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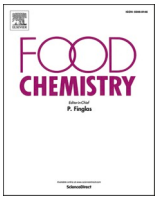

# A new approach to detecting sugar syrup addition to honey: Stable isotope analysis of hexamethylenetetramine synthesised from honey monosaccharides (fructose and glucose)

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ABSTRACT

One of the most common types of adulteration of honey involves the addition of invert sugar syrups. A new method was developed to measure the stable isotope ratios of carbon and carbon-bound non-exchangeable (CBNE) hydrogen from specific molecular positions in fructose and glucose in honey. This was achieved through periodate oxidation of the sugars to produce formaldehyde, followed by reaction with ammonia to form hexamethylenetetramine (HMT). The preparation was simplified, optimized, and validated by isotopic analysis of replicate syntheses of HMT from fructose, glucose, sugar syrup and a representative authentic honey sample. The optimized method had a repeatability standard deviation from 1.5‰ to 3.0‰ and from 0.1‰ to 0.4‰ for $\delta^2$H and $\delta^{13}$C, respectively. This methodology has advantages over alternative isotopic methods, for measuring CBNE hydrogen isotope ratios in sugars, in terms of time, sensitivity and operability and offers a complementary method to differentiate authentic honey from invert sugar syrups.

## 1. Introduction

Honey adulteration has been a persistent problem in the world market, dating back to the 1970s when high fructose corn syrup (HFCS) was introduced to the food industry and misused to extend honey (Tura & Seboka, 2019). Over the years, this fraudulent practice has evolved to include the use of invert syrups from various plant sources, which has caused economic and reputational damage to the honey industry and beekeepers. It is also one of the most significant issues facing honey producers and distributors. Recent European Union investigations by the Joint Research Centre reported that of the 320 honey samples taken at EU borders, 46% were suspected of being non-compliant with EU regulations (Ždiniaková et al., 2023). Consequently, there is an ongoing requirement for reliable methods to protect consumers, producers, and traders from the negative impacts of food fraud, such as reputational damage and the possibility of related food safety incidents, through eating adulterated honey.

To address this issue, stable isotope analysis has emerged, amongst other instrumental techniques, to detect economically motivated adulteration (EMA) of honey with invert syrups. Undoubtedly, stable carbon isotope ratio analysis (SCIRA), first reported by White and Doner in 1978 (White & Doner, 1978), has been an important technique for detecting honey adulteration with $C_4$ sugars syrups derived from corn or cane plants (Padovan et al., 2003). The principle of this method is based on the differences in $^{13}$C enrichment between $C_3$ plants (the predominant nectar source of honey) and $C_4$ plants (e.g., corn, cane) due to the different photosynthetic pathways used to assimilate carbon dioxide and water to synthesize sugars. As a result, the typical average $\delta^{13}$C value (i.e. the $^{13}$C/$^{12}$C isotope ratio related to Vienna Pee Dee Belemnite (V-PDB) as standard reference material, expressed in ‰) of honey sugar is −25.4‰ compared with the accepted average $\delta^{13}$C value of −9.7 ‰ for $C_4$-plant derived sugars, which may be used to adulterate honey (White

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Jr & Doner, 1978). The limit of detection for this method was further improved and lowered to 7% by using honey protein as an internal isotopic standard, which overcame the problem of the suspect sample "grey area" with $\delta^{13}C$ values between –23.5 ‰ and –21.5 ‰ (White & Winters, 1989). This internal standard stable carbon isotope ratio analysis (ISCIRA) method was validated for worldwide application and adopted by the Association of Official Analytical Chemists (AOAC) for the detection of honey adulteration with $C_4$-plant sugars (Cunniff, 1999). However, one of the drawbacks of this method is that it is more challenging to use with honeys that have a naturally low protein content (e.g. acacia honey) and honeys where there is the potential for co-precipitation of other endogenous compounds (e.g. methylglyoxal in Manuka honey) (Rogers et al., 2014). Moreover, the AOAC method cannot be applied to detect honey adulteration with $C_3$-plant-derived sugars such as invert syrups produced from hydrolyzed rice, potato and beet starch. This problem has been overcome to a large extent by the use of Liquid Chromatography coupled with Isotope Ratio Mass Spectrometry (LC-IRMS). LC-IRMS permits the $\delta^{13}C$ value of fructose, glucose, and di-, tri- and oligosaccharides in honey to be complemented with AOAC 998.12 data and provide a more robust method for the detection of $C_3$– and $C_4$-plant-derived sugar syrups in honey (Elflein & Raezke, 2008). In addition, high-field Proton Nuclear Magnetic Resonance Spectroscopy ($^1H$ NMR) has been successfully used to characterize authentic honeys and invert syrups to try to identify specific marker compounds and build chemometric models relating to multiple $^1H$ chemical shifts, indicating the presence of $C_3$-plant sugar syrups (Spiteri et al., 2015). Whilst these methods exhibit greater reliability, they are at the forefront of honey testing technology. However, they are not readily accessible to many laboratories with 'entry level' stable isotope equipment, such as Elemental Analyzer-Isotope Ratio Mass Spectrometry (EA-IRMS), or laboratories with no access to high-field $^1H$ NMR facilities.

Bulk hydrogen stable isotope analysis, measured by high-temperature thermal conversion EA-IRMS or chromium reduction EA-IRMS, is an effective technique to supplement carbon stable isotope analysis in many areas of food authentication (Rossmann, 2001). Consequently, it is reasonable to assert that some of the disadvantages of the ISCIRA method may be overcome by measuring $\delta^2H$ of the sugars present in honey due to protium and deuterium being more markedly fractionated and varying more widely in the biosphere. However, the technical challenge of using hydrogen stable isotope analysis is that it requires the measurement of the carbon-bound non-exchangeable (CBNE) hydrogen isotopes ($\delta^2H_{NE}$) rather than the labile portion, which can readily exchange with ambient water vapour in the environment (i.e., amine, carboxyl and hydroxyl hydrogen atoms). Site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR) has been successfully applied to the measurement of non-exchangeable hydrogen isotope ratios for the detection of added sugar, after fermentation to ethanol, in fruit juices (Martin et al., 1986; Martin et al., 1996) and, for some specific cases in honey, dependent on floral type and geographical origin (Cotte et al., 2007). However, SNIF–NMR analysis is relatively time consuming as it requires at least three days for the fermentation step to convert sugars to ethanol, high-yield distillation of the ethanol, and corrections for the deuterium content of the water present during fermentation, which need to be applied to ensure the accuracy of ethanol-methyl group hydrogen stable isotope ratio measurements, known as $(D/H)_I$ by convention. Other researchers managed to solve this problem by removing the exchangeable hydrogen atoms, which are connected to oxygen in hydroxyl groups of carbohydrates and then measuring the isotopic ratios of derivatives such as calcium acetate (Werner & Rossmann, 2015) and calcium formate (Krueger, 1995) measured by isotope ratio mass spectrometry (IRMS). However, these methods can still be labor-intensive and require highly corrosive reagents, such as concentrated nitric acid, 'chromic' acid or perchloric acid, respectively. More recently, Abrahim et al. (2020) developed a novel approach that substitutes the hydroxyl group with a trifluoroacetate group, which makes the sugar derivatives sufficiently volatile to be separated and measured by Gas Chromatography coupled to Isotope Ratio Mass Spectrometry (GC-IRMS). The challenge of applying this technique is that GC-IRMS is a relatively specialized instrument that is not as widely available as EA-IRMS. In 2003, Kelly et al. (2003) reported a method of periodate oxidation of fructose isolated from apple juice to produce formaldehyde, followed by reaction with ammonia to form hexamethylenetetramine (HMT), which retained position-specific hydrogen isotopic information from only the carbon-bound hydrogen atoms in the parent fructose molecule. Periodic acid has been used since the 1930s to elucidate the structure of carbohydrates through oxidation of the vicinal diols to form carbonyl compounds such as formaldehyde and formic acid (Jackson & Hudson, 1938). The reaction between formaldehyde and ammonia in aqueous solution generally leads to the formation of HMT without any by-products (Polley et al., 1947). Despite its successful application in identifying authentic apple juices adulterated with beet and cane invert sugar syrups, this method had the drawback of requiring semi-preparative high-performance liquid chromatography for isolation of fructose and GC-IRMS for $\delta^{13}C$ and $\delta^2H$ measurement (Kelly et al., 2003).

The objective of the research presented here was the development of a simplified method for the preparation and detection of stable isotopes of carbon and CBNE hydrogen in HMT synthesised from honey sugars and invert sugar syrups, based on a simplified formation and instrumental analysis of HMT, using a standard EA-IRMS system. We measured the $\delta^2H$ and $\delta^{13}C$ values of HMT synthesized from the monosaccharides fructose and glucose present in honey and invert syrup samples, collected from various botanical and geographical origins, using the proposed method to demonstrate its potential for detecting adulteration of honey with exogenous sugar syrups added illegally for economic gain.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals and isotopic reference materials

HMT standard material (99.7% w/w), formaldehyde solution (39% w/v) and ammonia solution (32% w/w) were obtained from VWR Chemical Co. Stable isotope reference materials USGS61 (caffeine) and USGS62 (caffeine) were obtained from the Reston Stable Isotope Laboratory (RSIL), United States Geological Survey (USGS). The reagent periodic acid ($H_5IO_6$, for synthesis, ≥98.0% w/w) was purchased from the Sigma-Aldrich Chemical Company (MA, USA). The solvent dichloromethane (DCM) (ACS reagent, ≥99.8% w/v) for HMT extraction was purchased from Merck Co. (Germany). Laboratory general purpose reagent (GPR) grade sodium hydrogen carbonate ($NaHCO_3$, ≥99.0% w/w), fructose (≥99.0% w/w) and glucose (≥99.5% w/w) were obtained from the Sigma-Aldrich Chemical Company (MA, USA).

#### 2.1.2. Authentic honey, reference materials and syrup samples

Genuine honey samples were collected from project partners in IAEA Coordinated Research and Technical Cooperation Projects, where there was a high degree of certainty regarding their authenticity. IAEA project partners collected the samples directly from reputable beekeepers and producers, with whom they had established working relationships, in New Zealand ($n = 34$), Chile ($n = 17$) and Malaysia ($n = 10$). Authentic honey reference materials USGS82 (honey from tropical Vietnam) and USGS83 (honey from a prairie in Canada) were obtained from RSIL, USGS. Commercial rice syrups ($n = 9$), corn syrup ($n = 2$) and Agave syrup ($n = 5$) were purchased from various commercial suppliers.

### 2.2. Methods

In this study, HMT was first synthesised from GPR-grade fructose and glucose, and its purity was assessed through LC-MS/MS. Stable isotope analysis of hydrogen and carbon was performed on the synthesised

HMT, with appropriate calibration and quality control procedures, to monitor the instrument's performance and determine the long-term intra-laboratory precision of the isotopic measurements. The HMT synthesis conditions (reaction time, pH) were then optimised to obtain a consistent yield and high-purity product, and the optimised method was applied on multiple syntheses of HMT from various sugar sources, including GPR grade fructose and glucose, a 1:1 mixture of these two monosaccharides (approximating to their relative presence in honey), as well as honey and syrup samples, to assess the repeatability of the entire HMT procedure. Finally, $\delta^2$H and $\delta^{13}$C measurements were performed on HMT produced from 63 authentic honey samples and 16 invert sugar syrups from $C_3$, $C_4$ and CAM plants through the optimized methodology.

#### 2.2.1. Synthesis of HMT

HMT was synthesised through periodate oxidation of sugars, followed by reaction of the produced formaldehyde with ammonia. The oxidising reagent was prepared by mixing an amount of 8% w/v $NaHCO_3$ solution with 3 mL of 30% w/v $H_5IO_6$ solution under stirring until effervescence stopped. The exact amount 8% w/v $NaHCO_3$ solution used was established through the optimization experiments described below. A sample of 100–200 mg of fructose, glucose, 1:1 glucose-fructose mixture, honey or syrup, was accurately weighed and dissolved in 5 mL of deionized water. The sample solution was added to the oxidising reagent, and stirred continuously at room temperature for 45 min. 3.5 mL of 32% w/w ammonia solution was then added and left to react with the formaldehyde produced during the oxidation reaction (the optimal reaction time was defined through the optimization). The reaction mixture was then rotary evaporated to dryness, using acetonitrile to form a lower-boiling azeotrope to facilitate water removal. The HMT was extracted from the dried residual reagents and reaction products by shaking with 30 mL DCM. The filtered DCM extracts were combined before rotary-evaporating to a less than 5 mL volume and transferred to a 5 mL glass vial. The remaining DCM was removed by passing dry nitrogen gas over the HMT/DCM solution until no apparent solvent remained. The vial was then heated at 105 ℃ until a constant weight of HMT was obtained.

#### 2.2.2. LC-MS/MS analysis of synthesised HMT to assess its purity

HMT can be accurately quantified using HPLC/DAD (Lim et al., 2014) or LC-MS/MS (Molognoni et al., 2018). In this study, a LC-MS/MS system consisting of a Waters Alliance 2695 liquid chromatograph coupled to a triple quadrupole mass spectrometer (Waters Co., Milford, MA, USA) was employed to evaluate the purity of the HMT synthesised from honey monosaccharides fructose and glucose. The system was equipped with an electrospray ionisation source, working in positive mode ($ESI^+$) with multiple-reaction monitoring (MRM). An Alltima C18 5 μm-column (150 mm × 3.0 mm i.d., Alltech, USA), maintained at 40 ℃, was used for chromatographic separation. The mobile phase consisted of an aqueous solution containing 10 mmol/L ammonium formate and 0.1% formic acid (mobile phase A) and methanol acidified with 0.1% formic acid (mobile phase B). The linear gradient elution was performed as follows: 0–1 min, 95% A; 3 min, 40%; 3.1–11 min, 95% A. The flow rate and injection volume were 0.25 mL/min and 10 μL, respectively. The MRM transitions for the detection of HMT were *m/z* 141.09 → 112.01 (quantitative ion, collision energy, 12 eV) and qualitative ions were as follows *m/z* 141.09 → 41.91 (collision energy, 23 eV); *m/z* 141.09 → 70.80 (collision energy, 17 eV); *m/z* 141.09 → 84.39 (collision energy, 15 eV). The cone voltage for each MRM transition was set at 35 V.

#### 2.2.3. Stable isotope analysis of hydrogen and carbon in HMT

*2.2.3.1. IRMS measurement.* The $\delta^2$H value of HMT was determined using elemental analyser - chromium reduction - isotope ratio mass spectrometry (EA-CR-IRMS). The EA-CR-IRMS system consisted of a Thermochemical Conversion – Elemental Analyser (TC-EA, Thermo Fisher Scientific, Bremen, Germany) fitted with a Uniprep autosampler (Eurovector, Milan, Italy) coupled to a Delta V IRMS (Thermo Fisher Scientific, Bremen, Germany). The TC-EA housed a chromium-chip packed alumina reactor, maintained at 1050 °C, to retain carbon and nitrogen, yielding hydrogen gas for determination of the mass distribution of its isotopologues ($^2H^1H$ and $^1H^1H$) by IRMS (Kelly et al., 2001). 0.4 mg of the prepared HMT samples were weighed into silver capsules (5 mm × 3.5 mm) for EA-IRMS measurement. $\delta^2$H values were measured with respect to the hydrogen monitoring (cylinder) gas using the proprietary software Isodat version 3.0 (Thermo Fisher Scientific, USA) and then subsequently normalized to the VSMOW-SLAP scale using reference materials USGS61 and USGS62, which are caffeine standards without exchangeable hydrogen atoms and contain the hetero-atom nitrogen. $H_3^+$ corrections were calibrated using increasing intensities of the monitoring gas and applied automatically to $\delta^2$H values by the Isodat software. A quality control in-house reference material (IHRM) of HMT (99.7% w/w, VWR) was also analyzed within each batch of HMT samples to monitor the repeatability of the EA-CR-IRMS measurement. The $\delta^{13}$C value of HMT was measured by EA-IsoLink system (Thermo Fisher Scientific, Bremen, Germany), using typical combustion conditions, i.e., a tungsten oxide and copper-filled quartz reactor maintained at 1050 °C. About 0.2 mg of HMT samples were weighed in tin capsules (5 mm × 3.5 mm) for carbon stable isotope measurement. Similarly, the $\delta^{13}$C results were normalized to the V-PDB scale using reference materials USGS61 and USGS62. The HMT IHRM was also measured within each batch to monitor the ongoing performance of the $\delta^{13}$C measurement. The isotopic abundance ratios ($^2H/^1H$ and $^{13}C/^{12}C$) were expressed in the delta-notation according to Eq. (1):

$$\delta = \left(R_{Sample}/R_{Reference} - 1\right) \quad (1)$$

R represents the ratio of the heavy stable isotope relative to the light stable isotope of the element of interest.

*2.2.3.2. Quality control.* Monitoring the long-term stability of stable isotope measurements is of paramount importance when developing and using authenticity methods that rely on comparison between a database of reference values, from genuine samples, and those of an adulterant ingredient. In this case, comparing CBNE hydrogen and carbon stable isotope measurements of HMT prepared from authentic honey samples against invert sugar syrups to ensure the long-term validity of authenticity interpretations. To monitor the instrument's performance and determine the long-term intra-laboratory precision of the isotopic measurements, the $\delta^2$H value of the HMT IHRM was measured in triplicate on 15 different batch-analysis-days over six months. Whilst the $\delta^{13}$C value of the HMT IHRM was measured in triplicate on six different batch-analysis-days over three months. Control charts were created and evaluated using warning limits (mean ± 2σ) and action limits (mean ± 3σ) based on the "Westgard rules" adapted from the IUPAC guidelines (Thompson and Wood, 1995).

## 3. Results

### 3.1. Quality control of stable isotope analyses

As shown in Fig. 1, the HMT IHRM $\delta^2$H measurement precision in our study ranged from 0.08 to 1.74 ‰ (n = 3), which is consistent with expectations for hydrogen stable isotope analysis through a high-flow EA inlet; typically ≤ 3‰ (Hilkert et al., 1999). The average sample standard deviation ($\sigma_{n-1}$) for the $\delta^{13}$C value of the HMT IHRM was 0.14 ‰ over six batch-analysis-days, which indicates good stability for the measurement of carbon isotopes using the standard EA-IRMS combustion technique. All measured delta values of the HMT IHRM fell within the control limits and complied with the "Westgard rules".

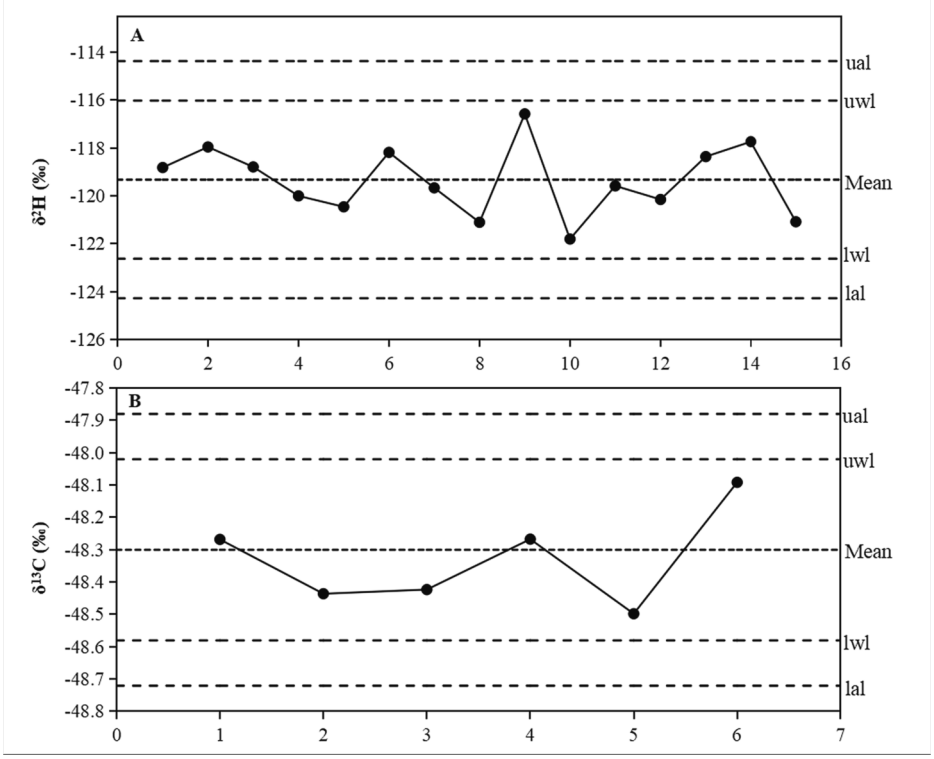


**Fig. 1.** Control chart for $\delta^2$H (a) and $\delta^{13}$C (b) measurements of the in-house reference material hexamethylenetetramine (IHRM HMT); ual = upper action limit (mean + 3σ), uwl = upper warning limit (mean + 2σ), lwl = lower warning limit (mean − 2σ), lal = lower action limit (mean − 3σ).

### *3.2. Optimisation of the reaction conditions for the synthesis of HMT*

Formaldehyde is known to react with ammonia in the aqueous phase to form the heterocyclic "cage-like" organic compound HMT (Ogata & Kawasaki, 1964), which takes approximately 3 h according to previously published literature (Kelly et al., 2003). We conducted experiments using formaldehyde and ammonia laboratory reagent grade solutions, with different reaction times (0.5, 1, 2 and 3 h), to verify optimum conditions for HMT synthesis. To ensure the quality of isotopic data obtained from HMT derived from honey, it was important to obtain a consistent yield and high purity product whilst minimising the time taken for reaction. Consistent high yields of HMT reduced the possibility of introducing isotopic fractionation, or limiting the expression of any kinetic isotope effect, in the carbon and/or CBNE hydrogen present in the HMT product. Consequently, the yield and purity of the synthesised HMT were determined by LC-MS/MS. All treatments yielded similar amounts of HMT product, ranging from 25.15 mg to 26.01 mg, compared with the theoretical HMT yield of 28 mg, from the amount of sugars used, equivalent to 90 – 93 % yield. Furthermore, the measured purity of the HMT produced ranged from 89.0% w/w to 98.0% w/w. Statistical analyses (one-way ANOVA and LSD test) were performed using PASW Statistics 18. There was no significant difference in the purity of HMT with different reaction times ($p > 0.05$), as shown in Fig. 2A. Therefore, one hour was selected as a conservative estimate of the minimum reaction time required between formaldehyde and ammonia to form high purity HMT with a consistent and acceptable yield.

The pH of the periodic acid solution is critical in the oxidation of carbohydrates due to the sensitive acetal and ester linkages present or formed during the reaction. To evaluate the effect of the pH value on the purity of the HMT product, we prepared sodium bicarbonate ($NaHCO_3$) solutions of varying concentrations. These were mixed with periodic acid to control the pH of the oxidizing agent solution prior to pure sugar, honey or invert syrup addition. Fig. 2B and 2C show that the purity of the HMT product varied significantly with the pH of the oxidizing agent solution when synthesised from both fructose and glucose. The purity of HMT from fructose and glucose prepared with oxidizing agent solutions containing periodic acid only (pH = 0.82) was 50.4% and 67.4%, respectively. We observed that the HMT purity initially decreased and then increased with increasing pH of the periodic acid oxidising agent solution. Ultimately, the yield of the HMT product was more stable and purer when the pH was over 6.0. Consequently, the optimal reaction conditions were obtained with a pH value = 6.5 using 10 mL of 8% w/v sodium hydrogen carbonate solution to produce the oxidising agent solution.

### *3.3. Repeatability of $\delta^2$H and $\delta^{13}$C measurements of HMT synthesised from various sugar sources*

We applied the optimized method to make quintuple syntheses of HMT from various sugar sources, including GPR grade fructose, GPR grade glucose, a 1:1 mixture of these two materials (approximating to their relative ratio amount in honey), authentic honey, and rice invert syrup, to assess the repeatability of the entire HMT procedure. Table 1 summarizes the $\delta^2$H and $\delta^{13}$C values and the purity of HMT prepared from the different sugar starting materials. The standard deviation of $\delta^2$H for five separate preparations ranged from 1.5 ‰ to 3.0 ‰, while the standard deviation of $\delta^{13}$C for five preparations ranged from 0.09 ‰ to 0.42 ‰. Additionally, the synthesized HMT obtained from the different sugar sources possessed a consistently high purity, ranging from 87.5% to 98.5% w/w. These data demonstrated that the method produced acceptable precision for repeat preparation and isotopic measurement and performed favorably compared to the published GC-IRMS method for HMT analysis, which gave sample standard deviations for hydrogen and carbon of ± 2.5 ‰ (n = 6) and ± 0.5 ‰ (n = 6), respectively (Kelly et al., 2003).

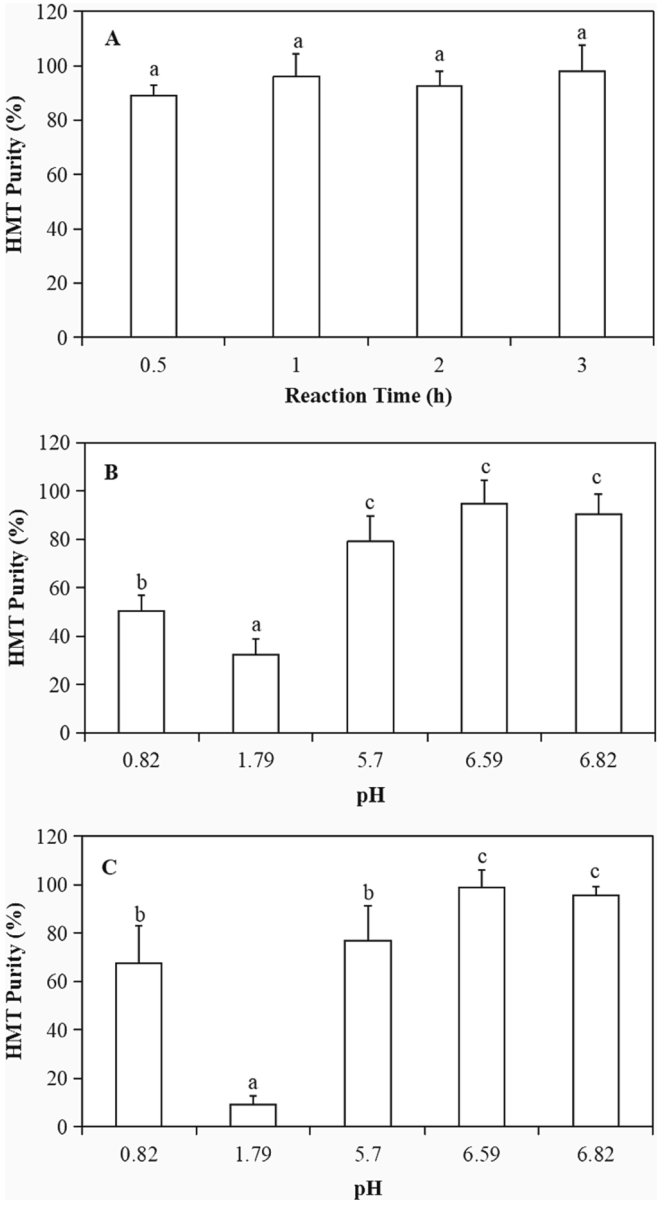


**Fig. 2.** Variation in HMT purity with different derivatization times (A, reaction with ammonia) and pH values of the oxidising solution (B, fructose; C, glucose).

**Table 1**
Repeatability of HMT synthesis procedure – purity, hydrogen and carbon stable isotope data of quintuple syntheses of HMT prepared from different sugar sources.

| Sugar source | n | Purity of HMT (%w/w) | $\delta^2$H ± SD (‰) | $\delta^{13}$C ± SD (‰) |
|---|---|---|---|---|
| GPR Fructose Standard | 5 | 98.5 ± 6.6 | −29.2 ± 2.3 | −13.2 ± 0.2 |
| GPR Glucose Standard | 5 | 91.4 ± 3.4 | −35.7 ± 3.0 | −11.5 ± 0.4 |
| Mixture of GPR Fructose/ Glucose (1:1) | 5 | 93.4 ± 2.3 | −30.9 ± 2.8 | −12.3 ± 0.2 |
| Clover Honey from New Zealand | 5 | 90.7 ± 7.2 | –233.0 ± 1.5 | −31.2 ± 0.2 |
| Rice Invert Syrup | 5 | 87.5 ± 3.0 | −170.4 ± 1.8 | −31.8 ± 0.1 |

SD = sample standard deviation $\sigma_{(n-1)}$.

### 3.4. $\delta^2$H and $\delta^{13}$C values of HMT synthesised from authentic honey and sugar syrup samples

The optimized HMT synthesis procedure described above was applied to 63 authentic honey and 16 invert sugar syrup samples. The $\delta^2$H and $\delta^{13}$C values of HMT prepared from authentic honey produced in New Zealand, Chile, Malaysia, Vietnam and Canada, and invert sugar syrups derived from rice ($C_3$ plant), corn ($C_4$ plant) and agave (CAM plant), are summarized in Table 2.

The HMT derived from honey produced in different countries exhibited a range of $\delta^2$H values as expected, due to a combination of factors, such as the systematic fractionation of hydrogen isotopes through the global hydrological cycle; and physiological, morphological and biochemical differences in the nectar–source plants (Estep and Hoering, 1980). Malaysian honeys possessed $\delta^2$H values that were relatively enriched, with a mean value of −174.8 ‰, compared with the average $\delta^2$H values of −194.5 ‰ and −183.1 ‰ of honey from Chile and New Zealand, respectively. The $\delta^2$H value of the USGS82 honey reference material, which originated from Ho Chi Minh City, Vietnam, was relatively close to the $\delta^2$H values of the samples from Malaysia. This is reasonable finding since these two countries are located in tropical areas with similar climatic conditions. Furthermore, in both countries the honeys were produced by bees located in forests, with a potentially diverse range of nectar sources. The USGS83 honey reference material sample from Saskatoon, Canada, possessed a heavily depleted deuterium $\delta^2$H value of −290.6 ‰, indicating both the latitude and inland effect on the fractionation of hydrogen isotopes in the source water used for the metabolism of nectar carbohydrate. It is also reasonable to assume that plant morphology and physiology influenced the heavily depleted value, as small prairie-type plants, such as clover, with a small leaf area leads to relatively limited evapotranspiration and low enrichment of leaf-water hydrogen prior to assimilation through photosynthesis (White, 1989). This phenomenon is also observed in the $\delta^2$H values of the honey samples from Chile and New Zealand derived from clover nectar sources, i.e., having relatively depleted hydrogen isotope values within their country's sample's range of $\delta^2$H values (see supplementary data S1 for floral sources and individual $\delta^2$H values). The rice syrups were slightly enriched in deuterium compared to the honey samples from Chile and New Zealand, with an average $\delta^2$H value of −178 ‰. However, a large overlap of $\delta^2$H values of samples between rice syrup and these two countries, as well as the majority of samples from Malaysia, was observed, as shown in Fig. 3. The corn syrup and agave syrup were both enriched in deuterium compared to the honey and rice syrup. The overall mean HMT $\delta^2$H values for corn and agave syrup were −55.6 ‰ and –32.8 ‰, respectively. The $\delta^{13}$C value of the HMT results for authentic honey samples ranged from −35.3 ‰ to –31.9 ‰. The honeys from Malaysia were relatively depleted in $^{13}$C compared to the remaining honey samples. The corn invert syrup and agave-sap syrup, which were derived from $C_4$ plant and CAM plants, respectively, were enriched in $^{13}$C as expected, compared to honey and rice invert syrups that are derived from plants using the $C_3$ photosynthetic pathway.

Fig. 3 shows an X-Y scatter plot generated from the measured isotope ratio ($\delta^2$H-$\delta^{13}$C) data of HMT prepared from the honey samples and various sugar syrups. The 95% prediction interval (PI) ellipse is shown for authentic honey centred around a $\delta^2$H value of −180‰ and a $\delta^{13}$C value of –32 ‰. This representation assists in visualizing the potential of $\delta^2$H and $\delta^{13}$C values to differentiate the majority of authentic honey from invert sugar syrups analysed in this study. Overlapping isotope ratios between rice syrup and honey from New Zealand, Chile and Malaysia were observed. However, honey from Canada can be well separated from rice invert syrup based on stable hydrogen isotope measurements of HMT. Both hydrogen and carbon stable isotope values of HMT permit clear differentiation between authentic honey of various origins used in this study, and $C_4$ corn invert syrup and CAM agave syrup.

**Table 2**
Summary of $\delta^2H$ and $\delta^{13}C$ values of HMT prepared from authentic honey and sugar syrups.

| Sample types | Origin / Photosynthetic pathway | n | $\delta^2H$ (‰) | | | | $\delta^{13}C$ (‰) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | mean | SD | Max | min | mean | SD | max | min |
| Honey | New Zealand | 34 | −183.1 | 15.0 | −154.9 | −233.0 | −31.2 | 0.8 | −28.1 | −32.5 |
| | Chile | 17 | −194.5 | 19.4 | −176.4 | −235.9 | −32.5 | 0.6 | −31.6 | −33.7 |
| | Malaysia | 10 | −174.8 | 12.4 | −161.9 | −204.1 | −33.7 | 0.8 | −32.6 | −35.3 |
| | Vietnam (USGS82) | 1 | −174.3 | – | – | – | −29.2 | – | – | – |
| | Canada (USGS83) | 1 | −290.6 | – | – | – | −30.5 | – | – | – |
| Rice syrup | $C_3$ | 9 | −178.0 | 9.7 | −165.1 | −190.7 | −33.1 | 1.5 | −31.2 | −34.5 |
| Corn syrup | $C_4$ | 2 | −55.6 | 8.6 | −49.5 | −61.7 | −16.8 | 2.2 | −15.2 | −18.4 |
| Agave syrup | CAM | 5 | −32.8 | 9.5 | −21.1 | −46.6 | −14.3 | 0.6 | −13.7 | −15.1 |

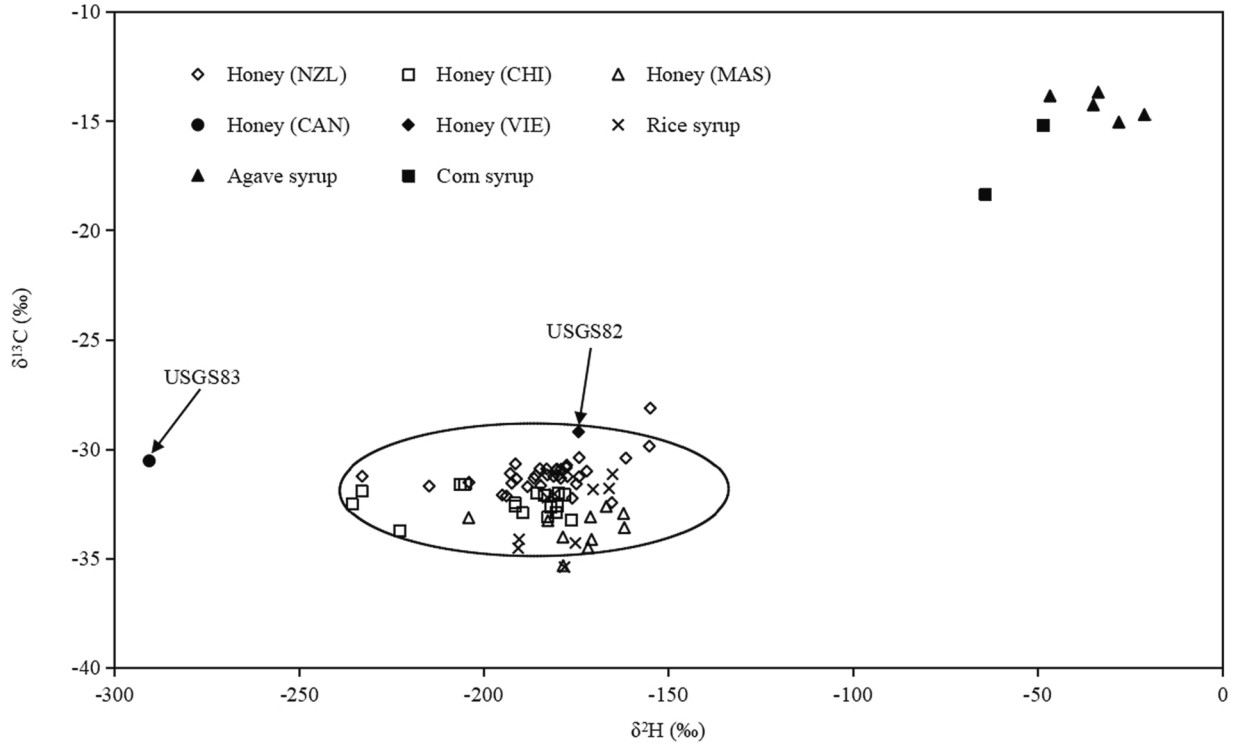


**Fig. 3.** X-Y scatter plot of $\delta^2H$ and $\delta^{13}C$ values of HMT synthesised from fructose and glucose derived from different sugar sources (95% prediction interval (PI) ellipse is shown for authentic honey).

## 4. Discussion

This study demonstrated that the EA-CR-IRMS system achieved acceptable precision for inter- and intra-day measurements of a standard reagent grade HMT IHRM, with the highest SD of 1.74 ‰ for $\delta^2H$ measurements. This is an improvement over the previous within laboratory HMT $\delta^2H$ measurement repeatability, which reported a precision of ±2.4 ‰ using GC-pyrolysis-IRMS. However, it is generally accepted as a less precise technique compared to EA-IRMS (Kelly et al., 2003). The high-temperature decomposition of nitrogen-containing compounds in an EA-IRMS system can produce hydrogen cyanide (HCN) as a thermally stable byproduct. The use of chromium metal as a reducing agent prevents the formation of HCN (Gehre et al., 2015). This is important to prevent incomplete conversion of HMT, to the measuring gas hydrogen, and the potential fractionation and consequent detrimental effects on the accuracy and repeatability of $\delta^2H$ determinations.

It is important to note that position-specific $\delta^2H$ values, from both fructose and glucose in the honey, are determined when applying the technique presented in this study. Fructose and glucose are oxidatively cleaved by periodic acid, which breaks the carbon–carbon bonds with α-glycol, α-hydroxy ketone, or glycolaldehyde groups (Jackson, 1944). During the reaction of a monosaccharide with periodic acid, the terminal methyl-hydroxy moieties are oxidised to formaldehyde. Whereas the intermediate H—C—OH moieties, or aldehyde groups, are oxidized to formic acid (Bobbitt, 1956). Consequently, one mole of glucose yields one mole of formaldehyde corresponding to the $C_6$ position, whilst one mole of fructose yields two moles of formaldehyde corresponding to $C_1$ and $C_6$ positions (see Fig. 4). Generally, both fructose and glucose require five equivalents of periodic acid or periodate for oxidation since they are six-carbon carbohydrates. In this study, the carbohydrate was oxidized with an excess of periodate, using the mole ratio of 1:7, to ensure that all vicinal diols in the carbohydrates were cleaved and degraded by the oxidant. This ratio was selected to maximize the yield of the HMT product, after the addition of ammonia, and to ensure that the oxidation was not reactant-limited and proceeded to completion.

As mentioned previously, the pH of the reaction medium is critical for the reaction efficiency when carrying out periodate oxidation. Eisenberg (1954) reported that the oxidation of potassium gluconate proceeds much more rapidly in sodium periodate ($NaIO_4$) buffered at pH 5.8 rather than in periodic acid ($H_5IO_6$) with a pH of 1. Specifically, it takes 45 min using periodate to approach the theoretical consumption of the oxidant, whilst complete oxidation of potassium gluconate by

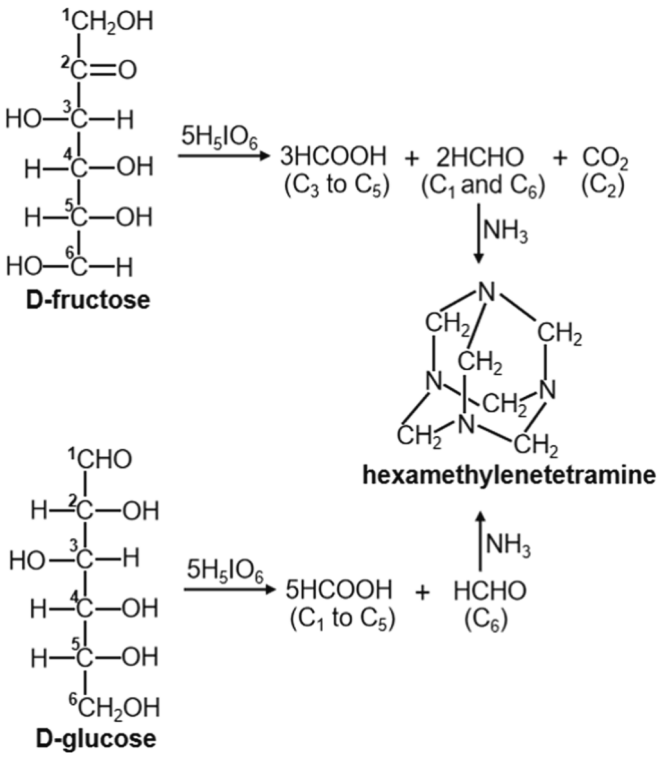


**Fig. 4.** Schematic representation of the synthesis of HMT from fructose and glucose (the predominant sugars present in honey).

periodic acid takes 20 h. In this study, different amounts of sodium bicarbonate mixed with periodic acid were used to achieve a pH range of 0.82 to 6.82 for the oxidation process. The experiments yielded similar results to previous reports on the optimum pH for the periodate oxidation of sugars as illustrated in Fig. 2B and 2C. The periodate oxidation of 1,2-diols involves the formation of a key intermediate, a cyclic periodate ester of the diol, which decomposes to the final products (Jackson, 1944). The pH effect observed in the periodate oxidation reaction is likely due to the salt effect on the dissociation of periodic acid, as suggested by Buist et al., (1966). It is also important to note that there is no exchange of CBNE hydrogen with the water in the reaction medium during the synthesis of HMT. This was previously demonstrated by Kelly et al., (2003) by producing HMT from a standard fructose using water with a $\delta^2$H value of −44.3 ‰ and a water enriched to a $\delta^2$H value of 31,000 ‰, which had no significant effect on the HMT $\delta^2$H value.

Until recently, there was a lack of effective techniques to measure the CBNE hydrogen isotope ratios of sugar in honey, leading to a limited amount of data for CBNE $\delta^2$H values, apart from those obtained previously using the SNIF-NMR® technique (Cotte et al., 2007). However, the approximate distribution of hydrogen isotope values in honey from different origins can be observed by measuring the hydrogen isotopes in bulk honey samples and/or honey protein samples. For example, a study involving 516 honey samples from 20 European regions showed that the $\delta^2$H values of honey protein were higher in regions close to the Atlantic Ocean (e.g. Algarve: −73 ‰) than in regions further from the sea (e. g. Allgau: −121 ‰) (Schellenberg et al., 2010). Banerjee et al., reported the $\delta^2$H of bulk honey collected from Canada and Costa Rica ranged from −117 ‰ to −57 ‰ (Banerjee et al., 2015). Furthermore, the $\delta^2$H value of the reference material USGS82 collected from tropical Vietnam was calibrated at −43.1 ‰ for the bulk sample, while the other honey reference material, USGS83, collected from the prairies in Canada, was assigned a $\delta^2$H value of −110.5 ‰ (Schimmelmann et al., 2020). In this study, the measured HMT $\delta^2$H value of honey sugars prepared from USGS82 and USGS83 were −174.3 ‰ and −290.6 ‰, respectively. These reflect the relatively enriched and depleted values reported by Schimmelmann et al. However, the HMT values are significantly more separated (116 ‰) compared to the $\delta^2$H values of the bulk samples (67 ‰), which include water from the plant nectar. Schimmelmann et al. reported that the moisture content of the Vietnamese honey used to produce USGS82 was reduced from 22% w/w to 14% w/w by evaporative drying to prevent microbial spoilage. The water content of the honey from Saskatoon, used to produce USGS83, was 9.4% w/w (Schimmelmann et al., 2020). This demonstrates that measuring the position-specific HMT CBNE hydrogen isotope values of honey sugar provides a better indicator of the botanical and geographical source than the bulk honey hydrogen isotope ratios, which includes the extrinsic water from exchangeable hydrogen of the sugar hydroxyl groups and the remaining nectar water present in the honey. Furthermore, the data presented in Fig. 3 from this feasibility study indicate that, on average, the addition of corn syrup and agave syrup to honey was easily detected through the CBNE hydrogen and carbon isotope ratios using the HMT method. It is known that the carbon isotopic compositions are highly related to the photosynthetic pathway of the botanical source. These pathways ($C_3$, $C_4$, and CAM) have different water-use efficiencies and rates of transpiration, which can influence the way hydrogen isotopes are fractionated during water uptake and evapotranspiration processes (Smith & Ziegler, 1990). Furthermore, different plant groups have distinct strategies to access water, such as shallow-rooted versus deep-rooted plants. These plants may uptake water with varying isotopic compositions based on the depth of the water source and seasonal effects on precipitation $\delta^2$H values, which can affect the overall hydrogen isotopic composition in plant materials (Dawson, 1993). These factors are in addition to the global hydrogen isotopic ratio variation observed in precipitation and groundwater, which systematically varies due to fractionation in the hydrological cycle (Gat et al., 2011; Coplen et al, 2002). As a result, agave syrup, derived from the botanical plant cultivated in hot and dry regions of Mexico, exhibits a higher abundance of deuterium. Plants from arid environments often have specialized structures (e.g., thick leaves or sunken stomata) to reduce water loss, which may lead to different patterns of hydrogen isotopic fractionation compared to plants from wet or relatively humid environments. Moreover, the large size of corn leaves facilitates strong leaf-water evapotranspiration, resulting in the enrichment of deuterium in the leaf water and in the plant's photosynthetic products. The HMT $\delta^2$H values of agave syrup ranged from −46.6 ‰ to −21.1 ‰, and corn syrup ranged from −61.7 ‰ to −49.5 ‰, and were found to be comparable to those reported in previous literature for bulk honey (agave syrup: −43 ‰; corn syrup: −21 ‰ to −14 ‰) (Banerjee et al., 2015). Moreover, the HMT $\delta^2$H values are in line with expectations for hydrogen isotope data from $C_4$ plants compared to the $(D/H)_I$ value of ethanol from $C_4$ syrups obtained using the SNIF-NMR® technique and reported by Cotte et al. (2007) as 108.6 ppm (–30.3 ‰ $_{V\text{-}SMOW}$), which are derived from carbon-bound hydrogen at the 1, 6 and 6′ position of honey glucose (Martin et al., 1986). As expected, the addition of CAM-derived agave syrup and $C_4$-dervied corn syrup can be easily distinguished from the majority of authentic honey based on hydrogen and carbon isotope ratios. However, it can be quite challenging to differentiate between rice syrup and most authentic honey due to the overlap in the $\delta^2$H and $\delta^{13}$C values, as shown in Fig. 3. This is because the $\delta^{13}$C values of rice syrup are similar to those of authentic honey, as they both utilize the Calvin ($C_3$) Cycle to fix carbon. Additionally, the range of $\delta^2$H values of rice syrup lies within the range of most of the authentic honeys used in this study. The stable hydrogen isotope fractionation in plants can be affected by their botanical origin, i.e., the specific plant species or group a plant belongs to, and the geographical location of the plant. Different plant species or groups may exhibit variations in their physiological and biochemical processes, leading to differences in hydrogen isotope fractionation patterns (Gao et al., 2014). Nevertheless, it is possible to differentiate deuterium-depleted honey produced at high latitudes, or very inland regions such as Saskatoon, Canada, which is resolved from rice syrup (-290.6 ‰ versus −190.7 ‰, the lowest value for rice syrup,

respectively). This latter observation specifically highlights the importance of considering both the geographic and botanical origin when applying this HMT technique to detect exogenous $C_3$-derived invert sugar added to honey.

## 5. Conclusion

In its current stage of development, the HMT method offers a reliable complementary solution of detecting adulteration of honey with commercial invert syrups using stable isotope analysis of CBNE hydrogen and carbon. The advantage of this synthetic method is the absence of side reactions, relatively gentle reaction conditions and a high yield of the target HMT compound. Because hydrogen stable isotope fractionation in plants is influenced by botanical origin linked to variations in water sources, plant physiology, morphology, and biochemical processes, further work is required to establish honey and sugar syrup databases from various botanical and geographical origins. In this way, it will be possible to identify when the HMT method can be reliably applied to detect $C_3$ rice invert syrup in honey. From the preliminary results reported here, it appears that the method has potential to detect $C_3$ rice invert syrup when applied to honeys that have been produced at higher latitudes and more inland areas, which possess more depleted $\delta^2$H values. In such a case where a specific floral and geographical origin for a honey is claimed, then specific databases can be applied rigorously. Compared to existing methods for determining $\delta^2$H values of CBNE in honey sugars, this technique offers some distinct advantages regarding ease of use, analysis time and position-specific information. Additional commercial rice syrup samples need to be evaluated to characterize their natural $\delta^2$H variation and other $C_3$ invert syrup sources such as sugarbeet and chicory (inulin). There is also potential to examine the correlation between the $\delta^2$H value of HMT prepared from honey fructose and glucose with the $\delta^2$H value of extracted honey protein as an internal isotopic standard. However, the challenge of routinely and rapidly measuring the latter cannot be overlooked for many of the aforementioned reasons.

## CRediT authorship contribution statement

**An Li:** Writing – original draft, Investigation, Methodology, Formal analysis, Funding acquisition. **Aiman Abrahim:** Investigation, Methodology, Writing – review & editing. **Marivil Islam:** Methodology, Writing – review & editing. **Enrique Mejías:** Resources, Writing – review & editing. **Nur Hafizati Abdul Halim:** Resources, Writing – review & editing. **Russell Frew:** Resources, Writing – review & editing. **Christina Vlachou:** Supervision, Writing – review & editing, Resources. **Simon D. Kelly:** Conceptualization, Investigation, Methodology, Writing – review & editing, Supervision, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137451.

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