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Detection of Sugar Syrups in Apple Juice by δ^2H‰ and δ^{13}C‰ Analysis of Hexamethylenetetramine Prepared from Fructose

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An improved procedure for determining ^{13}C and ^{2}H isotope ratios, using gas chromatography−isotope ratio mass spectrometry (GC−IRMS), has been developed for identifying the addition of low cost commercial sugar syrups to apple juices and related products. Isotopic techniques are commonly used to identify the addition of low cost sugars to fruit juices and are difficult to circumvent as it is not economically viable to change the isotopic ratios of the sugars. The procedure utilizes the derivative hexamethylenetetramine, which is produced through chemical transformation of a sugar degradation product and provides position-specific ^{13}C and ^{2}H ratios that relate to the parent sugar molecule. The new procedure has advantages over methods using nitro-sugar derivatives in terms of analysis time and sensitivity. The differences between the δ^2H‰ and δ^{13}C‰ values of the 100 authentic apple juices and beet and cane commercial sugar syrups permit their addition to be reliably detected.

KEYWORDS: Apple juice; authenticity; hydrogen isotope; GC−IRMS; pyrolysis

INTRODUCTION

Stable carbon isotope ratios are used widely for the authentication of foodstuffs, especially for the detection of added cane sugar in fruit juices (*1*, *2*), spirits (*3*, *4*), and honey (*5*). These methods resulted from the discovery that cane and corn, which are the source materials for many commercial sugar syrups, both utilized the Hatch/Slack (C_4) photosynthetic pathway to fix atmospheric CO_2 which results in δ^{13}C‰ values between −10 and −12‰, whereas the majority of other important agricultural crops use the Calvin (C_3) pathway resulting in δ^{13}C‰ values between −23 and −28‰ (*6*). The addition of added cane or corn syrup to fruit juices produces a deviation from the normal δ^{13}C‰ value expected for a C_3 plant, which can be detected using ^{13}C stable isotope ratio analysis (SIRA).

The major deficiency of stable carbon isotope analysis is that it cannot be used to detect the addition of beet sugar syrups to C_3 crops, as the beet plant also uses the Calvin photosynthetic pathway. However, the addition of commercial sweeteners such as beet medium invert syrup can be detected by isotope ratio mass spectrometry if the abundance of deuterium in the sugars is determined. The natural abundance of deuterium varies widely in the biosphere due to its low mass and the large difference between the mass of the two stable isotopes ^{2}H and ^{1}H of hydrogen. Physicochemical and biological fractionation effects are much more pronounced than for the heavier bio-elements carbon, nitrogen, oxygen, and sulfur. Generally, the deuterium content of plant material decreases with increasing latitude. Since beet sugar is normally grown further from the equator than tropical fruits, beet sugar contains less deuterium than the sugars present in many fruit crops. In addition, differences in the relative rates of evapo-transpiration between ground plants such as beet sugar, which has a relatively small surface area of leaves, and aerial plants such as fruit trees, results in a significant difference in the ^{2}H/^{1}H ratio of these species. These differences can be exploited to detect the addition of beet medium invert syrup to fruit juices.

In the early 1980s, quantitative measurement of D/H ratios using nuclear magnetic resonance (NMR) was developed (*7*, *8*). In the form of site-specific natural isotope fractionation−nuclear magnetic resonance (SNIF-NMR), this proved to be a powerful technique in the field of wine analysis and was adopted by the European Union as the official method for detecting illegal addition of beet sugar to wine. Subsequently, the approach was modified to permit the detection of beet sugar in fruit juices (*9*, *10*) and to determine the authenticity of flavors (*11*).

For all of these applications, it is necessary to quantitatively extract a marker molecule from the food and to determine the deuterium/hydrogen ratio (D/H) at specific chemical sites of this molecule by high-resolution NMR. For example, the D/H ratio at the methyl site of ethanol extracted from wine, $(D/H)_1$, is indicative of the botanical origin of the starting sugars (beet or grape). For fruit juices, the sample preparation is more

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complicated. Direct analysis of extracted sugars is not possible due to a poor NMR response. Therefore, it is necessary to ferment the sugars to ethanol under controlled laboratory conditions. The resulting ethanol is then quantitatively recovered by distillation and the deuterium content at the methyl $(D/H)_1$ site determined as for wine. The fermentation procedure makes the overall preparation relatively slow. Also, while $(D/H)_1$ measurements from fruit juice predominantly reflect the botanical origin of sugar, it is known that this parameter is also influenced by the deuterium content of the water present during fermentation. To minimize the influence of the water, a normalization factor has been developed to allow all $(D/H)_1$ results to be normalized to a nominal mean water D/H value of ~156 ppm. To apply the normalization, the D/H ratio of the residual water after ethanol extraction must be measured.

The determination of the $^2H/^1H$ ratio of the nonexchangeable hydrogen atoms in sugars, after conversion to the nitro-ester derivative, has also been used to authenticate wine (*12*) and fruit juices (*13*, *14*). Although these methods are effective, they have not been widely adopted because measuring stable hydrogen isotope ratios of nitro-sugar derivatives poses certain technical problems. The methods tend to be time-consuming, potentially hazardous, and do not readily lend themselves to high sample throughput on a routine basis. Some preliminary data have been reported on the use of calcium formate as an alternative derivative, produced by oxidative cleavage of sugar molecules by cerium salts (*15*), which obviate the need to use explosive nitro-sugar derivatives. Nevertheless, this method still involves the difficult manipulation of small quantities of water and off-line combustion and reduction stages.

Recent instrumental developments which proceed by high-temperature pyrolysis (Py) of organic material and measurement of the resulting hydrogen gas by continuous-flow isotope ratio mass spectrometry (CF−IRMS) have, to a large extent, overcome these difficulties and greatly increased the applicability of this technique. In addition, the availability of commercial GC−Py−IRMS systems capable of measuring $^2H/^1H$ ratios in the presence of a helium carrier gas, means that it is now possible to routinely measure compound-specific ^2H abundances in organic molecules after chromatographic separation.

In this study, we investigated the chemical transformation of fructose, present in 100 authentic apple juice samples, into hexamethylenetetramine which retains position-specific ^{13}C and ^2H isotopic information from the carbon and nonexchangeable hydrogen atoms in the parent sugar. The $^2H/^1H$ and $^{13}C/^{12}C$ ratios of the hexamethylenetetramine were determined by GC−Py−IRMS and GC−combustion−IRMS, respectively. This method enables the addition of commercial sugar syrups to apple juice to be reliably detected.

MATERIALS AND METHODS

Chemicals and Isotopic Reference Materials. Hexamethylenetetramine standard material (99.5% purity), fructose standard material (99.5% purity), and *n*-hexadecane (C16) (99.5% purity) were obtained from the Sigma-Aldrich Chemical Co. The *n*-hexadecane was used as an isotopic reference and was calibrated for δ^2H‰ analysis by elemental analyzer−pyrolysis−IRMS (*16*) and $\delta^{13}C$‰ analysis by EA−combustion−IRMS, against NBS22 mineral oil. The NBS22 was obtained from the International Atomic Energy Agency, Vienna, Austria. The NBS22 had an assigned δ^2H‰ value of -118.5 ± 2.8‰ versus Vienna-Standard Mean Ocean Water (V-SMOW) and an assigned $\delta^{13}C$‰ value of -29.7 ± 0.2‰ versus Pee Dee Belemnite (PDB).

Authentic Apple Juice Samples. Samples were obtained from a wide range of geographical locations including the major apple juice producing countries (Germany, England, United States, New Zealand, and Australia). The majority of the samples were obtained in the UK as whole fruits from a reputable fruit importer. Additional samples were received via government officials in other countries. Juice samples were frozen in solid carbon dioxide directly after collection. Whole fruits were squeezed either before transporting to the laboratory or shortly after arrival at the laboratory. In all cases, the juice from whole fruits was frozen immediately after squeezing (either in cardice or in the laboratory freezer). Once at the laboratory, all juices were stored frozen (−20°C) until thawed before sample preparation. Samples were refrozen after subsampling.

Semipreparative HPLC of Fructose from Apple Juice and Sugar Syrups. Concentrated apple juices and sugar syrups were diluted to approximately 12° Brix with deionized water. Single strength apple juices and diluted concentrates (20 mL) were passed through a 0.7 μm filter. Fruit acids and minerals were removed by passing 10 mL of the filtered juice through a column of AG 1-X8, 100−200 mesh, chloride form, anion-exchange resin (Bio-Rad) followed by a column of AG 501-X8, 20−50 mesh mixed-bed resin. Both columns were washed with aliquots of water (3×10 mL). The eluant was collected and its volume was reduced to approximately 5 mL by rotary evaporation. The concentrated and deionized juice was transferred to a 5 mL volumetric flask and diluted to volume with water. This solution (500 μL) was injected onto a 250×21 mm i.d. YMC polyamine II column 5 μm 120 Å eluted at 12 mL/min with mobile phase containing 82% acetonitrile and 18% water. The column eluant was monitored with a refractive index detector to ensure baseline resolution of fructose, glucose, and sucrose. The retention times of fructose, glucose, and sucrose were 25, 34, and 70 min, respectively. An automated fraction collector (Superfrac, Pharmacia LKB Biotechnology, Uppsala, Sweden) was used to collect the fructose fraction eluting between 23 and 31 min. Two to three injections per sample were normally required to recover approximately 100 mg of fructose. The collected fractions were combined and transferred to a round-bottomed flask and dried by rotary evaporation.

Preparation of Hexamethylenetetramine from Fructose. The dried fructose fraction or 100 mg of fructose standard material was dissolved in deionized water (5 mL). A solution containing 8 g of potassium hydrogen carbonate dissolved in 10 mL of water was added to the fructose solution and stirred. A 15% w/v periodic acid solution was then added slowly with stirring. After reaction for 45 min at 20 °C, the fructose was quantitatively degraded to formaldehyde (C_1 and C_6) and formic acid (C_2 to C_5). A total of 5 mL of a 35% w/v ammonia solution was added to the reaction mixture. After 3 h at 20 °C, the reaction of formaldehyde with ammonia produced the 6:4 tricyclic complex hexamethylenetetramine. The reaction mixture was filtered through a Whatman GF/F filter using vacuum filtration equipment and rotary evaporated to dryness. The hexamethylenetetramine was extracted from the reaction residue by shaking with 3×10 mL portions of chloroform. The three chloroform extracts were combined and rotary evaporated to recover the hexamethylenetetramine, which was dissolved in a chloroform solution containing *n*-hexadecane internal standard (2.5 mg/mL) to give a concentration of hexamethylenetetramine of 10 mg/mL. This solution was used for isotopic analysis.

Hydrogen Isotope Analysis of Hexamethylenetetramine. The separation of the hexamethylenetetramine from the chloroform solvent and *n*-hexadecane internal standard was achieved using a HP5890, series II gas chromatograph (Hewlett-Packard, Palo Alto, CA). The GC was fitted with a SGE BPX5 wide-bore capillary column (25 m × 0.53 mm i.d.) of fused silica coated with 95% dimethyl−5% diphenyl polysiloxane (film thickness 0.1 μm). Helium was used as a carrier gas and injection was in splitless mode, to avoid isotopomer discrimination in the injector. The split valve was closed for 0.75 min following injection, and then purged at 100 mL/min. Injection was via an automatic sampler (Eurovector, Milan, Italy). The GC oven was temperature programmed to obtain optimum resolution between the hexamethylenetetramine and *n*-hexadecane internal standard.

The GC column effluent was passed into the pyrolysis interface coupled to the IRMS (PDZ-Europa, Crewe, UK). The pyrolysis interface and mass spectrometer have previously been described in detail (*17*, *18*). The manufacturer's proprietary software (PDZ-Europa, Orchid

Postprocessor) was used for peak identification, ratio calculation, and H_3^+ correction. The hydrogen isotope ratios of hexamethylenetetramine were calculated with reference to the assigned value of the *n*-hexadecane (C16) internal standard and reported in ‰ relative to the V-SMOW standard.

Carbon Isotope Analysis of Hexamethylenetetramine. The separation and carbon isotopic analysis of the hexamethylenetetramine and *n*-hexadecane internal standard were carried out with a GC−combustion−IRMS (GC−C−IRMS) continuous-flow system consisting of a HP5890, series II gas chromatograph fitted with a SGE BPX5 capillary column (0.32 mm i.d. × 25 m length) of fused silica coated with 95% dimethyl−5% diphenyl polysiloxane (film thickness 0.1 μm); a GC-combustion interface II and a delta S IRMS (Finnigan MAT, Bremen, FRG). The carbon isotope ratios of hexamethylenetetramine were calculated with reference to the assigned value of the C16 internal standard and reported in ‰ relative to the PDB standard.

RESULTS AND DISCUSSION

The formation of hexamethylenetetramine requires a vigorous oxidative cleavage of the fructose molecule (*19*). Many (bio)-chemical reactions have associated kinetic isotope effects which lead to a fractionation of the isotopomers of the reaction product, if the conversion is not quantitative (*20*). However, this does not necessarily preclude isotopic analysis of the reaction product if the reaction proceeds in a controlled and repeatable manner, or the isotope effect is negligible. Furthermore, physical processes such as distillation and chromatography can also produce fractionation effects if they are not quantitative. Chromatography produces a separation of the isotopomers of a given compound that results in the relative depletion of the "tail" of a chromatographic peak (*21*). To establish whether the hexamethylenetetramine preparation and analysis were proceeding in a controlled and repeatable manner, the following validation experiments were performed to optimize method conditions and determine the limits of repeatability of the method.

Precision of the GC−IRMS δ^2H‰ and δ^{13}C‰ Measurements. The first experiment was designed to establish whether the GC separation, pyrolysis, and subsequent MS measurement could be performed in a repeatable manner. A solution of hexamethylenetetramine was prepared using chloroform containing C16 internal standard. It was analyzed by GC−Py−IRMS to determine the "internal precision" of the δ^2H‰ measurement. The standard hexamethylenetetramine solution was analyzed five times and had a mean value of −99.4‰ (SD 2.4‰). The hexamethylenetetramine solution was also analyzed five times by GC−C−IRMS to determine the repeatability of δ^{13}C‰ measurements. A mean δ^{13}C‰ value of −47.3‰ with a SD of 0.2‰ was obtained. These data demonstrated that both the GC−Py−IRMS and GC−C−IRMS systems achieved acceptable precision for repeat measurement of a standard hexamethylenetetramine solution. In particular, the precision of the hydrogen isotope measurement compared favorably with other reported values of ^{2}H GC−Py−IRMS measurement precision at natural abundance levels (*22*)

Effect of the Periodate Degradation Reaction on the δ^2H‰ and δ^{13}C‰ Measurement Precision. The mean δ^2H‰ result for six preparations of hexamethylenetetramine from standard fructose was −238.8‰ (SD 2.5‰). The inclusion of the degradation step did not lead to a significant increase in the SD of hexamethylenetetramine δ^2H‰ measurements compared to those obtained for a standard hexamethylenetetramine solution under repeatability conditions (SD 2.4‰). Similarly, the mean δ^{13}C‰ value determined from the same set of six hexamethylenetetramine samples prepared from fructose was −37.2‰ (SD 0.5‰). These data clearly demonstrated that any isotope effects associated with the periodate degradation of fructose and formation of hexamethylenetetramine were sufficiently well controlled to obtain repeatable δ^2H‰ and δ^{13}C‰ results. In addition, the δ^2H‰ SD was of the same order of magnitude as previously reported for repeat analysis of sucrose as its nitro-derivative using off-line preparation (*13*). The long-term precision of the periodate degradation was also monitored to ensure that it remained under control. Hexamethylenetetramine was produced from the fructose standard with each batch of authentic apple juice samples over a two-and-a-half-month period. The mean δ^2H‰ and δ^{13}C‰ values of 33 hexamethylenetetramine samples prepared from the fructose standard was −237.4‰ (SD 4.8‰) and −37.2‰ (SD 0.7‰), respectively. As expected, the inter-batch results were less precise than those under repeatability conditions. However the mean values of −237.4‰ for δ^2H‰ analysis and −37.2‰ for δ^{13}C‰ analysis agree well with those obtained from the initial repeatability studies for the fructose standard material of −238.8‰ and −37.2‰ respectively.

Effect of the Semipreparative Isolation of Sugars on the δ^2H‰ Measurement Precision. High performance liquid chromatography can produce fractionation effects if the recovery of the analyte is not quantitative. By analyzing hexamethylenetetramine samples produced from the fructose standard before and after being subjected to the semipreparative chromatographic process, it was possible to establish whether significant fractionation was occurring during automated fraction collection. The mean value for six samples without chromatography was −232.2‰ (SD 2.3‰). The mean value for six samples after chromatography was −234.2‰ (SD 3.6‰). The difference between the two mean values was within the limits of experimental error. These experiments demonstrated that there was no significant fractionation introduced by the chromatographic separation of the fructose prior to conversion to hexamethylenetetramine. In addition, the introduction of the chromatographic step did not significantly affect the overall δ^2H‰ measurement precision.

Effect of the Entire Procedure on the δ^2H‰ and δ^{13}C‰ Measurement Precision of Apple Juice. Replicate preparations of hexamethylenetetramine were made from subsamples of an apple juice in-house reference material prepared from bulked retail apple juice. These experiments were conducted to establish the short-term δ^2H‰ and δ^{13}C‰ measurement precision for a "real" apple juice sample and to monitor the long-term measurement stability of the entire procedure. The mean value of five hexamethylenetetramine samples, prepared from apple juice under repeatability conditions, was −192.4‰ (SD 2.7‰) for δ^2H‰ analysis and −36.9‰ (SD 0.9‰) for δ^{13}C‰ analysis. The short-term repeatability of the reference material showed good precision, and the SDs were comparable to those obtained for δ^2H‰ and δ^{13}C‰ measurements of hexamethylenetetramine prepared from standard fructose. The ion-exchange step, to remove the fruit acids, therefore had a negligible effect on the overall δ^2H‰ and δ^{13}C‰ measurement precision. In addition, there were no other significant "matrix effects" introduced by analyzing a "genuine" apple juice. The mean hexamethylenetetramine δ^2H‰ and δ^{13}C‰ results obtained from seven subsequent preparations of the reference material prepared over a two-and-a-half-month period were −190.0‰ (SD 5.7‰) and −36.6‰ (SD 1.0‰), respectively. The mean values of the long-term analyses agree well with the values obtained from the initial repeatability study of −192.4‰ for δ^2H‰ and −36.9% for δ^{13}C‰. The long-term variability of δ^2H‰ measurements was

Table 1. Summary of δ^2H‰ and δ^{13}C‰ Values of Hexamethylenetetramine Prepared from Authentic Apple Juices and Sugar Syrups

country/ies of origin of apple juice	hexamethylenetetramine δ^2H‰ analysis (‰ V-SMOW)					hexamethylenetetramine δ^{13}C‰ analysis (‰ V-PDB)				
	n	mean	SD	max	min	*n*	mean	SD	max	min
England	25	–183.4	9.9	–167.9	–210.1	24	–33.9	2.9	–25.8	–37.8
Germany	23	–181.9	8.6	–159.6	–196.9	23	–34.8	2.8	–28.8	–41.1
Ukraine	11	–195.7	15.3	–162.7	–215.3	11	–34.3	1.1	–31.8	–36.3
France, Italy, Belgium, and Austria	10	–175	8.2	–161.5	–189.2	10	–33.6	5.0	–27.8	–43.9
New Zealand and Australia	9	–171.9	6.1	–158.7	–179.7	9	–38.0	3.1	–31.8	–42.6
South Africa	8	–157.9	13.5	–143.9	–180.5	8	–37.0	3.7	–31.0	–41.9
United States and Canada	6	–199.9	7.8	–187	–209.6	6	–33.9	3.3	–27.8	–43.9
Brazil and Chile	3	–192.8	9.5	–186.6	–203.8	3	–33.7	4.5	–29.2	–38.2
Czech Republic, Hungary, and Poland	5	–188.2	12.3	–167.1	–199.3	5	–33.2	3.4	–27.8	–36.0
overall statistics for apple juice	100	–182.0	14.3	–143.9	–215.3	99	–34.6	3.6	–25.8	–43.9
beet medium invert syrup (BMIS)	6	–258.1	4.1	–250.5	–261.7	6	–32.5	1.8	–30.9	–35.7
high fructose corn syrup (HFCS)	3	–49.4	5.8	–44.0	–55.6	3	–16.3	2.1	–14.8	–18.7

3.3‰ higher than the repeatability study; however, this variability was less than two times the standard deviation of δ^2H‰ repeatability measurements and so was deemed to be acceptable.

Exchange of Carbon-Bound Hydrogen during Synthesis of Hexamethylenetetramine. Due to the aggressive reaction conditions used to prepare hexamethylenetetramine from fructose, the possibility of exchange between the carbon-bound hydrogen and the water in the reaction medium was considered. A sample of hexamethylenetetramine was prepared from the fructose standard using groundwater with a δ^2H‰ value of −44.3‰ and a second sample of hexamethylenetetramine was prepared from the fructose standard using deuterium enriched water with a calculated δ^2H‰ value of 31000‰. The mean δ^2H‰ results obtained for the groundwater prepared sample and deuterium enriched water prepared sample were −238.8‰ and −235.6‰, respectively. The final results differ by 3.2‰, a difference less than two times the standard deviation of δ^2H‰ repeatability measurements. Therefore, no significant difference is seen between the two samples, and no enrichment in the hexamethylenetetramine has occurred during the synthesis due to exchange of hydrogen with deuterium in the reaction medium.

***δ*²H‰ and *δ*¹³C‰ Values of Hexamethylenetetramine Derived from Authentic Apple Juice.** The δ^2H‰ and δ^{13}C‰ values of the hexamethylenetetramine prepared from the fructose of 100 authentic apple juices are summarized in **Table 1**. In addition, **Table 1** contains the data obtained from commercial sugar syrups derived from beet and cane sucrose. The apple juice δ^2H‰ and δ^{13}C‰ results are grouped by country of origin and include the "individual country" and "all countries"; mean, SD, maximum and minimum values. Each country exhibited a different range of δ^2H‰ values as expected, due to the "geographical influence" on the D/H ratio of agricultural products. As a result, there was a correlation between the latitude of European, New Zealand, Australian, and South African growing locations and the D/H ratio of the apple juices. A general increase in the D/H ratio was observed with decreasing latitude of the country of origin. This confirms similar observations made previously on the nitrate esters of orange juice sugars (*23*).

Juice from apples cultivated in Western European countries exhibited similar δ^2H‰ and were located in the middle of the overall spread of results at approximately −180‰. Australian and New Zealand apple juices possessed similar δ^2H‰ values that were relatively enriched, at about −170‰, compared with the Western European countries. Juice from Eastern European apples produced in the Ukraine, Poland, Hungary, and the Czech Republic had similar values, tending toward the minimum observed, at about −200‰. The results for North and South America were also similar. These values were depleted in deuterium in comparison to the Western European apples. This has previously been observed for apples from the Washington state area of the United States (unpublished data) and is thought to be due to irrigation with depleted water from the Cascade mountain range. Although hydrogen atoms from the juice water are not incorporated into hexamethylenetetramine, the depleted irrigation water remains the starting point for leaf water, and subsequent evapo-transpiration, before inclusion in sugars during photosynthesis. South African samples had the highest δ^2H‰ values. These ranged between approximately −140‰ and −160‰. These samples were enriched with deuterium in comparison to the Western European apples. This is due to the warmer, dryer climate, which increases the rate of evapotranspiration and thus enrichment of the leaf water in deuterium prior to inclusion into plant carbohydrate.

Generally, the δ^2H‰ values for hexamethylenetetramine, derived from apple juice sugars, were very different from those published for the nitrate esters of apple sugars. The overall mean hexamethylenetetramine δ^2H‰ value was −182‰ compared to −69‰ obtained from the analyses of 15 nitro-sugar derivatives produced from different apple juices (*14*). A similar observation was made previously (*15*) and may be explained in terms of the different carbon-bound hydrogens being measured in the two types of derivative. All of the nonexchangeable hydrogen atoms in a sugar molecule contribute to the D/H ratio of the corresponding nitrate ester. However, periodic acid cleaves the diol functions of sugars, to produce formic acid and formaldehyde. One mole of periodate is consumed for each pair of adjacent hydroxyl groups. For fructose, there are two possible pathways. When fructose is in aqueous solution, it equilibrates to both pyranose and furanose forms. The predominant confirmation, D-fructopyranose (60%), yields one mole of formaldehyde and four moles of formic acid and the other conformation, D-fructofuranose (40%), gives two moles of formaldehyde and three moles of formic acid (*24*). The D/H ratio of hexamethylenetetramine therefore relates to a mixture of the carbon-bound hydrogens attached to the C1 position of D-fructopyranose or C1 and C6 of D-fructofuranose. Through the validation experiments described above, the fructose equilibrium has been shown

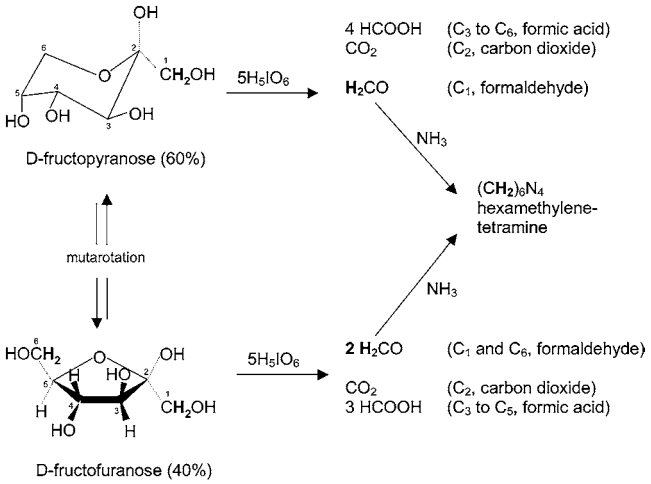

Figure 1. Schematic representation of the periodate degradation of fructose – the periodioc acid cleaves the diol functions of sugars, to produce formic acid and formaldehyde. One mole of periodate is required for each pair of adjacent hydroxyl groups. In aqueous solution, fructose equilibrates to both pyranose and furanose forms. The predominant confirmation D-fructopyranose yields one mole of formaldehyde and four moles of formic acid, and the other conformation, D-fructofuranose, gives two moles of formaldehyde and three moles of formic acid. The ^2H abundance of hexamethylenetetramine relates to a mixture of the carbon-bound hydrogens attached to the C_1 and C_6 positions of fructose only.

to be sufficiently repeatable, under the specified reaction conditions, to obtain precise δ^2H‰ and δ^{13}C‰ data. Consequently, hexamethylenetetramine only contains between two and four of the seven available carbon-bound hydrogens present in fructose. Due to the nonstatistical distribution of isotopes in natural products the difference in δ^2H‰ values can therefore be explained (*25*). A schematic representation of the degradation of fructose to formaldehyde and subsequent conversion to hexamethylenetetramine is shown in **Figure 1**.

The δ^{13}C value of the hexamethylenetetramine results ranged between −25.8‰ and −43.9‰. The apple juice samples from South Africa, New Zealand, and Australia were relatively depleted in ^{13}C compared to the remaining samples. This may be a result of local climatic or altitude effects despite the fact that C_3 plants tend to possess a narrow range of carbon isotope ratios. The mean δ^{13}C‰ value obtained for all the apple juice samples was −34.6‰ (SD 3.6%). The δ^{13}C‰ values of 40 American apple juices have previously been reported (*1*). In this case, the mean value obtained was −25.4‰ (SD 1.2‰). As with the hydrogen isotope ratios, a significant difference exists between the results from the two different techniques. The data reported by Doner were obtained from the total organic carbon present in the apple, which is predominantly sugars. As mentioned above, the δ^{13}C‰ value of hexamethylenetetramine relates specifically to the C_1 and C_6 positions of apple juice fructose only. It has been reported that a depletion of about five δ-units exists at the C_6 position of glucose arising from isotope effects during carbohydrate biosynthesis (*25*). It is therefore reasonable to expect different δ^{13}C‰ results from bulk and position-specific isotope analysis.

Detection of Sugar Syrup Addition to Apple Juice. Significant differences in the δ^2H‰ values of hexamethylenetetramine synthesized from beet medium invert syrup and apple fructose exist. The difference between the observed values for beet medium invert syrup and the minimum apple juice values, from the different geographical locations, ranged from 40 and 70 ‰. Of the 17 countries considered, the smallest differences were associated with the Ukranian, Canadian, American, and South American samples, as these populations contained apple juices with the lowest δ^2H‰ values. This compares favorably with the previously reported difference between apple and beet sugar nitro-ester derivatives of about 50‰ (*14*). As expected, the average carbon isotope ratios of the hexamethylenetetramine derived from high fructose corn syrup were relatively enriched compared to those from the apple juice, by approximately 18‰.

A "controlled adulteration" of apple juice with beet medium invert syrup (BMIS) was performed to confirm that the δ^2H‰ values varied linearly with increasing concentrations of BMIS. Nine calibration solutions were produced using the apple juice reference material and a diluted BMIS solution of the same Brix value. The solutions were prepared as % w/w of total sugars present, equivalent to 0, 5, 25, 40, 65, 80, 90, 95, and 100% BMIS. The fructose was isolated, converted to hexamethylenetetramine, and the δ^2H‰ value was measured by GC−Py−IRMS. The δ^2H‰ values corresponding to the calibration solutions were −192.5, −191.4, −202.9, −210.0, −222.5, −244.7, −251.3, −254.6, and −250.5‰, respectively. The sensitivity of the method was calculated as the rate of change

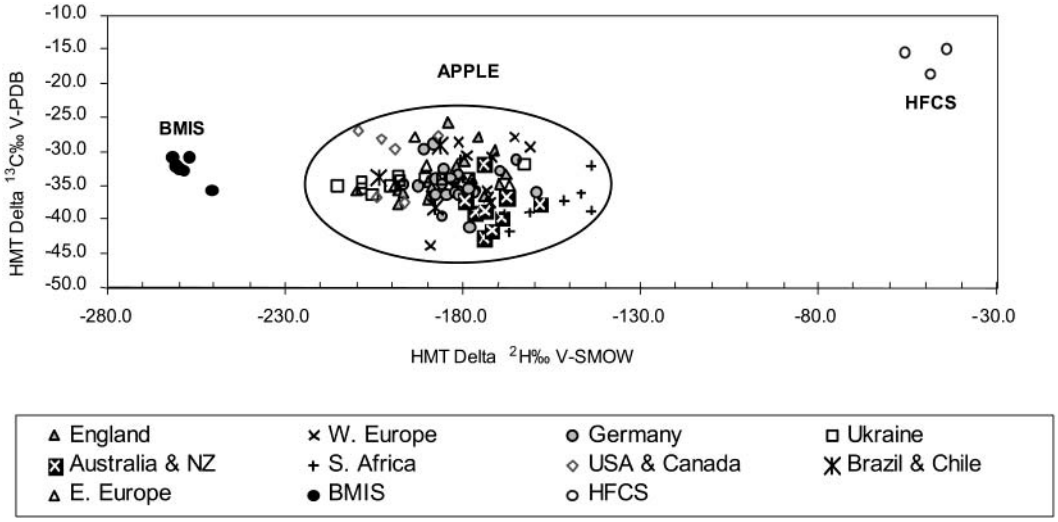

Figure 2. X–Y scatter plot of hexamethylenetetramine δ^2H‰ and δ^{13}C‰ data from authentic apple juice and commercial sugar syrups. The 99.9% confidence limit of the apple juice data is shown as an ellipse described by the equation in the text.

in the δ^2H‰ value with increasing BMIS concentration and was found to be −0.66‰ per unit BMIS (%w/w) added.

To quantify the discrimination between the apple juices and the two types of sugar syrup investigated, the following formula was derived describing the 99.9% confidence ellipse around the authentic apple juice δ^2H‰ and δ^{13}C‰ data points.

$$\left[\frac{\delta^2\text{H‰} + 182.0\text{‰}}{14.3\text{‰}}\right]^2 + \left[\frac{\delta^{13}\text{C‰} + 34.6\text{‰}}{3.6\text{‰}}\right]^2 < 9$$

This approach to defining the limits of authentic fruit juice samples has been employed previously (*13*). Assuming that the entire apple juice population follows a normal distribution, then authentic samples should generate a value < 9 from this equation. **Figure 2** shows an X−Y scatter plot generated from the 100 authentic apple juice and sugar syrup data points. The 99.9% confidence ellipse is also shown. Theoretically, only one in 1000 authentic apple juices will lie outside the ellipse. However, the sensitivity of the method for detecting adulteration ultimately depends on the difference in deuterium content of apple and beet and is more pronounced for Western European and South African samples. This in effect increases the sensitivity of the technique for detecting the presence of beet sugar in these products. In its current stage of development, the methodology offers an alternative and reliable means of detecting adulteration of apple juice with commercial beet and cane sugar syrups. To further improve the detection of the quantity of added sugar an internal, or intermolecular, isotopic reference may be used to improve the sensitivity of the technique. This potential improvement in the methodology is analogous to that used in the detection of C_4 sugars in honey, by exploiting the internal isotopic correlations between the δ^{13}C‰ value of the honey protein and sugars (*5*).

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