was demonstrated by measuring an HMT in-house-reference material (IHRM) 31 times over a 10 day period. The maximum number of replicate measurements of the IHRM on any one day was five. The mean $\delta^2H\%_{00}$ value obtained was $+57.4\%_{00}$, s.d. $3.1\%_{00}$.

Water samples

Owing to the inertness of the pyrolysis tube packing,[21] it was necessary to deposit an 'active' carbon surface on the glassy carbon in order to obtain quantitative results for water analysis. This was achieved by pyrolysing 10×2 μl portions of n-hexadecane prior to analysis, thus permitting the conversion of water to CO and H_2 according to the Schütze–Unterzaucher reaction:[22,23]

$$H_2O + C \rightarrow CO + H_2 \qquad (2)$$

International reference materials VSMOW, GISP and SLAP were used to calibrate the measurement of a local tap water sample, used as a standard in our laboratory. VSMOW was used to determine the H_3^+ correction. A high linear correlation was obtained between measured $^2H/^1H$ ratios and accepted reference water $^2H/^1H$ ratios ($r^2 = 0.998$). The results of the $^2H/^1H$ analysis of the local tap water standard using the CF-IRMS technique was $-39.4\%_{00}$, s.d. $3.0\%_{00}$, n = 4. The value obtained by the off-line zinc reduction technique was $-38.5\%_{00}$, sd $1.5\%_{00}$, n = 14. The measurement accuracy and precision obtained by CF-IRMS compare well with those for the zinc reduction method.

Memory effects

Natural organic substances may vary widely in their deuterium isotope abundance. In order to avoid the necessity for 'dummy samples' to condition the apparatus and remove any effect on $\delta^2H\%_{00}$ measurements from previous samples, it is necessary to ensure that

Table 3. $^2H/^1H$ results versus VSMOW for HMT samples obtained with the elemental analyser-IRMS continuous-flow technique and calibrated with the Laboratory D-NMR hexamethylenetetramine standard

Sugar source material	CF-IRMS D/H (ppm)	s.d. (ppm)	n	D-NMR D/H (ppm)	s.d. (ppm)	n
Beet sugar	118.9	0.2	4	118.1	2.5	10
Cane sugar	146.0	0.1	4	148.6	1.4	3
Apple juice sugars	132.7	0.2	4	130.2	1.5	6

Table 4. 'Memory effect' test for the elemental analyser-IRMS continuous-flow technique demonstrated by the $\delta^2H\%_0$ measurement of HMT samples

Analysis No.	Material	$\delta^2H\%_0$	Mean $\delta^2H\%_0$	s.d. (‰)	n
1	HMT standard	−50.4	−50.0	1.2	4
2	HMT standard	−51.2			
3	HMT standard	−48.4			
4	HMT standard	−50.1			
5	Apple juice sugars	−149.1	−147.7	1.5	4
6	Apple juice sugars	−147.5			
7	Apple juice sugars	−148.5			
8	Apple juice sugars	−145.7			
9	HMT standard	−51.0			
10	Beet sugar	−234.7	−236.3	1.2	4
11	Beet sugar	−236.7			
12	Beet sugar	−236.3			
13	Beet sugar	−237.5			
14	HMT standard	−51.2			
15	Cane sugar	−62.7	−61.8	0.9	4
15	Cane sugar	−61.4			
17	Cane sugar	−60.8			
18	Cane sugar	−62.3			

memory effects are insignificant when compared with the measurement precision. The HMT samples analysed by the CF-IRMS system ranged from −50.8‰ to −236.3‰, a range of ~185‰. Each HMT sample was analysed four times interspersed with the standard HMT sample. These results are shown in Table 4. The good precision achieved over a 185‰ range demonstrates the absence of memory effects at the natural abundance level. In addition, all of the subsequent interspersed measurements of the HMT standard fell within one s.d. of the first four measurements of the same material.

CONCLUSION

Comparison of $\delta^2H\%_0$ values obtained by pyrolytic CF-IRMS with accepted reference values, off-line measurements and deuterium NMR demonstrate the satisfactory performance of the new system. Provided that appropriate reference compounds are carefully chosen, this system provides rapid and accurate $^2H/^1H$ analysis of both organic and water samples with precisions of <3.3‰ with no memory effect. In addition, the system is fully automated and only small quantities of samples equivalent to <300 µg H are required.

The method described therefore offers a rapid alternative to the experimentally difficult off-line IRMS measurement of organic hydrogen and water samples. Furthermore, it may be used instead of deuterium NMR when the analyte is pure and contains 'equivalent' hydrogen atoms or when an average molecular $\delta^2H\%_0$ value provides sufficient infomation for accurate authenticity assessment. The system therefore has the potential for food studies where only small quantities of pure analyte are available. The use of water standards to calibrate measurements of organic samples may be made possible by using catalysts such as nickelized carbon in the pyrolysis tube or changing to a combustion/reduction reactor. However, the benefits of using water standards should be balanced against the potential problems of memory effects within such systems. As CO is the other primary product of the pyrolysis of organics which contain oxygen, obtaining $^2H/^1H$ and $^{18}O/^{16}O$ isotope ratios in one analysis may also be possible in the future. This will require reproducible magnetic field and source parameter switching between the elution of H_2 and CO from the GC column, which is not currently commercially available.

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