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Quantitative Analysis of Sugar Ingredients in Beverages and Food Crops by an Effective Method Combining Naphthimidazole Derivatization and ^{1}H-NMR Spectrometry

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ABSTRACT

Background: Though carbohydrates are needed for health, excessive uptake of sugar may induce obesity, decayed teeth, and chronic diseases. The Taiwan Food & Drug Administration has recently proposed to regulate common sugars, a list that includes glucose, galactose, fructose, lactose, maltose, and sucrose in beverages and dietary foods. Accordingly, rapid and effective methods are needed for the quantitative analysis of sugar ingredients in beverages and foods.

Objective: To establish a convenient method of quantifying sugar ingredients in beverages and foods by using one-dimensional ^{1}H-NMR spectroscopy via a simple treatment with naphthimidazole (NAIM) labeling kit.

Methods: The sample of beverages or the hydrolysate of food crops were treated with an NAIM labeling kit that contains 2,3-naphthalenediamine and iodine in acetic acid solution. After the complete conversion of the reducing sugars to sugar-NAIM derivatives, the mixture solution was subjected to rotary evaporation under reduced pressure, and the residue was dissolved in D_2O

solution with dimethylsulfoxide (DMSO) as an internal standard for the ^{1}H-NMR spectrometric analysis.

Results: An aldose existing in two anomeric isomers is completely converted to the corresponding NAIM derivative that shows a single characteristic vinyl proton at a distinct position to facilitate the quantitative analysis by NMR spectrometry. Even 10 μmol of common sugar ingredients in 50 μL of beverage or in the hydrolysate prepared from 1 mg of food crop can be quantified.

Conclusion: The results suggest that a simple treatment of beverage with the NAIM labeling kit provides a convenient method for the quantification of sugar ingredients by ^{1}H-NMR spectrometry. This method combining NAIM derivatization and ^{1}H-NMR analysis is also useful for the profiling and fingerprinting of food crops.

Keywords: sugar, beverage, food crop, naphthimidazole, ^{1}H-NMR spectrometry, quantitative analysis.

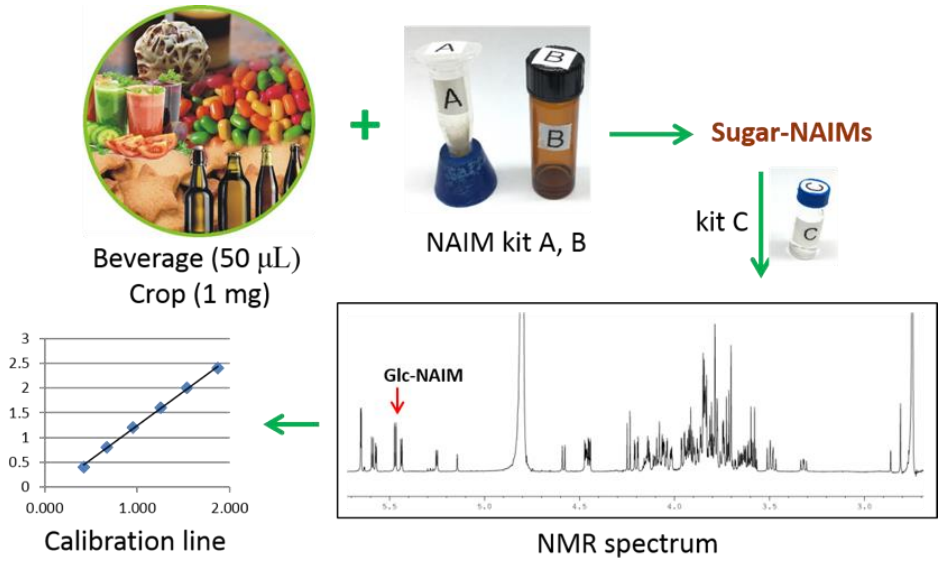
INTRODUCTION

Carbohydrates are found in various beverages and dietary foods, including rice, milk, fruit, juice, and vegetables. Glycans are the polymer forms of sugar occurring as starch, amylopectin, cellulose, and fiber in crop foods. Carbohydrates are also used as "added sugar" in soft drinks, cookies, candies, and foods. For example, the added sugar in beverage can be sucrose, high-fructose corn syrup (HFCS), and other sweeteners. Differential sugar profiling plays an essential role in energy intake [1]. Though carbohydrates are needed for health, an excessive uptake of sugar may induce obesity, decayed teeth, and chronic diseases [2−4]. It is important to know the sugar ingredients in foods. For this reason, foods of low glycemic index (GI) are suggested for diabetes patients. Furthermore, many countries have introduced the sugar tax and soft-drink tax in order to reduce sugar consumption [5]. The appropriate sugar intake is 25 grams per day according to the scientific recommendation by the World Health Organization (WHO) [6]. Since August 2015, Taiwan Food & Drug Administration (TFDA) has proposed to regulate common sugars in foods, including glucose (Glc), galactose (Gal), fructose (Fru), lactose (Lac), maltose (Mal), and sucrose (Suc) [7]. The amounts of sugars must be labeled in the "Nutrition Facts Panel" for the products of beverages and foods. Even though the information of sugar content surely benefits consumers, this regulation will impose challenges to the food industry concerning the identification and quantification of specific sugar in beverages and crop foods.

Chemical, enzymatic, and instrumental techniques have been utilized in the analysis of sugars in foods. For an example of chemical analysis, the Luff−Schoorl method was used to determine the total content of reducing sugars by treating with excess $CuSO_4$ in alkaline boiling solution, followed by the iodometric titration of the remaining Cu(II) ions [8]. Although this chemical method shows high accuracy in sugar analysis, it cannot distinguish individual sugars. In contrast, enzymatic methods for sugar analysis are specific. The concentration of a specific sugar (e.g. glucose) is measured by stoichiometry of the reaction catalyzed a particular enzyme (e.g. glucose oxidase), and the change in the accompanied coenzyme system (e.g. NADH/NAD^{+}) is often evaluated by photometric methods (e.g. the change of UV absorption at 340 nm for NADH). Calibration with standards is required for enzymatic assays because the enzyme activity may vary with reaction conditions (e.g. pH, temperature, and solvent).

Chromatographic methods, especially reversed-phase high-performance liquid chromatography (HPLC), have been widely used in sugar analysis [9]. However, carbohydrate molecules lack responsive chromophores; thus, the prior introduction of chromophore/fluorophore to carbohydrates are usually required for the detection in chromatography and electrophoresis [1, 10]. For example, analyses of carbohydrates are often performed by labeling with appropriate reagents of 2-aminobenzamide (2-AB) [11], 2-aminopyridine (2-AP) [12], phenylhydrazine [13], 1-phenyl-3-methyl-5-pyrazolone (PMP) [14] or 2,3-naphthalenediamine [15] to form the derivatives that are responsive to ultraviolet-visual (UV-vis) or fluorescence detection. We have previously demonstrated that labeling aldoses with 2,3-naphthalenediamine via an iodine-promoted oxidative condensation reaction to form the naphthimidazole (NAIM) derivatives is a highly sensitive method for HPLC, capillary electrophoresis (CE), and mass analyses [16−18]. For example, the sugar composition in beverages and dietary foods can be determined by HPLC analysis via their NAIM derivatives [18]. This kind of derivatization of carbohydrates may also increase hydrophobicity to improve ionization for mass spectrometric analysis [16]. Though high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) can be used to analyze original carbohydrates without derivatization [1, 10], the instrument is expensive and the decay of the column and electrode is a concern in the system with strong alkaline elution.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful method for simultaneous identification and quantification of low molecular weight compounds in complex mixtures [19]. Due to a long acquisition time and the nuclear overhauser effect, ^{13}C-NMR spectra are less commonly used than ^{1}H-NMR spectra in the routine quantitative analysis of sugars [20]. However, the simultaneous quantification of all sugar ingredients from a single one-dimensional (1D) ^{1}H-NMR spectrum is still difficult due to the overlap of the proton signals. This problem may be circumvented by using two-dimensional (2D) NMR spectroscopy along with special software, such as independent component analysis (ICA) [21] and pulse length based concentration determination (PULCON) [22] to resolve the overlapping signals. Alternatively, we present in this study a convenient method for the routine quantification of six common sugars in beverages and food crops via NAIM derivatization for 1D ^{1}H-NMR spectrometric analysis (Scheme 1).



Scheme 1. The materials and method in NAIM tagged sugars for beverages and crops analysis using kits and ^{1}H-NMR.

MATERIALS AND METHODS

General

All chemicals and solvents were of analytical grade and used without further purification. Sugars (glucose, galactose, mannose, maltose, lactose, fructose, and sucrose), iodine, acetic acid (AcOH), 2,3-naphthalenediamine, and deuterium solvents were purchased from Merck & Co., Inc. (Darmstadt, Germany). Beverages were purchased from tea drinking stores and the crop samples, e.g. soybean (non-GMO), rice (common rice), and wheat (low gluten flour), were purchased from traditional local markets in Taipei city. The NAIM labeling kit used in this study was a gift from Sugarlighter Co., Inc. (New Taipei City, Taiwan). This kit is suitable for analysis of sugar ingredients up to 5 mg of the total amount in a sample.

General procedure for preparation of sugar-NAIM derivatives

According to the previously reported procedures [15], glucose (2.0 mg, 11 μmol), 2,3-naphthalenediamine (2.0 mg, 13 μmol), and iodine (2.0 mg, 8 μmol) in AcOH (1.0 mL) was stirred at room temperature. The reaction was completed in less than 3 h as indicated by the thin-layer chromatography (TLC) analysis. The mixture was concentrated by rotary evaporation under reduced pressure to give the sample of Glc-NAIM derivative, which was directly subjected to ^{1}H-NMR measurement without further purification. This reaction protocol is applicable to prepare other sugar-NAIM derivatives, including those of mixed sugars, in smaller quantities.

Alternatively, mono and disaccharides were converted to the sugar-NAIM samples by using a NAIM labeling kit that consists of three vials (Sugarlighter Co., New Taipei City, Taiwan). In brief, vial A containing 2,3-naphthalenediamine (10.0 mg) and vial B containing iodine (2.0 mg) in AcOH solution (1.0 mL) was used for conversion of reducing sugars to the NAIM derivatives. Vial C containing D_2O (1.0 mL) and a small amount (0.03−0.1%) of dimethylsulfoxide (DMSO) as the internal standard was used in recording ^{1}H-NMR spectra.

General procedure for analysis of common sugars in beverage

Beverage (50 μL) was taken and directly treated with a NAIM labeling kit. Pretreatment or dilution of the beverage sample is not required in this typical analysis. The components of reducing sugars in beverage were converted to the corresponding NAIM derivatives at room temperature using the reagents from vials A and B of the NAIM labeling kit. The resulting solution was concentrated under reduced pressure by rotary evaporation, and the residue was dissolved in vial C for ^{1}H-NMR measurement.

General procedure for analysis of the monosaccharides released from the glycan of food crops

In a typical procedure, food crop (1.0 g) was ground for homogenization and washed with water (10 mL × 2) to remove free monosaccharides. The dried material (1.0 mg) was treated with trifluoroacetic acid (TFA) (1 mL of 4 M aqueous solution) at 110 ^{o}C for 4 h. The resulting aqueous solution was concentrated by rotary evaporation under reduced pressure at room temperature. The residue containing the released monosaccharide components was subsequently treated with a NAIM labeling kit (vials A and B) at room temperature. The sample was dissolved in D_2O solution containing DMSO (vial C) as an internal standard for the ^{1}H-NMR measurement.

^{1}H-NMR analysis

The ^{1}H-NMR spectra were recorded on a Bruker AV600 MHz NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with a 5 mm dual cryoprobe DCI ^{1}H/^{13}C. The above-prepared NAIM-derivatized sample was dissolved in D_2O solution containing DMSO (vial C) as an internal standard. Quantification of sugars was based on the integral areas of the characteristic proton signals (e.g. H-2 in Glc-NAIM) by comparison with that of DMSO (integral region from δ 2.792 to 2.727 ppm for six protons of the two methyl groups). ^{1}H-NMR acquisition parameters: 90^o pulse, P1 = 9.95 μs, PL1 = −0.8 dB; relaxation delay D1 = 2 sec; number of acquisition aq = 1.9530824 (s); type of baseline correction: quad; window function: EM; LB = 0.5 Hz; software for spectral processing and regression analysis: TopSpin 3.0.

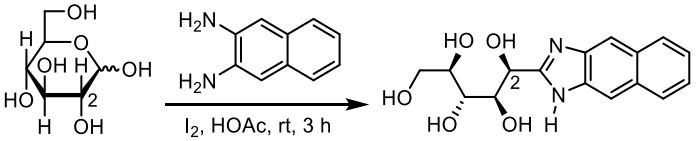
HPAEC–PAD analysis

Beverage was 100-fold diluted in double-distilled water (dd-H_2O), and 10 μL of the sample was injected for HPAEC–PAD analysis. Alternatively, food crop (1 mg) was hydrolyzed and concentrated. The residue was 100-fold diluted in dd-H_2O and 10 μL of the sample was injected for HPAEC–PAD analysis. The above-prepared carbohydrate samples were analyzed using a Dionex^{TM} ICS-3000 DC equipment containing a gradient pump and an eluent degas module. Separation of carbohydrate molecules was carried out on a CarboPac PA-10 anion-exchange column (250 × 2 mm). The mobile phase contained 100 mM NaOH (eluent A) and 500 mM NaOAc (eluent B) in gradients. Eluent A was constant (100%) during 0–10 min and gradient (100% to 0%) was produced during 10–30 min with eluent B. The flow rate was 0.25 mL min^{-1}. Carbohydrates were detected by pulsed amperometric detection (PAD) with a gold working electrode and a hydrogen reference electrode. The temperature was set at 25 ^{o}C and all analyses were carried out in duplicate.

RESULTS

Derivatization and NMR spectrometric analysis of aldo-sugars

An aldose molecule exists inherently in solution as a mixture of the □□and □□anomeric isomers, displaying a rather complicated ^{1}H-NMR spectrum. The transformation of both aldose anomers to a single NAIM compound (Eq. 1) would simplify the ^{1}H-NMR analysis. An aldose (2 mg) was generally converted to the NAIM derivative at room temperature in less than 3 h by using a NAIM labeling kit that contains the reagents of 2,3-naphthlenediamine and iodine in acetic acid. Completion of the oxidative condensation reaction of aldose was monitored by TLC analysis and later confirmed by the ^{1}H-NMR spectrum. After the removal of acetic acid by rotatory evaporation under reduced pressure, the residue of sugar-NAIM derivative without further purification was dissolved in D_2O for recording the ^{1}H-NMR spectrum. Instead of using the conventional but less accessible reagent, trimethylsilylpropanoic acid (TMSP sodium salt, $Me_3SiCD_2CD_2CO_2Na$), the readily available and cost-effective reagent DMSO was applied as an internal standard, which showed the two methyl groups as a singlet at δ 2.73 ppm. The NAIM derivatives of several mono and disaccharides, including glucose (Glc), galactose (Gal), mannose (Man), rhamnose (Rha), arabinose (Ara), glucuronic acid (GlcUA), *N*-acetylglucose (GlcNAc), maltose (Mal), and lactose (Lac) were individually prepared and subjected to ^{1}H-NMR spectral analyses. Table 1 lists the characteristic proton signals of these sugar-NAIM compounds.



(Eq. 1)

Table 1. ^{1}H-NMR data (600 MHz, D_2O) of the NAIM derivatives prepared from common mono and disaccharides[a].

Compound	Chemical shift (δ, ppm)				
	H-2	H-3	H-4	H-5	H-6
Glc-NAIM	5.38	4.40	3.85	3.77	3.75, 3.62
Gal-NAIM	5.54	4.15	4.06	3.95	3.80, 3.80
Man-NAIM	5.59	4.69	4.28	3.93	3.87, 3.73
Rha-NAIM	5.13	4.33	3.95	3.72	–
Ara-NAIM	5.49	4.11	3.97	3.96, 3.80	–
GlcUA-NAIM	5.53	4.41	4.13	4.31	–
GlcNAc-NAIM	5.44	4.51	3.90	3.84	3.73, 3.60
Mal-NAIM	5.52	3.89	3.66	4.11	3.93, 3.82
Lac-NAIM	5.56	4.43	4.14	4.08	3.93, 3.84

[a] The signal of HDO was set at δ 4.80 ppm, and the internal standard $(CH_3)_2SO$ (0.1%, v/v) occurred at δ 2.73 ppm.

Glucose exists as a mixture of α and β anomers, which revealed the C-1 protons at δ 5.24 and 4.66 respectively (Figure 1A). In comparison, the ^{1}H-NMR spectrum of Glc-NAIM (Figure 1B) was significantly simplified, and the H-2 shifted downfield to δ 5.38 as a doublet (J = 5.4 Hz). The characteristic H-2 of Gal-NAIM (Figure 1C) appeared at δ 5.54 (d, J = 1.8 Hz). The disaccharide derivative Mal-NAIM (Figure 1D) exhibited H-2 at δ 5.52 (d, J = 1.8 Hz) and the glycosidic proton (H-1′) at δ 5.25 (d, J = 3.6 Hz), whereas Lac-NAIM (Figure 1E) displayed H-2 at δ 5.56 (d, J = 4.2 Hz) and H-1′ at δ 4.59 (d, J = 7.8 Hz). These sugar-NAIM derivatives consistently showed the characteristic patterns of C-2 protons in the region of 5.1–5.6 ppm in addition to other well recognizable proton signals in the ^{1}H-NMR spectra. Thus, the parental sugars could be easily inferred from their corresponding NAIM derivatives using ^{1}H-NMR spectrometry. Specifically, this ^{1}H-NMR method is versatile to distinguish glucose from mannose (C2-epimer) and galactose (C4-epimer). Maltose and lactose were also readily differentiated by the ^{1}H-NMR spectra of their NAIM derivatives.

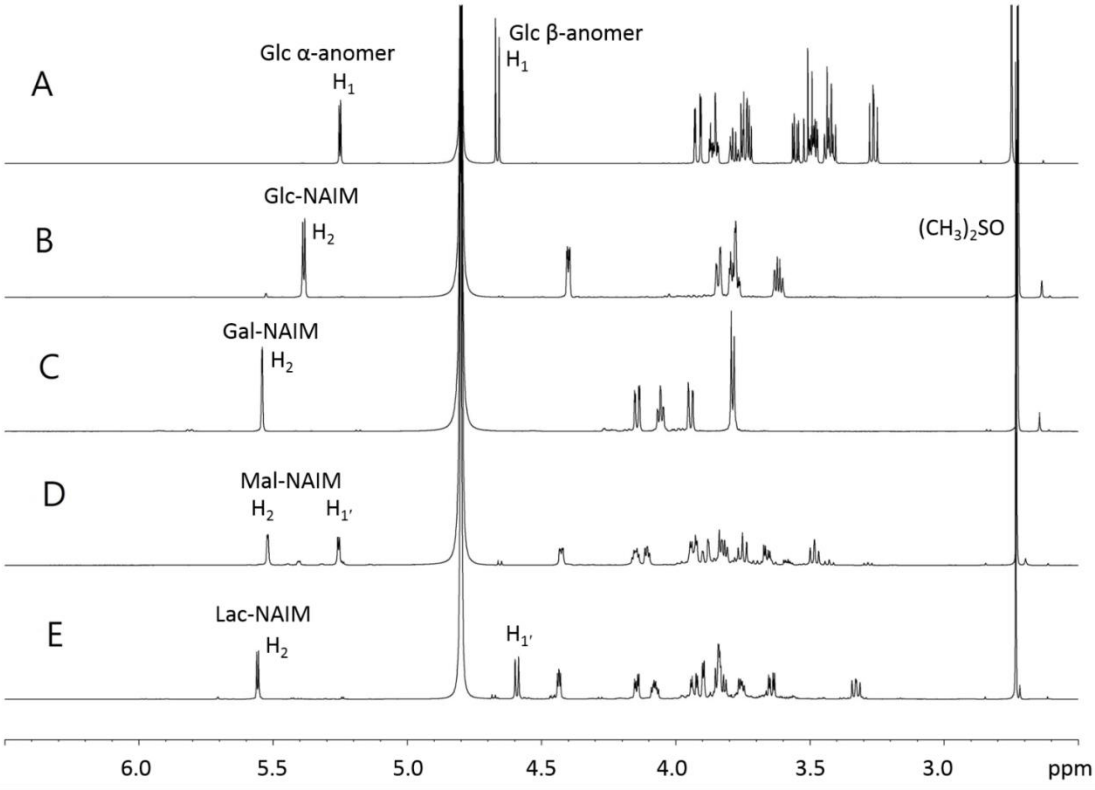


Figure 1. ^{1}H-NMR spectra (600 MHz) in D_2O solution (1.0 mL) containing 0.1% $(CH_3)_2SO$ as internal standard: (A) Glc, (B) Glc-NAIM, (C) Gal-NAIM, (D) Mal-NAIM, and (E) Lac-NAIM. The aromatic protons of NAIM derivatives in the range of δ 7.2–8.2 ppm are not shown for clearance. The signal of HDO was set at δ 4.80 ppm, and the signal of $(CH_3)_2SO$ occurred at δ 2.73 ppm.

To demonstrate the advantage of using NAIM derivatives in ^{1}H-NMR analysis of sugar mixture, a sample containing 4 aldoses (Glc, Gal, Mal, and Lac) in equal amounts (5 mg each) was subjected to NAIM derivatization using a NAIM labeling kit, followed by quantitative analysis using ^{1}H-NMR spectrometry. The parental sugars Glc, Mal, and Lac could not be easily quantified because their C-1 protons overlapped on the same position (Figure 2A). In contrast, the sugar-NAIM derivatives were easily distinguished by their C-2 protons with the diagnostic patterns at distinct chemical shifts, which may have small variation due to intermolecular interactions (Figures 2B−2E). Taking the integration area of the $(CH_3)_2SO$ peak from δ 2.792 to 2.727 ppm for the two methyl groups (6 protons) as a reference, one could calculate the amount of each sugar-NAIM derivative from its H-2 signal. The glycoside protons (H-1′) of Mal-NAIM (at δ 5.25) and Lac-NAIM (at δ 4.59) could also be used for quantitative analysis. The sugar-NAIM mixture in small amounts as low as 0.25 mg of each component could be detected with S/N ≥ 5 by ^{1}H-NMR spectroscopy (Figure 2E).

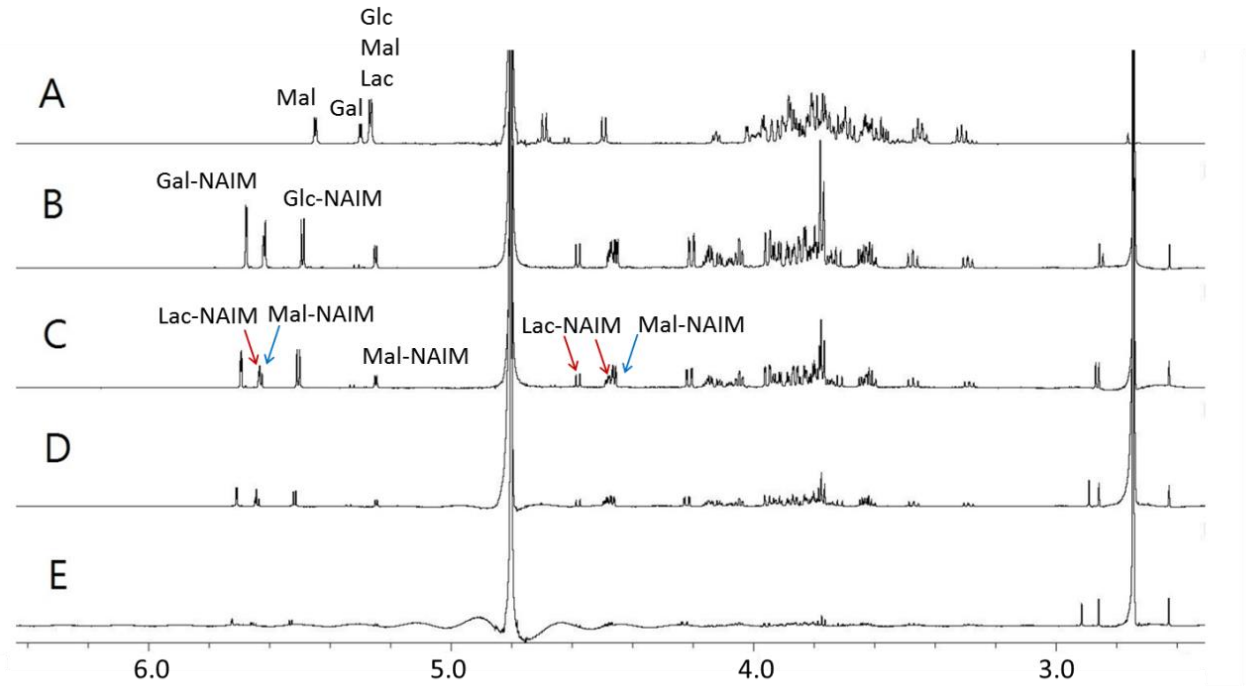


Figure 2. ^{1}H-NMR spectra (600 MHz) in D_2O (1.0 mL) containing 0.1% $(CH_3)_2SO$ as an internal standard: (A) a mixture of 4 aldoses (Glc, Gal, Mal and Lac, 5 mg of each sugar). The mixtures of sugar-NAIMs were prepared from the corresponding aldose mixtures, which contain each aldose in 5 mg (B), 2.5 mg (C), 1.25 mg (D), and 0.25 mg (E) respectively. The aromatic protons of NAIM derivatives in the range of δ 7.2–8.2 ppm are not shown for clearance. The signal of HDO was set at δ 4.80 ppm and the signal of $(CH_3)_2SO$ occurred at δ 2.73 ppm.

NMR spectrometric analysis of six common sugars: We first examined the ^{1}H-NMR spectrum of a mixture containing six common sugars (Glc, Gal, Fru, Mal, Lac, and Suc). In this spectrum (Figure 3A), fructose has no distinct peak for identification. Furthermore, glucose and maltose could not be quantified because their anomeric protons (H-1α and H-1β) overlapped. Alternatively, the aldose components including Glc, Gal, Mal, and Lac in the sample were converted to the corresponding NAIM derivatives on the treatment with a NAIM labeling kit. The sugar-NAIM derivatives were readily distinguished by their characteristic signals in the ^{1}H-NMR spectrum (Figure 3B). Taking the integration areas of the characteristic proton signals, one can calculate the amount of each sugar-NAIM derivative, for example, from the H-2 signals of Glc-NAIM at δ 5.47, Gal-NAIM at δ 5.65, Mal-NAIM at δ 5.57, and Lac-NAIM at δ 5.59. The signals at δ 5.25 and 4.59 were also diagnostic for Mal-NAIM and Lac-NAIM respectively. It was noted that some resonances for the parental sugars and their NAIM derivatives in Figure 3 showed slight variations of chemical shifts by comparison with those in Figure 1 and Figure 5 (see below), though the signal patterns remained without changes. This phenomenon might be caused by intermolecular interactions at different degrees in the compound mixtures.

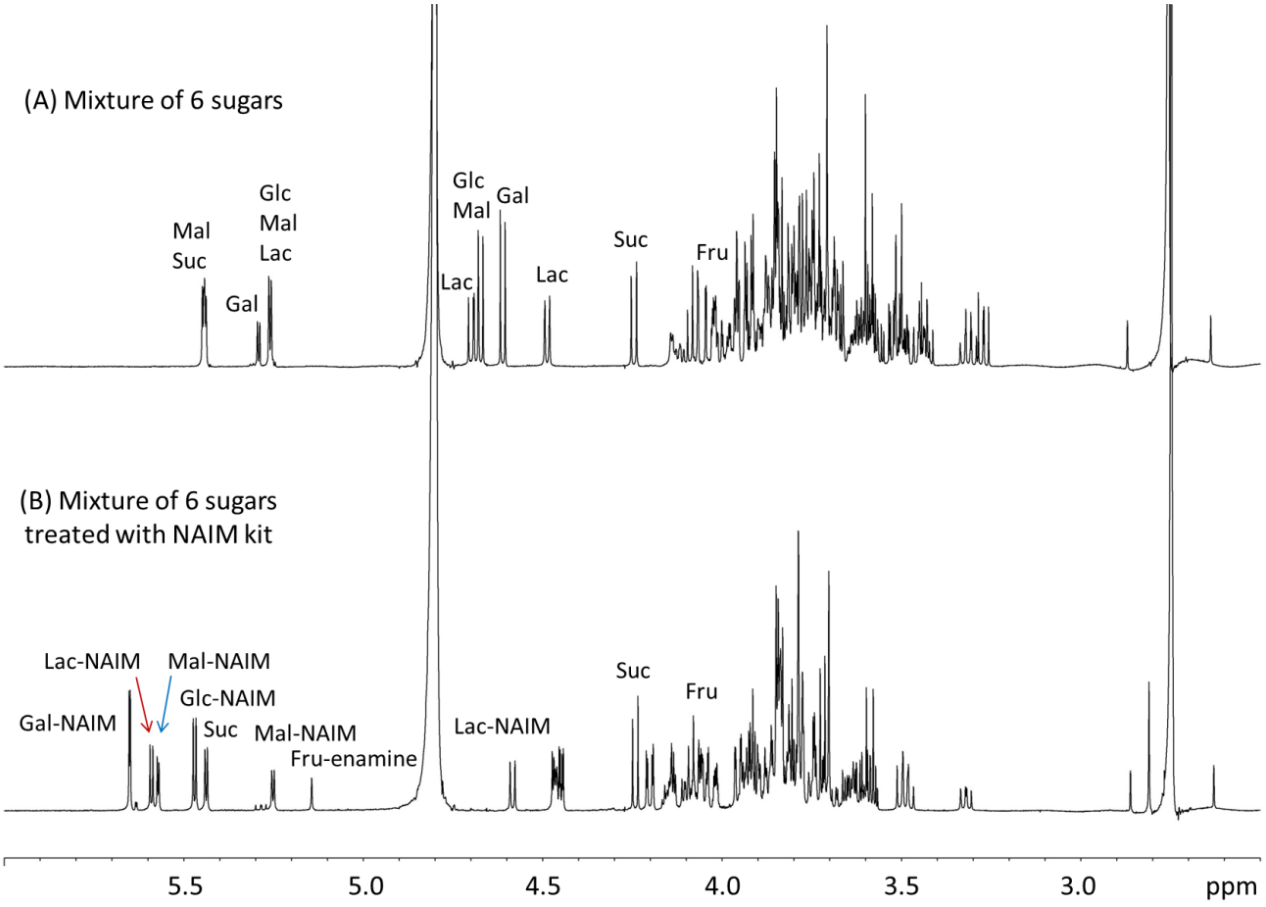


Figure 3. ^{1}H-NMR spectra (600 MHz) in D_2O (1.0 mL) solution containing 0.1% $(CH_3)_2SO$ as internal standard: (A) a mixture of 6 sugars (Glc, Gal, Fru, Mal, Lac, and Suc, with 5 mg of each sugar), and (B) a mixture containing four sugar-NAIMs, unmodified Suc, and partial conversion of Fru to Fru-enamine [**A**]. The aromatic protons of NAIM derivatives in the range of δ 7.2–8.2 ppm are not shown for clearance. The signal of HDO was set at δ 4.80 ppm and the signal of $(CH_3)_2SO$ occurred at δ 2.73 ppm.

Furthermore, we established the calibration lines for individual sugar component based on their characteristic proton signals in D_2O solution (1.0 mL) containing DMSO as the internal standard (Figure 4A−F). For example, the quantity of glucose (y) is calculated from the relative integration (x) of the selected proton signal of Glc-NAIM at δ 5.47 (Figure 3B and Figure 4A): $y = 1.3756x - 0.1399$ with a high coefficient of determination ($R^2 > 0.99$).

Sucrose, a nonreducing sugar, was retained without oxidative condensation by 2,3-naphthalenediamine under such reaction conditions. Nonetheless, sucrose can be identified by its glycosidic proton (H-1) at δ 5.44 and another proton at δ 4.23 (Figure 3B). Accordingly, the calibration line is established for quantification of sucrose: $y = 1.666x + 0.138$ using the selected proton signal at δ 5.44 (Figure 4E).

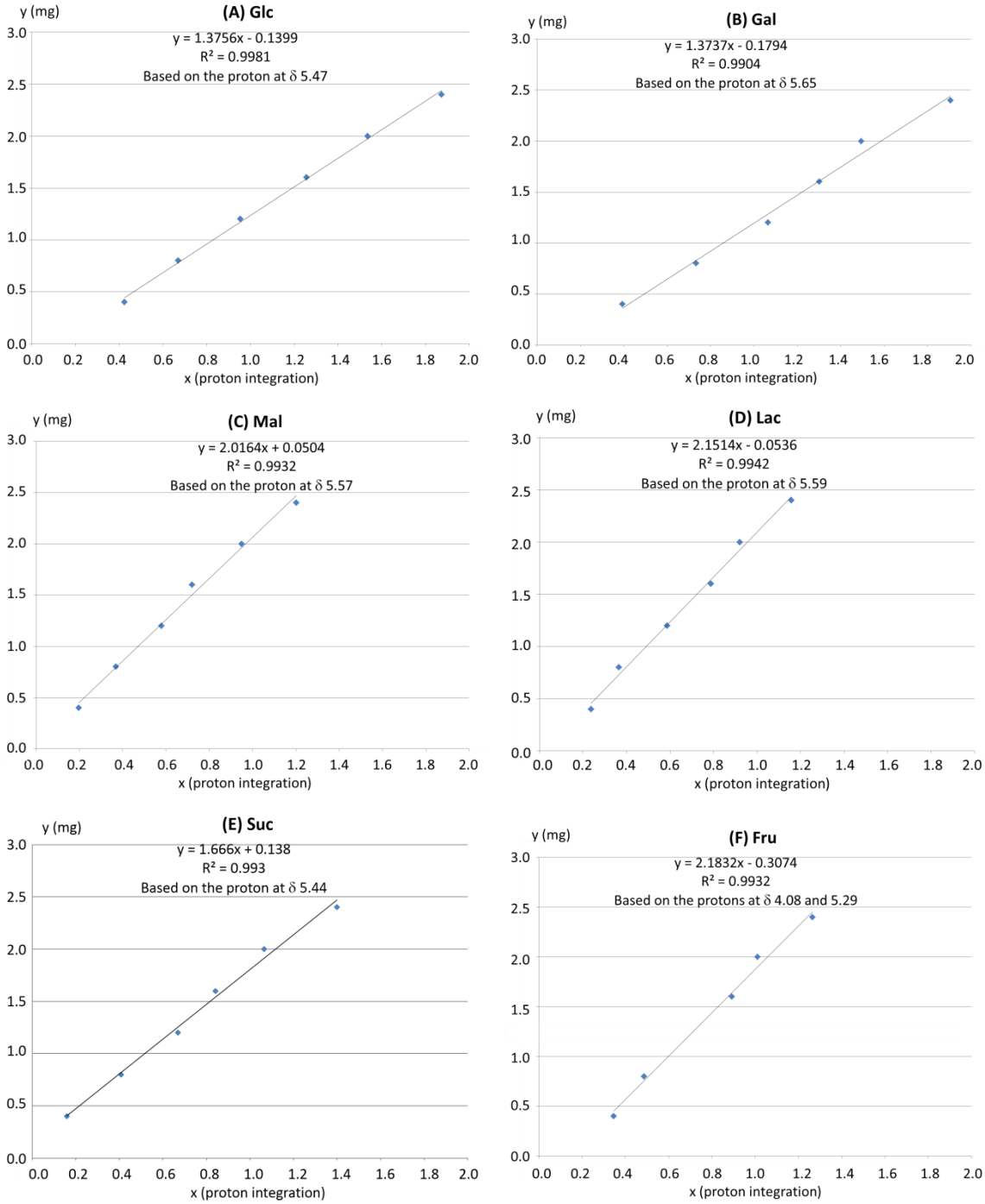


Figure 4. Calibration lines of Glc, Gal, Mal, Lac, Suc, and Fru ingredients: x is the relative integration of the selected proton in D_2O solution (1.0 mL), proportional to the 6 protons of $(CH_3)_2SO$ (4.3 μmol) at 0.03% (v/v) concentration in the ^{1}H-NMR spectrum; and y is the weight (in mg) of the parental sugar.

Interestingly, some small but distinct peaks were also observed at δ 5.14 (s), 5.29 (dd), and 5.64 (d) in Figure 3B. Though fructose could not form a NAIM derivative, we surmised that these peaks might belong to the intermediates derived from the reaction of fructose with 2,3-naphthalenediamine. Accordingly, we performed a separate experiment by treating fructose with

2,3-naphthalenediamine in D_2O solution (without addition of iodine) and recorded the ^{1}H-NMR spectrum (Figure 5B). In comparison with the spectrum of fructose (Figure 5A), the emerging proton signals occurring in Figure 3B also appeared in Figure 5B, though there were slight variations in chemical shifts. The signals at δ 5.20 (s), 5.32 (dd), and 5.66 (d) were tentatively ascribed to the structure of fructose-enamine [**A**] (containing the *E* and *Z* isomers), while the much smaller signals at 5.10 (s), 5.44 (t), 5.63 (d), and 9.24 (s) might be attributable to the α-amino aldehyde [**B**] as a tautomer of [**A**] (Eq. 2). Taking the combined integration (x) of the signals at δ 4.08 (d) for unchanged fructose and δ 5.29 (s) for the enamine [**A**] derivative, the original content of fructose (y) could be estimated by the following equation: $y = 2.183x - 0.307$ (Figure 4F).

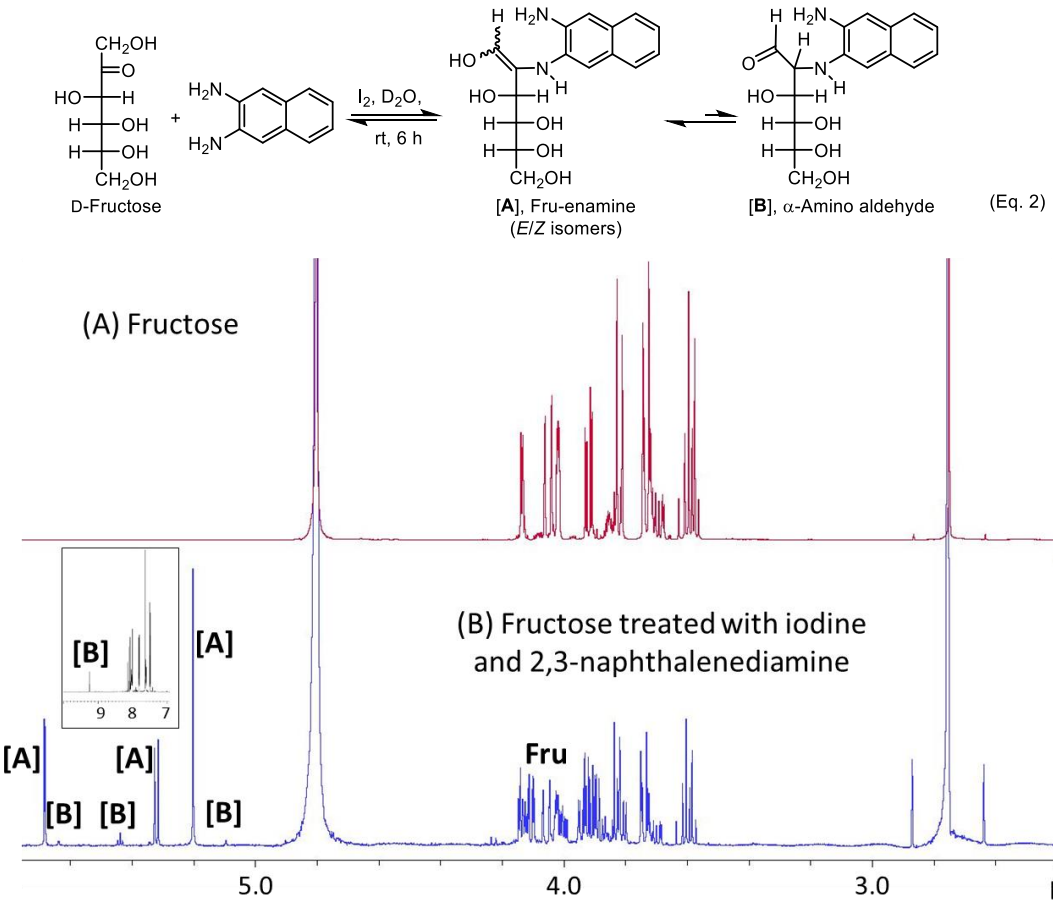


Figure 5. ^{1}H-NMR spectra (600 MHz) in D_2O (1.0 mL) solution containing 0.1% $(CH_3)_2SO$ as internal standard: (A) fructose, and (B) fructose treated with 2,3-naphthalenediamine. Inset: aromatic protons in the range of δ 7.4–8.2 ppm, and a singlet at δ 9.24 ppm that may be attributable to an aldehyde proton. The signal of HDO was set at δ 4.80 ppm and the signal of $(CH_3)_2SO$ occurred at δ 2.73 ppm.

NMR spectrometric analysis of six common sugars in beverage: The samples of milk tea and soymilk were purchased from shops and street vendors respectively. These samples are marked as H (high), M (medium), L (low), and F (free) according to their sugar contents. An

individual beverage sample (50 μL) was taken and directly treated with a NAIM labeling kit at room temperature. After removal of acetic acid by rotary evaporation under reduced pressure at room temperature, the residue of sugar-NAIM derivatives, without further purification, was dissolved in D_2O (1.0 mL) containing 0.1% DMSO as an internal standard for the ^{1}H-NMR analysis. Table 2 shows the quantities of individual sugar ingredients in each sample. In all the test samples of milk tea and soymilk, no glucose or fructose was found. However, in 100 mL of any kind of milk tea samples we discovered 1.0 g of lactose and 0.1 g of galactose due to the added milk. In contrast, there was no lactose or galactose found in the soymilk samples. Maltose was detected in all the soymilk samples which may come from the fermentation of soybean starch. Sucrose appeared to be the sole sugar added by vendors to milk tea and soymilk. The content of sucrose was as high as 18.4 g in 100 mL of milk tea (H) compared to no sucrose in the original milk tea (F). It was noted that even the allegedly sugarless soymilk (F) from markets still contained a noticeable amount of sucrose (1.8 g). In contrast, we also performed HPAEC–PAD analysis to determine the sugar contents in the above-mentioned beverages, and found that the results (data not shown) were consistent with that of ^{1}H-NMR analysis. According to our present analyses, one may still intake excessive sugar, predominating in sucrose, over the daily need (25 g) as recommended by WHO and nutritionists, by drinking a cup (300 mL) of the allegedly low-sugar-content milk tea or medium-sugar-content soymilk.

Table 2. Quantitative analysis of common sugars in beverages using ^{1}H-NMR spectrometric measurement (600 MHz) in D_2O (1.0 mL) solution containing 0.1% $(CH_3)_2SO$ as internal standard.[a]

Sample (100 mL)[a]	Glc (g)	Gal (g)	Fru (g)	Mal (g)	Lac (g)	Suc (g)	Total sugar (g)
Milk tea (H)	0	0.1	0	0	1.0	18.4	19.5
Milk tea (M)	0	0.1	0	0	1.0	13.5	14.6
Milk tea (L)	0	0.1	0	0	1.0	10.9	12.0
Milk tea (F)	0	0.1	0	0	1.0	0	2.1
Soymilk (H)	0	0	0	0.1	0	12.6	13.5
Soymilk (M)	0	0	0	0.1	0	9.3	9.4
Soymilk (F)	0	0	0	1.0	0	1.8	2.8

[a] The sample was subjected to treatment with NAIM labeling kit, and the amounts of sugar ingredients were deduced from the ^{1}H-NMR spectrometric analysis. The value of sugar content is the average of two measurements. The sugar contents in the samples of milk tea and soymilk are denoted as H (high), M (medium), L (low), and F (free) in parentheses respectively.

NMR spectrometric analysis of sugar composition in food crops: We also investigated the feasibility of using the ^{1}H-NMR spectrometric method to quantify the monosaccharides released from the glycans in food crops. A food crop (1 mg) was hydrolyzed in 4 M trifluoroacetic acid at 110 ^{o}C for 4 h. The crude hydrolysate was treated with a NAIM labeling kit at room temperature to give a sample containing the corresponding monosaccharide-NAIM derivatives. The sample was dissolved in D_2O solution (1.0 mL) containing 0.1% (v/v) of DMSO as an internal standard for the ^{1}H-NMR measurement. As shown in Table 3, starch is the main ingredient in all the tested food crops, yielding 1.4–11.2% (w/w) glucose after hydrolysis. Galactose was also found in 1.0% and 0.3% in soybean and potato respectively. Although soybean had the lowest sugar content among the test food crops, it still contains a noticeable amount of arabinose (3.7%). In the crop hydrolysate, there are also other types of monosaccharides and oligosaccharides such as maltose and its oligomers, presumably due to incomplete hydrolysis. However, no lactose or sucrose was found in all the crop samples. In this study, only a small amount (1 mg) of the raw material was required for the NAIM derivatization and ^{1}H-NMR analysis to quantify the monosaccharide contents. This method can be potentially utilized in the profiling and fingerprinting of food crops.

Table 3. Quantitative analysis of the glycan composition in food crops (1.0 mg) using ^{1}H-NMR spectrometric measurement (600 MHz) in D_2O (1.0 mL) solution containing 0.1% $(CH_3)_2SO$ as the internal standard[a].

Crop (1 mg)[a]	Glc (μg)	Gal (μg)	Ara (μg)	Others (μg)	Total sugar (μg)
Rice	112.2	N.D.[b]	N.D.[b]	N.D.[b]	112.2
Soybean	14.0	10.3	37.3	11.3	72.9
Wheat	102.7	N.D.[b]	9.5	13.4	125.6
Potato	82.9	2.8	4.4	N.D.[b]	90.1
Corn	89.1	N.D.[b]	2.0	2.2	93.3
Mung bean	91.2	N.D.[b]	N.D.[b]	N.D.[b]	91.2
Yam bean	77.0	0.2	8.2	2.5	88.0
Taro	93.7	N.D.[b]	3.4	N.D.[b]	97.0
Banana	65.3	N.D.[b]	4.7	N.D.[b]	70.0

[a] The sample was subjected to acidic hydrolysis, followed by NAIM labeling. The value of sugar content is the average of two measurements. [b] N.D. is not detected.

DISCUSSION

In this study, we demonstrate an efficient ^{1}H-NMR spectrometric method for routine quantification of six common sugars in beverages. In this typical analysis, pretreatment or dilution of the beverage sample is not required. The beverage sample (50 μL), i.e. milk tea in this study was simply treated with a NAIM labeling kit containing the reagents of 2,3-naphthalenediamine and iodine in acetic acid to convert the four reducing sugars (i.e. glucose, galactose, lactose and maltose) to their corresponding sugar-NAIM derivatives, along with unchanged sucrose and partially modified fructose. After rotary evaporation under reduced pressure, a 1D ^{1}H-NMR spectrum of the residual sample, without further purification, was sufficient for quantitative determination of the six common sugars because the sugar-NAIM compounds exhibited the diagnostic H-2 protons in much better resolution than their parental sugars, which are complicated due to their having both α and β anomers. In this particular case, we further established the calibration lines and empirical equations (Figure 4) for facile quantification of each common sugar by the integral area of the selected proton signal relative to that of DMSO (0.03% in D_2O solution). DMSO appears to be a suitable internal standard that is more cost-effective than TMSP. In this study, we also demonstrate that the monosaccharides released from the glycans in food crops can be easily identified and quantified by the NAIM derivatization for ^{1}H-NMR spectrometric analysis.

The quantitative NMR (qNMR) technique has been designed for direct quantification of multiple components in a mixture without the requirement of any sample preparation steps [23−25]. In comparison, our current method still requires NAIM derivation, which may take two to three hours. On the other hand, recording a qNMR spectrum would take a longer acquisition time than a routine ^{1}H-NMR spectrum. An even longer time would be necessary when the 2D spectrum is acquired for deconvolution of overlapped signals using the purpose-tailored software [21, 22, 26]. Whether the qNMR, 2D-NMR, or our present method is suitable to quantify individual sugar ingredients, which would supposedly be conducted in the presence of other non-sugar components in the beverage and food samples, requires further experiments to assess this on a case-by-case basis.

We have also made a previous report using the HPLC method to quantify the six common sugars in beverages [18]. Though the NAIM derivatives of Glc, Gal, Lac, and Mal can be quantified by using UV detector, a less sensitive refractive index (RI) detector is needed to quantify the unmodified Fru and Suc that have no chromophore. The HPLC analysis usually takes a longer elution time than recording an ^{1}H-NMR spectrum, and the HPLC method also requires parental sugars and sugar-NAIM standards for calibration in every quantitative analysis.

In this study, we also confirm that the results for qualification of sugar ingredients in the beverage using our present ^{1}H-NMR spectrometric method are consistent with that using the conventional HPAEC–PAD analysis without prior NAIM derivatization. HPAEC–PAD method is a very sensitive method in carbohydrate analysis; however, the decay of electrodes may cause problems in calibration and quantitative measurement. Due to its high sensitivity, HPAEC–PAD analysis is usually operated with minute amounts (10^{-12} to 10^{-15} mol) of the beverage sample. Then even a small experimental error may be amplified to a large deviation in backward counting to the real sugar content. This problem is less obvious by using ^{1}H-NMR spectrometry that is usually operated with 10^{-5} mol of carbohydrate sample.

CONCLUSION

To echo the recent proposal of the TFDA on listing the amounts of six common sugars on "Nutrition Facts Panel" of beverages and foods, we demonstrate in this study that 1D ^{1}H NMR spectroscopy can be effectively utilized to quantify the common sugar ingredients via a simple treatment with a NAIM labeling kit. The NAIM reaction is smoothly performed at room temperature, and the product without further purification is directly subjected to the ^{1}H-NMR analysis. This operation renders the anomeric isomers of an aldose to a single NAIM derivative that shows the characteristic H-2 signal at downfield for diagnosis and quantitative analysis by ^{1}H-NMR spectrometry. Sucrose is unchanged under such NAIM reaction conditions and is readily identified by its glycosidic proton (H-1) at δ 5.44. The content of fructose ingredient can be estimated from the calibration line that is established by taking the combined integration of the proton signals at δ 4.08 (d) for unchanged fructose and at δ 5.29 (s) for the enamine derivative [**A**]. Thus, even small amounts of sugar ingredients in 50 μL of the beverage and in the hydrolysate from 1 mg of food crop can be quantified by the method using NAIM derivatization for ^{1}H-NMR analysis. Consequently, this method is potentially useful for the profiling and fingerprinting of food crops.

List of Abbreviations: 2-AB, 2-aminobenzamide; 2-AP, 2-aminopyridine; Ara, arabinose; dd-H_2O, double-distilled water; DMSO, dimethylsulfoxide; Fru, fructose; Fuc, fucose; Gal, galactose; GI, glycemic index; Glc, glucose; GlcUA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GMO, genetically modified objects; HFCS, high fructose corn syrup; HPAE−PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high performance liquid chromatography; ICA, independent component analysis; Lac, lactose; Man, mannose; Mal, maltose; NAIM, naphthimidazole; NMR, nuclear magnetic resonance; PMP, 1-phenyl-3-methyl-5-pyrazolone; PULCON, pulse length based concentration determination; qNMR, quantitative NMR; Rha, rhamnose; RI, refractive index; Suc, sucrose; S/N, signal-to-noise ratio; TFA, trifluoroacetic acid; TFDA, Taiwan Food & Drug Administration; TLC, thin-layer chromatography; TMSP, trimethylsilylpropanoic acid; UV-vis, ultraviolet-visible; WHO, World Health Organization.

Competing Interests: The authors declare no competing financial interest.

Authors' Contributions: Y.-T. Chen and W.-T. Hung contribute to execution of experiments, data acquisition and statistical analysis. S.-H. Wang provides administrative, technical and material support. J.-M. Fang contributes to methodology development and writing manuscript. W.-B. Yang is the principle investigator for this research, and contributes to design of experiments and preparation of manuscript.

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REFERENCES

1.Ruhaak LR, Zauner G, Huhn C, Bruggink C, Deelder AM, Wuhrer M: Glycan labeling strategies and their use in identification and quantification. Anal Bioanal Chem 2010, 397: 3457–3481.

2. Nishida C, Uauy R, Kumanyika S, Shetty P: The joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases: process, product and policy implications. Public Health Nutrition 2004, 7: 245–250.

3. Mannucci E, Lauria A, Dicembrini I, Pozzilli P: Is glucose control important for prevention of cardiovascular disease in diabetes? Diabetes Care 2013, 36: 259–263.

4. Drake EN: Application of nmr to biochemical kinetics. A laboratory experiment in physical biochemistry. J Chem Educ 1977, 54: 124–127.

5. Falbe J, Rojas N, Grummon AH, Madsen KA: Higher retail prices of sugar-sweetened beverages 3 months after implementation of an excise tax in Berkeley, California. Am J Public Health 2015, 105: 2194–2201.

6. [http://www.marksdailyapple.com/how-much-sugar-is-recommended-per-day/]

7. Ku AN, Wu PW, Chen WY, Shen YR, Huang TY, Tsai CH, Chang YH, Tseng SH, Kao, YM, Chou HK, Cheng HF: Simultaneous determination of glucose, fructose, sucrose, maltose, lactose and galactose in foods using high performance liquid chromatography and high performance anion-exchange chromatography. Ann Rep Food Drug Res 2015, 6: 22–35.

8. Dekker KD: The Luff−Schoorl method for determination of reducing sugar in juices, molasses and sugar. South African Sugar J 1950, 34: 157−171.

9. Montero CM, Dodero MCR, Sánchez DAG, Barroso CG: Analysis of low molecular weight carbohydrates in food and beverages: a review. Chromatographia 2004, 59: 15–30.

10. Harvey DJ: Derivatization of carbohydrates for analysis by chromatography; electrophoresis and mass spectrometry. J Chromatography B: Anal. Technol. Biomed. Life Sci. 2011, 879: 1196–1225.

11. Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB: Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal Biochem 1995, 230: 229–238.

12. Fan JQ, Huynh LH, Lee YC: Purification of 2-aminopyridine derivatives of oligosaccharides and related compounds by cation-exchange chromatography. Anal Biochem 1995, 232: 65–68.

13. Lattová E, Perreault H: Method for investigation of oligosaccharides using phenylhydrazine derivatization. Methods Mol Biol 2009, 534: 65–77.

14. Saba JA, Shen X, Jamieson JC, Perreault H: Effect of 1-phenyl-3-methyl-5-pyrazolone labeling on the fragmentation behavior of asialo and sialylated N-linked glycans under electrospray ionization conditions. Rapid Commun Mass Spectrom 1999, 13: 704–711.

15. Lin C, Lai PT, Liao SKS, Hung WT, Yang WB, Fang JM: Using molecular iodine in direct oxidative condensation of aldoses with diamines: an improved synthesis of aldo-benzimidazoles and aldo-naphthimidazoles for carbohydrate analysis. J Org Chem 2008, 73: 3848–3853.

16. Lin C, Hung WT, Chen CH, Fang JM, Yang WB: A new naphthimidazole derivative

for saccharide labeling with enhanced sensitivity in mass spectrometry detection. Rapid Commun. Mass Spectrom 2010, 24: 85–94.

17. Kuo CY, Wang SH, Lin C, Liao KS, Hung WT, Fang JM, Yang WB: Application of 2,3-naphthalenediamine in labeling natural carbohydrates for capillary electrophoresis. Molecules 2012, 17: 7387–7400.

18. Hung WT, Chen YT, Wang SH, Liu YC, Yang WB: A new method for aldo-sugar analysis in beverages and dietary foods. Func Food Health Dis 2016, 6: 234–245.

19. Duus JØ, Gotfredsen CH, Bock K: Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. Chem Rev 2000, 100: 4589–4614.

20. Kazalaki A, Misiak M, Spyros A, Dais P: Identification and quantitative determination of carbohydrate molecules in Greek honey by employing ^{13}C NMR spectroscopy. Anal Methods 2015, 7: 5962−5972.

21. Monakhova YB, Tsikin AM, Kuballa T, Lachenmeier DW, Mushtakova SP: Independent component analysis (ICA) algorithms for improved spectral deconvolution of overlapped signals in ^{1}H NMR analysis: application to foods and related products. Magn Reson Chem 2014, 52: 231–240.

22. Ackermann SM, Dolsophon K, Monakhova YB, Kuballa T, Reusch H, Thongpanchang T, Bunzel1 M, Lachenmeier DW: Automated multicomponent analysis of soft drinks using 1D ^{1}H and 2D $^{1}H-^{1}H$ J-resolved NMR spectroscopy. Food Anal Methods 2017, 10: 827–836.

23. Cao R, Nonaka A, Komura F, Matsui T: Application of diffusion ordered-^{1}H-nuclear magnetic resonance spectroscopy to quantify sucrose in beverages. Food Chem 2015, 171: 8–12.

24. Beyer T, Diehl B, Holzgrabe U: Quantitative NMR spectroscopy of biologically active substances and excipients. Bioanal Rev 2010, 2: 1−22.

25. Garrido R, Vélez H, Vérez V: Nuclear magnetic resonance: New applications in the quantification and assessment of polysaccharide-based vaccine intermediates. VacciMonitor 2013, 22: 35−42.

26. Monakhova YB, Kuballa T, Leitz J, Andlauer C, Lachenmeier DW: NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. Dairy Sci Technol 2012, 92: 109−120.