Quantitative Analysis of D-(+)-Glucose in Fruit Juices Using Diffusion Ordered-¹H Nuclear Magnetic Resonance Spectroscopy

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This study works on D-(+)-glucose quantitative analysis using diffusion ordered-quantitative ¹H nuclear magnetic resonance spectroscopy (DOSY-qNMR), by which an analyte could be distinguished from interferences based upon a characteristic diffusion coefficient (*D*) in gradient magnetic fields. The *D* value of D-(+)-glucose in deuterium oxide at 30°C was 5.6×10^{-10} m²/s at a field gradient pulse of between 5.0×10^{-2} and 3.0×10^{-1} T/m, distinguished from fructose, sucrose and starch. Good linearity ($r^2 = 0.9998$) was obtained between D-(+)-glucose (0.5 – 20.0 g/L) and the ratio of the resonance area of α -C1 proton (5.21 ppm) in D-(+)-glucose to that of the β -C1 proton (5.25 ppm) in D-glucuronic acid (50.0 g/L) as an internal standard. The DOSY-qNMR method was successfully applied to quantify D-(+)-glucose in orange juice (18.3 ± 1.0 g/L), apple juice (26.3 ± 0.4 g/L) and grape juice (45.6 ± 0.6 g/L); the values agreed well with a conventional F-kit glucose method.

Keywords DOSY, qNMR, D-(+)-glucose, food analysis

(Received November 20, 2013; Accepted December 12, 2013; Published March 10, 2014)

Introduction

High-resolution nuclear magnetic resonance (NMR) spectroscopy has been increasingly applied to the quantitative analysis of analytes (qNMR analysis).^{1–3} qNMR analysis is based on a quantitative feature of the ¹H NMR signal intensity corresponding to the number of protons in a molecule, after normalization to an internal standard (IS). The use of an appropriate IS makes qNMR analysis extremely attractive for purity determination.⁴ In food analysis, some researchers have applied qNMR to the determination of interests in natural products,⁵ foods and beverages,^{6–10} because fewer tedious procedures are necessary prior to an assay. Sugimoto *et al.*⁸ have successfully measured quercetin in tartary buckwheat noodle by qNMR, and showed that hexamethyldisilane was effective as an IS in normalizing the proton at H-2' in quercetin, though the use of this IS was limited due to its poor solubility in aqueous solution.

Even though the typical qNMR is rapid, requires no tedious pretreatment and is specific, it presents problems in complex mixtures such as foods, because contaminants may cause an overlapped resonance with the analyte peak. Alternatively, diffusion-ordered NMR spectroscopy (DOSY-NMR) is a powerful tool that may overcome these issues. DOSY-NMR is a two-dimensional (2D) NMR approach, in which compounds with different diffusion coefficients (*D*) at an appropriate pulsed-

field gradient strength can be separately monitored at the 2D DOSY spectra.¹¹ Indeed, the DOSY-NMR method based on the pulse-gradient spin-echo technique has been challenged to the quantitative analysis of polystyrene,¹² poly(hydroxyethyl cellulose) and diethyleneglycol monobutylether,¹³ by overcoming the disadvantage of the algorithm, which is greatly influenced by the assay conditions such as solution viscosity and temperature, *etc.* However, there have so far been few reports on the DOSY method for food analysis. In the present work, we thus tried to apply the DOSY-NMR method aided by an appropriate IS technique to a quantitative D-(+)-glucose assay in fruit juices (orange, apple and grape juices). A data-inversion program, SPLMOD algorithm,¹⁴ was used for the present DOSY-qNMR method in juice assays, since factors affecting the algorithm were minimized to be constant throughout the DOSY-NMR measurements. The D-(+)-glucose content in fruit juices obtained by the DOSY-qNMR method was also compared to those obtained by conventional assays (enzymatic kit and high-performance liquid chromatography (HPLC)) to evaluate the validation of the proposed DOSY-qNMR method for food analysis.

Experimental

Reagents and chemicals

D-(+)-Glucose ($\geq 98\%$), D-(–)-fructose ($\geq 99\%$), sucrose ($\geq 99\%$) and starch were purchased from Nacalai Tesque Inc. (Kyoto, Japan). D-Glucuronic acid ($\geq 98\%$) was purchased from Sigma Chemical Co. (St. Louis, MO). 3-Trimethylsilyl-1-propanesulfonic acid-*d*₆ (DSS-*d*₆, 98 atom% D) was obtained

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from Wako Pure Chemical Ind. (Osaka, Japan). Deuterium oxide (D₂O, 99.8 atom% D) was acquired from Acros Organics (Fair Lawn, NJ). Orange, apple and grape juices used in this study were commercially available products from concentrated reduced juice raw material in the local market in Japan, and were stored at -20°C prior to use.

¹H NMR analysis

¹H NMR measurements were carried out on an ECS-400 spectrometer (JEOL, Tokyo, Japan) composed of fully digitized circuitry including an RF generator, an NMR lock, and a digital matrix shim, equipped with a z-field gradient unit operating at 400 MHz and 30°C. Samples were dissolved in D₂O and placed into 5 mm-sample tubes (Nihonseimitsu Scientific Co., Tokyo, Japan). All of the 1D ¹H NMR spectra were acquired by a single pulse-sequence without water suppression, 16384 acquisition points, a relaxation delay of 15 s, an acquisition time of 2.18 s, a receiver gain of 26 and 128 transients. The 90° pulse-width of D-(+)-glucose was determined under the same conditions as described above, with pulse-widths from 9.0 to 11.0 μs at 0.1 μs interval and non-spinning. The spin-lattice relaxation delay (*T*₁) value was measured on D-(+)-glucose of 10 g/L by an arrayed experiment under the same conditions as described above, using an inversion-recovery pulse sequence⁹ with a relaxation delay of 30 s and pulse-interval times in the range of 0.1 to 5.0 s. All spectra were referenced to the signal of DSS-*d*₆ at 0.0 ppm.

DOSY-qNMR analysis

2D DOSY-NMR measurements with a spin-echo method¹⁵ (pulse sequence beginning with a 90° pulse, after a time interval and followed by a 180° pulse) were performed using a diffusion time of 0.1 s, a field gradient pulse-width of 1.0×10^{-3} s and a field gradient pulse value of between 5.0×10^{-2} and 3.0×10^{-1} T/m at 128 scans of 16384 acquisition points, a 90° pulse-width of 10.1 μs, and a relaxation delay of 30.0 s at 30°C and non-spinning. Auto-shimming for each measurement was performed with the filed-gradient shimming at 4 scans, shimming sets of Z1 to Z6, and receiver gain 20. DOSY spectra were calculated in a data-inversion program, SPLMOD algorithm¹⁶ (double precision version 3DP, June 1988), with the display range being from 1.0×10^{-10} to 1.0×10^{-8} m²/s. The *D* value was calculated as follows:¹⁷

$$D = -\frac{1}{(\gamma G \delta)^2 (\Delta - \delta/3)} \ln \frac{I(G, \Delta)}{I(0, \Delta)} \quad (1)$$

Here, γ is the gyromagnetic ratio in the observation of nuclei, *G* the pulse-field gradient strength, δ the pulse-width of the field gradient, Δ the diffusion time, and *I* the signal intensity.

The quantitation of D-(+)-glucose was performed using D-glucuronic acid as IS, which was dissolved in 50 μL of D₂O (50.0 g/L) and loaded into a 5-mm insert capillary glass tube (a specialized NMR sample tube, N-502B, Nihonseimitsu Scientific Co.). The IS-loaded capillary tube was then inserted into a 5 mm-NMR sample tube containing 400 μL of the sample solution prior to the 2D DOSY-NMR measurements. The same IS-loaded capillary tube was used for all quantitative NMR experiments. The ratio of the resonance area of the analyte proton to that of the IS proton in sliced DOSY spectra taken from a *D* value of 5.6×10^{-10} m²/s was used for the qNMR assay.

Preparation of fruit juices for DOSY-qNMR analysis

For the determination of D-(+)-glucose in fruit juices,

commercially available orange juice, apple juice and grape juice were filtered through 0.45 μm COSMONICE filters (Nacalai Tesque) to remove any particulates, such as pectins interfering with the NMR resolution. Further, the filtrates were subjected to a 5-fold dilution with D₂O. The diluted solutions were then placed into 5 mm-NMR sample tubes, following inserting the D-glucuronic acid-loaded capillary tube. For recovery experiments, D-(+)-glucose with final concentration of 25 and 50 g/L was added into the orange juice, apple juice and grape juice respectively. The recovery was calculated as follows: recovery (%) = (D-(+)-glucose concentration found in glucose-added juices - D-(+)-glucose concentration found in original juices)/D-(+)-glucose concentration added $\times$ 100%.

Assay for D-(+)-glucose in fruit juices by conventional methods

Two conventional glucose assays, F-kit and HPLC methods, were used to validate the proposed DOSY-qNMR method for the D-(+)-glucose assay. For the F-kit enzymatic assay (F-kit glucose, Roche Diagnostics, Darmstadt, Germany), the D-(+)-glucose content in fruit juices filtered and diluted with water (200-fold of orange juice and apple juice; 400-fold of grape juice) were determined spectrophotometrically at 340 nm, according to the manufacturer's protocol. A standard calibration curve of D-(+)-glucose solution between 50.0 - 250 mg/L was used for the D-(+)-glucose assay in fruit juices using this kit.

The HPLC assay was conducted using a PU 2080 liquid chromatograph (Jasco Co., Tokyo, Japan) connected to a refractive-index (RI) detector (Jasco RI-930). LC separation was performed on a COSMOSIL Sugar-D column (4.6 $\times$ 250 mm, 5 μm, Nacalai Tesque) at 35°C with 75% (v/v) acetonitrile flowing at 1.0 mL/min. Prior to the HPLC assay, aliquots (1.0 mL) of the filtered orange juice, apple juice and grape juice were dried *in vacuo*. The dried samples were then dissolved in 1.0 mL of 75% (v/v) acetonitrile, and aliquots (100 μL) of solution were injected into the HPLC system. Under the HPLC conditions, D-(+)-glucose was eluted at a retention time of 7.6 min. A standard calibration curve of a D-(+)-glucose solution between 25.0 - 400 mg/L was used for the D-(+)-glucose assay in fruit juices using the HPLC method.

Statistics

Data are expressed as the mean $\pm$ standard deviation (SD). Five replicates were analyzed for each assay (*n* = 5: five parallel prepared samples from each juice). The statistical differences between the D-(+)-glucose content of orange juice, apple juice and grape juice obtained by the three assay systems were analyzed by the one-way analysis of the variance (ANOVA), followed by *post-hoc* Tukey-Kramer analysis. A *p* value of < 0.05 was considered to be statistically significant different. All analyses were performed using a Stat View J 5.0 (SAS Institute, Cary, NC).

Results and Discussion

Optimization of DOSY acquisition conditions

Since the signal intensity in the ¹H NMR spectrum corresponds to the number of protons of the same type in a molecule, qNMR normalized to an appropriate IS can allow a quantitative determination of the molecule.⁴ Thus, it is crucial to select a target proton in the D-(+)-glucose for this study. The ¹H NMR spectrum of standard D-(+)-glucose dissolved in D₂O revealed α , β -anomeric C1 protons distinct from other C2 - C6 protons at 3.0 - 4.0 ppm. Taking into consideration that D-(+)-glucose in water is an equilibrium mixture of α -pyranose and β -pyranose

Fig. 1 2D DOSY-NMR spectrum of a mixture of D-(+)-glucose, fructose, sucrose and starch, with the 1D sliced spectrum of D-(+)-glucose on the top. The four carbohydrates were dissolved in D₂O at a concentration of 10.0 g/L. 2D DOSY-NMR measurements were performed at a field gradient pulse value of between 5.0×10^{-2} and 3.0×10^{-1} T/m at 30°C.

with a constant ratio (%) of $\alpha:\beta = 36.4:63.6$ ¹⁸ which is mainly related to the interaction between the solvent and the free-electron pair of the anomeric oxygen,¹⁹ monitoring either α -C1 proton at 5.21 ppm or β -C1 proton at 4.64 ppm, but not both protons, was sufficient to quantitate D-(+)-glucose in D₂O by qNMR. As shown in Fig. S1 (Supporting Information), the ¹H NMR spectrum of intact fruit juices (only diluted with D₂O) provided some serious issues, in which interferences from juice contaminants overlapped with the β -C1 proton signal. Thus, the α -C1 proton in D-(+)-glucose was selected as the target proton in this study. The 90° pulse-width and T_1 value²⁰ in DOSY acquisition conditions were, thus, optimized to be 10.1 μ s and 3.0 s, respectively, for the α -C1 proton in D-(+)-glucose.

DOSY analysis of D-(+)-glucose

Using the optimized 90° pulse-width and the T_1 value, a DOSY-NMR measurement of D-(+)-glucose was performed (Fig. 1). The relaxation delay was set to 30.0 s (ten-times the T_1 value of 3.0 s) at a 90° pulse-width of 10.1 μ s. In this experiment, fructose, sucrose and starch, which are possible interfering carbohydrates of D-(+)-glucose (each concentration: 10.0 g/L), were added to the D-(+)-glucose standard solution to obtain information about the potential interferences of α -C1 proton in D-(+)-glucose in the 2D DOSY spectra. As shown in Fig. 1, completely isolated and selective detection of the α -C1 proton in D-(+)-glucose based on the 2D DOSY spectrum for a mixture of carbohydrates was obtained from its characteristic D value (5.6×10^{-10} m²/s), which reflects the molecular size.¹¹ Although the separation of molecules having the same molecular weight *i.e.*, D-(+)-glucose and fructose was difficult based on the D value in the DOSY-NMR method and artificial (or ghost) peaks caused a confused 2D DOSY spectrum (Fig. 1) due to their overlapped signals,¹¹ the characteristic and isolated α -C1 proton in D-(+)-glucose from fructose would allow for the selective measurement of D-(+)-glucose by DOSY-NMR.

DOSY-qNMR analysis of D-(+)-glucose

Normally, overlapping signals that show different decay

Fig. 2 1D sliced DOSY-NMR spectrum of D-(+)-glucose (10.0 g/L) in a D-glucuronic acid (50.0 g/L)-loaded capillary glass tube (A). The ratio of the resonance area of the α -C1 proton in D-(+)-glucose (0.5–20.0 g/L) to that of the β -C1 proton in D-glucuronic acid was used for the calibration curve (B). The sliced DOSY spectrum was taken at a D value of 5.6×10^{-10} m²/s. The ratio of the resonance area (α -C1 in glucose: β -C1 in IS) is the ratio of the resonance area of the α -C1 proton in D-(+)-glucose to that of the β -C1 proton in D-glucuronic acid.

behaviors during the DOSY experiment pose difficulties during the mathematical fitting procedures.¹³ However, for the well-resolved target peak in the 1D sliced DOSY spectrum extracted with an SPLMOD algorithm used in this study, the quantitative analysis would be appropriate, since the SPLMOD program automatically attempts to fit one-, two- or three-exponential attenuation functions to the data, and to evaluate the one that gives the best statistical fit,¹¹ by which the T_2 value can also be sufficed to describe the spin-spin relaxation behavior.²¹ With the aid of an IS, we tried to quantify the D-(+)-glucose content by the DOSY-qNMR method with the SPLMOD algorithm, in which the 1D sliced DOSY spectra from the D value of 5.6×10^{-10} m²/s were taken. In this study, D-glucuronic acid with a D value of 5.6×10^{-10} m²/s was used as an IS candidate, which was produced at low concentrations (< 0.01 g/L) by the tea fungus,²² but was not present in the fruit juices used in this study (Fig. S1 (Supporting Information) and later Fig. 4A). As shown in Fig. 2A, good resolution and detection of D-(+)-glucose was obtained by the present DOSY-qNMR method. The ratio of the resonance area of the α -C1 proton in D-(+)-glucose against the β -C1 proton in D-glucuronic acid (5.25 ppm) provided good linear regression at D-(+)-glucose concentrations of between 0.5 and 20.0 g/L ($r^2 = 0.9998$) (Fig. 2B), in accordance with the calibration curve of D-(+)-glucose obtained by the 1D ¹H NMR measurement (Fig. S2, Supporting Information). This finding strongly indicated that the resonance areas of the target protons in both D-(+)-glucose and D-glucuronic acid in the same sliced DOSY

Fig. 3 2D DOSY-NMR spectra of orange juice (A), apple juice (B) and grape juice (C) in a D-glucuronic acid (50.0 g/L)-loaded capillary glass tube. Each juice was 5-fold diluted with D₂O. IS, β -C1 proton in D-glucuronic acid; α -C1, α -C1 proton in D-(+)-glucose. 2D DOSY-NMR measurements were performed at field gradient pulse value of between 5.0×10^{-2} and 3.0×10^{-1} T/m at 30°C.

spectrum were comparable for the D-(+)-glucose assay by the SPLMOD algorithm.

Application of the DOSY-qNMR method to the quantitation of D-(+)-glucose in fruit juices

To validate our proposed DOSY-qNMR method for the quantitation of D-(+)-glucose, three commercial fruit juices (orange, apple and grape juices) diluted with D₂O (5-fold) were assayed by the proposed DOSY-qNMR method using D-glucuronic acid as IS. The representative 2D spectrum obtained from DOSY-qNMR measurements of each fruit juice (Fig. 3) revealed that the α -C1 proton in D-(+)-glucose and the β -C1 proton in IS were separated from other peaks in each juice according to their characteristic D value (5.6×10^{-10} m²/s). The constant D value (shown in Fig. 3) of the target protons in both D-(+)-glucose and IS convincingly demonstrated that the present DOSY acquisition conditions and algorithm successfully obtain full relaxation of the target peaks, excluding possible interferences from juice matrix. As shown in Fig. 4, the sliced spectra were applicable for D-(+)-glucose quantitation in fruit juices, since no interfering proton peak with the β -C1 proton in

Fig. 4 1D sliced DOSY-NMR spectrum for orange juices (A) in non D-glucuronic acid (or D₂O loaded)-capillary glass tube, and 1D sliced DOSY-NMR spectra for orange juice (B), apple juice (C) and grape juice (D) in D-glucuronic acid (50.0 g/L)-loaded capillary glass tube. Each sliced DOSY spectrum was taken at a D value of 5.6×10^{-10} m²/s.

Table 1 Validation data for D-(+)-glucose quantitation by DOSY-qNMR using glucuronic acid as an internal standard

Sample	Added/g L ⁻¹	Found/g L ⁻¹	Recovery, %	RSD, %
Orange juice	0	18.3 $\pm$ 1.0	—	5.7
	25	42.5 $\pm$ 1.6	96.9	3.7
	50	65.6 $\pm$ 3.3	94.7	5.0
Apple juice	0	26.3 $\pm$ 0.4	—	1.7
	25	51.9 $\pm$ 1.7	102.3	3.3
	50	76.7 $\pm$ 1.8	100.8	2.3
Grape juice	0	45.6 $\pm$ 0.6	—	1.2
	25	70.1 $\pm$ 1.7	98.0	2.4
	50	99.1 $\pm$ 2.9	107.0	2.9

Found was the mean of 5 different measurements $\pm$ SD.

The repeatability was defined as RSD (%) of 5 different measurements.

IS was observed in *e.g.*, orange juice (Fig. 4A).

In order to validate the proposed DOSY-qNMR method for D-(+)-glucose quantitation, recovery experiments by adding D-(+)-glucose (25 or 50 g/L) into each juice were performed using the calibration curves of standard solutions (Fig. 2B). As summarized in Table 1, at the optimized DOSY-qNMR assay conditions, the added D-(+)-glucose was recovered from each juice matrix with a recovery of > 94%, together with good repeatability (RSD < 6%), indicating the reliability of the proposed DOSY-qNMR method for D-(+)-glucose quantitation

Table 2 D-(+)-Glucose content in three fruit juices measured by DOSY-qNMR, F-kit and HPLC^a

Sample	D-(+)-Glucose/g L ⁻¹		
	DOSY-qNMR	F-kit method	HPLC
Orange juice	18.3 ± 1.0 ^a	17.7 ± 0.8 ^a	21.5 ± 0.2 ^b
Apple juice	26.3 ± 0.4 ^a	25.9 ± 0.4 ^a	29.7 ± 1.8 ^b
Grape juice	45.6 ± 0.6 ^a	43.9 ± 1.2 ^a	54.5 ± 4.3 ^b

a. Data are expressed as the mean of 5 different measurements ± SD. The means without a common letter in each group differed at $p < 0.05$ by the Tukey-Kramer's t -test.

in appropriate diluted fruit juices without showing any influence of the pH (pH values of 3.2 – 3.8 for the three juices) or the viscosity on the chemical shifts of the target protons or on the DOSY acquisition conditions such as relaxation time. The limits of detection (LODs) of D-(+)-glucose in orange, apple and grape juices were calculated from Table 1 to be 0.66, 0.26 and 0.37 g/L, respectively. Table 2 summarizes the results of the quantitative analyses of three fruit juices by the DOSY-qNMR method, together with the results of the conventional glucose assays: *i.e.*, F-kit and RI-HPLC. As a result of the proposed DOSY-qNMR method, the average D-(+)-glucose content ($n = 5$) in the orange juice was estimated to be 18.3 ± 1.0 g/L, which was consistent with that by the F-kit method (17.7 ± 0.8 g/L). Good agreement of the D-(+)-glucose content was also obtained for apple and grape juices between both the DOSY-qNMR and F-kit methods, with no significant difference at $p = 0.05$. In contrast, the D-(+)-glucose contents of the three fruit juices were significantly ($p < 0.05$) higher by the RI-HPLC method, compared to those by the DOSY-qNMR and F-kit methods. One possible explanation for the over-estimation of the results by the RI-HPLC method may be due to RI-detectable compounds in fruit juices that overlap with the D-(+)-glucose peak at 7.6 min and/or peak tailing under the isocratic RI-HPLC conditions.

Recently, the DOSY-NMR method has been extensively applied to food analysis in characterizing prominent components of foods. Gil *et al.*²³ have reported that the DOSY technique was useful for the simultaneous characterization of food compounds, such as organic acids, sugars, amino acids and polyphenols in fruit juices. Nilsson *et al.*²⁴ analyzed the quality of port wine based upon changes in the relative amount of organic acids using the DOSY technique. In this study, we demonstrated that the DOSY technique was useful for qNMR to quantitate D-(+)-glucose in fruit juices. Under the optimized DOSY conditions, D-(+)-glucose was separately detected in a mixture of carbohydrates based upon its characteristic D value. The DOSY-qNMR method was applicable to the quantitation of D-(+)-glucose in fruit juices, since D-glucuronic acid was an appropriate IS that achieved good linearity of the D-(+)-glucose content against the resonance area ($r^2 = 0.9998$). In addition, the proposed DOSY-qNMR method was well validated, and had some advantages, *i.e.*, the removal of tedious sample preparation (only dilution with D₂O), as well as the D-(+)-glucose detection of $> ca.$ 0.3 g/L in three juices. Besides, the recognition of signals at the same D value calculated in SPLMOD will possibly allow for the simultaneous quantitations of more compounds by the efficient DOSY-qNMR. Thus, the present DOSY-qNMR method will open a new field for food analyses in complex food matrices.

Acknowledgements

This study was supported in part by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (No. 24658125) to T. M.

Supporting Information

Figure S1 is the ¹H NMR spectra of orange juice (A), apple juice (B) and grape juice (C) in D₂O. The α -C1 proton in D-(+)-glucose was observed at 5.21 ppm. No peak of the β -C1 proton in D-glucuronic acid as IS was observed at 5.25 ppm. Figure S2 is the calibration curve obtained by the 1D ¹H NMR method using the ratio of the resonance area of the α -C1 proton in D-(+)-glucose (0.5 – 20.0 g/L) to that of the β -C1 proton in D-glucuronic acid. The ratio of the resonance area (α -C1 in glucose: β -C1 in IS): the ratio of the resonance area of the α -C1 proton in D-(+)-glucose to that of the β -C1 proton in D-glucuronic acid. The dotted curve is the calibration curve of D-(+)-glucose obtained by the DOSY-qNMR method, shown in Fig. 2(B). This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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