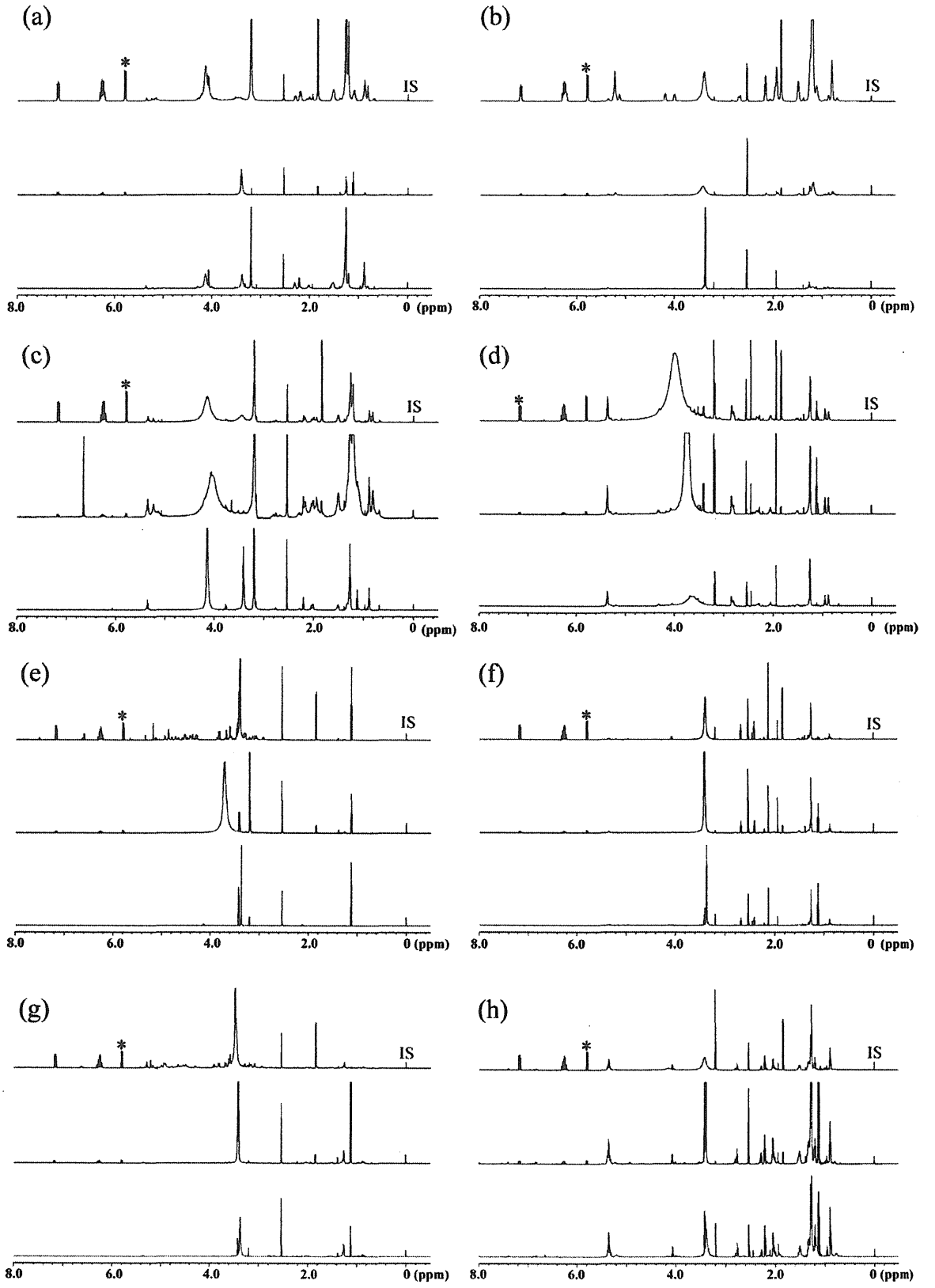

Fig. 3. ^{1}H NMR spectra (0–8 ppm) of each sample solution spiked with SA at the maximum usage level of each processed food (upper), at 0.13 g kg^{-1} (beverage containing *Lactobacillus* species, 0.013 g kg^{-1}) (middle), and blank (lower). (a) Cheese. (b) Fish paste. (c) Sausage. (d) Dried cuttlefish. (e) Syrup. (f) Vegetables pickled in soybean sauce. (g) Jam. (h) Soybean paste. (i) Noodle soup. (j) Ketchup. (k) Beverage containing *Lactobacillus* species. Signals marked with asterisks were used for quantification and the recoveries were calculated. IS, internal standard (DSS-d_6).

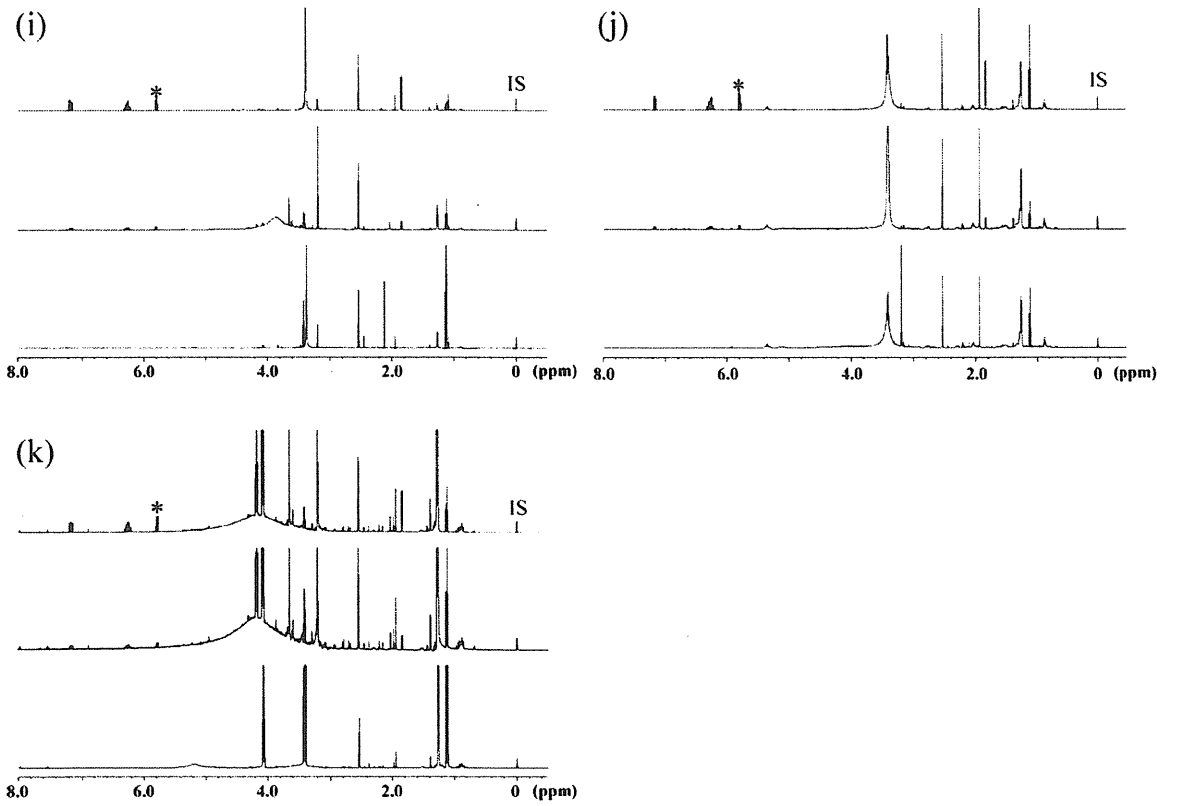

Fig. 3. (*Continued*)

the 11 processed foods, excluding dried cuttlefish and the beverage containing *Lactobacillus* species, was determined from the signal at δ_H 5.79, which showed relatively low multiplicity. By contrast, the SA quantities in dried cuttlefish and the beverage containing *Lactobacillus* species were determined from the signal at δ_H 7.18 because increased baseline distortion was observed in the signal at δ_H 5.79.

As shown in Table 3, the recoveries of samples spiked at the maximum usage level ranged from 89.1 to 100.2%, and the relative standard deviation (RSD) values ranged from 0.5 to 3.1%. At a concentration of 0.13 g kg^{-1} (beverage, 0.013 g kg^{-1}), the recoveries were larger than 80% with an RSD of 0.6–6.8%. At 0.063 g kg^{-1} (beverage, 0.0063 g kg^{-1}), the recoveries were 56.9–83.5% with an RSD of 0.2–8.9%, which were lower than those at the other two concentrations tested. On the basis of these results and the linearity of the quantification signal, we conclude that this method efficiently quantifies SA in processed foods at concentrations greater than 0.063 g kg^{-1} (beverage, 0.0063 g kg^{-1}), which can be taken as the limit of quantification. As the maximum levels of SA permissible in processed foods in Japan are 0.050–3.0 g kg^{-1}, this method would be effective in 2.2–12.5% of the maximum allowed concentrations.

3.5. Inter-day precision

To investigate the inter-day precision and accuracy of the proposed method, we performed recovery tests on five different days using two concentrations: the maximum usage level of each processed food and 0.13 g kg^{-1}. We selected two food samples (sausage and noodle soup), and prepared them for the inter-day precision test using an extraction method with and without a degreasing step.

Table 3
Recoveries of SA from processed foods.

Sample	0.063 g kg^{-1} spiked (0.0063 g kg^{-1} spiked)[a]		0.13 g kg^{-1} spiked (0.013 g kg^{-1} spiked)[a]		Maximum usage level spiked		
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Level (g kg^{-1})	Recovery (%)	RSD (%)
Cheese	56.9	2.4	98.8	1.6	3.0	97.1	3.1
Fish paste	61.7	8.9	84.4	4.0	2.0	100.2	0.5
Sausage	61.2	4.5	81.1	4.3	2.0	89.1	2.1
Dried cuttlefish	60.1	5.8	99.7	0.6	1.5	94.5	0.8
Syrup	83.5	5.9	96.2	1.8	1.0	99.5	0.9
Vegetable pickeled in soybean sauce	59.7	1.2	80.3	1.0	1.0	99.6	0.5
Jam	65.3	8.9	98.9	2.8	1.0	99.2	0.8
Soybean paste	75.0	0.2	90.5	6.7	1.0	92.8	2.3
Noodle soup	78.5	2.9	86.3	4.6	0.50	97.7	0.7
Ketchup	79.5	1.2	93.5	6.8	0.50	98.7	1.2
Beverage containing *Lactobacillus* species	71.2	3.4	86.4	2.0	0.050	93.3	3.0

Each recovery value represents the mean of three independent experiments on the same day. RSD, intra-day relative standard deviation.
[a] Beverage containing *Lactobacillus* species.

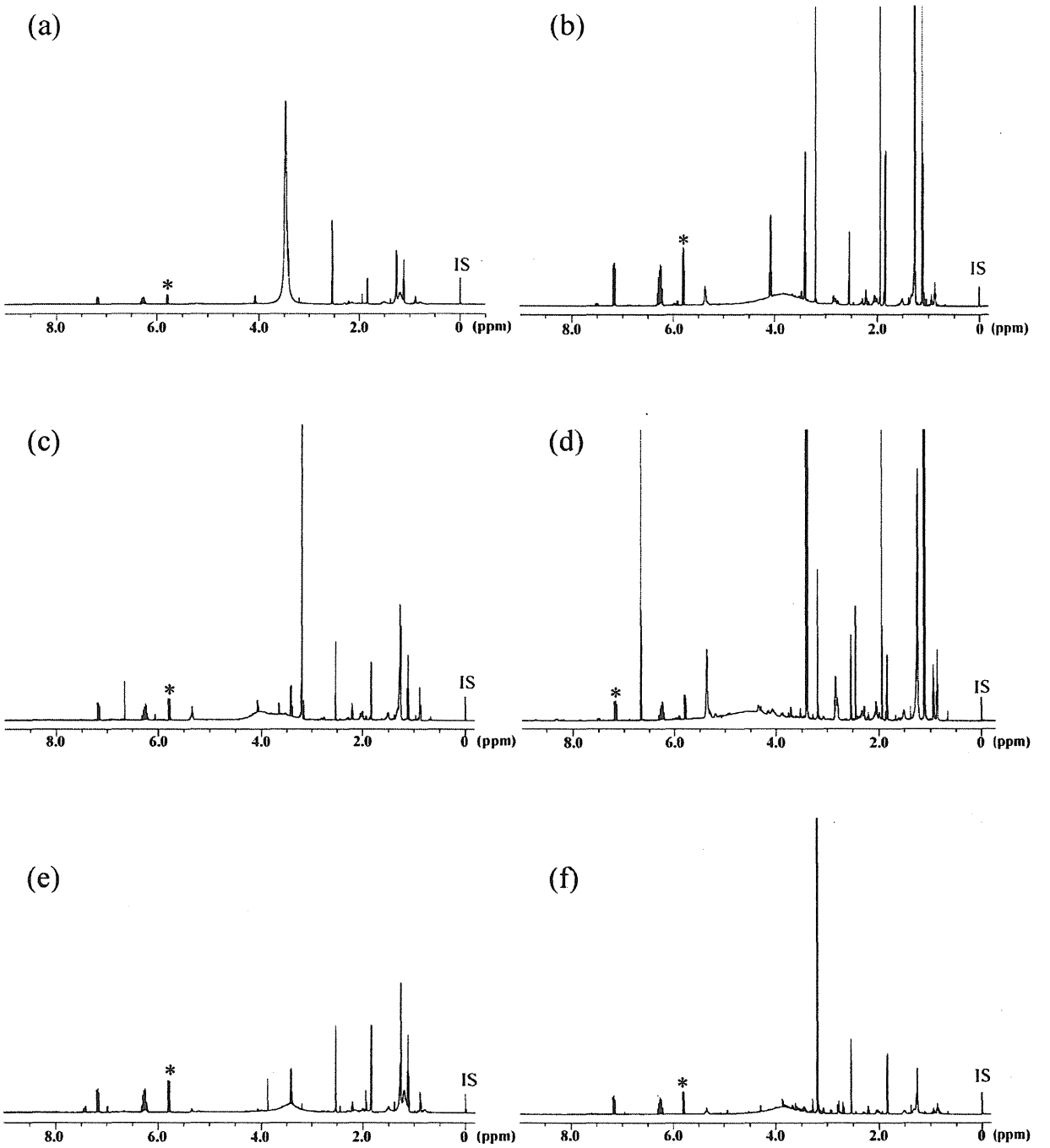

Fig. 4. ^{1}H NMR spectra of each sample solution from commercially produced food with SA. (a) Cheese. (b) Fish paste. (c) Sausage. (d) Dried cuttlefish. (e) Syrup. (f) Jam. Signals marked with asterisks were used for quantification and the contents were calculated. IS, internal standard (DSS-d_6).

Precise inter-day data were obtained from both spiked samples, for which the RSD ranged from 3.6 to 6.9% (Table 4). The recovery ranged from 81.3 to 91.7%, indicating that this method is both highly reliable and reproducible.

3.6. Determination of SA contents in processed foods

To investigate the applicability of the proposed method, we used it to examine the SA content of six commercial processed foods containing SA as a food additive, and compared the results with those obtained using steam distillation extraction and HPLC quantification analysis. As shown in Fig. 4, the signals at δ_H 7.18 for dried cuttlefish and at δ_H 5.79 for five other foods (cheese, fish paste, sausage, syrup, and jam) were clearly separated from those signals derived from impurities in qHNMR analysis, and so could be used to determine the SA content of processed food.

As shown in Table 5, the results of the proposed method of SA content were in good agreement with those of the conventional method for five of the processed foods (cheese, fish paste, sausage, syrup, and jam), but not for dried cuttlefish. The observed differences were less than 10%, indicating that the proposed method was useful for determining the SA content directly in these

Table 4
Inter-day recoveries and precisions of SA in two samples.

Sample	0.13 g kg^{-1} spiked		Maximum usage level spiked	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Noodle soup	83.6	5.1	91.7	3.6
Sausage	84.6	6.9	81.3	4.7

Each recovery value represents the mean of analysis in duplicate on five different days. RSD, relative standard deviation.

Table 5
Comparison of SA contents in commercial foods determined by two methods.

Sample	Proposed method (solvent extraction/qHNMR)		Conventional method (steam distillation/HPLC)	
	Content (g kg^{-1})	RSD (%)	Content (g kg^{-1})	RSD (%)
Cheese	0.25	5.5	0.27	4.3
Fish paste	1.46	2.9	1.42	3.1
Sausage	0.68	3.4	0.75	1.2
Dried cuttlefish	0.72	1.4	0.62	0.5
Syrup	0.66	1.6	0.66	0.9
Jam	0.62	2.8	0.59	2.8

Each value represents the mean of three independent experiments. RSD, relative standard deviation.

processed foods. In dried cuttlefish, the SA content determined by the proposed method (0.72 g kg^{-1}) was higher than that measured using the conventional method (0.62 g kg^{-1}). To understand this discrepancy, we examined the recovery experiment at a concentration level of 0.75 g kg^{-1} potassium sorbate. The recoveries were 94.5% for our method ($n = 3$) and 84.8% for the conventional method ($n = 2$), which could have been responsible for the observed difference in SA content. Moreover, the higher recovery for the proposed method indicates that this method is more accurate for the quantitative analysis of SA in dried cuttlefish than the conventional method.

4. Conclusion

Here an analytical method using a combination of solvent extraction and qHNMR analysis was applied and validated to determine SA levels in processed foods and we proved that the proposed method is useful for quantification of SA. This is the first report of the successful quantification of SA in processed foods using qHNMR. The proposed method has good accuracy, precision, selectiveness, and linearity in the assessed concentration range. In addition, it is an absolute quantification method with SI-traceability. The conventional method using steam distillation and HPLC require a long time for sample preparation (65 min), HPLC analysis including analysis of standard for creating calibration curve (50 min), and creation of calibration curve and determination of SA content (10 min). While, the proposed method takes 55 min (sample preparation: 35 min; qHNMR analysis: 15 min; data processing and determination of SA content: 5 min). In addition, the proposed method did not require multi-step purifications in pretreatment and a calibration curve to quantify SA in processed foods. Therefore, the proposed method is more rapid and simple than the conventional method. The LOQ was less than 12.5% of the maximum usage levels of all food regulated in Japan and by the Codex General Standard for Food Additives and also low enough for the purposes of monitoring SA. Therefore, the proposed method is applicable to the monitoring of SA in processed foods at the inspection center, regulatory laboratory, and quarantine stages.

Consequently, we consider that the proposed method is enabled to be an important and reliable alternative to the conventional method for the quantification of food additives in processed foods. In order to obtain the accurate data, different proton signals of SA in different processed foods should be selected to determine SA level, because the spectrum patterns are different depending on the processed food.

The proposed method could be applied to the identification and quantification of SA in samples with complex matrices, such as beverages, fruits, pharmaceutical formulations, and cosmetic products, as well as processed foods. Moreover, the results of the present study will aid in the development of future research, in fields such as food chemistry, pharmacognosy, natural product chemistry, and pharmacology, into the absolute quantification of main components or impurities in mixtures.

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Absolute quantification for benzoic acid in processed foods using quantitative proton nuclear magnetic resonance spectroscopy

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ABSTRACT

The absolute quantification method of benzoic acid (BA) in processed foods using solvent extraction and quantitative proton nuclear magnetic resonance spectroscopy was developed and validated. BA levels were determined using proton signals (δ_H 7.53 and 7.98) referenced to 2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt (DSS-d_6) after simple solvent extraction from processed foods. All recoveries from several kinds of processed foods, spiked at their specified maximum Japanese usage levels (0.6–2.5 g kg^{-1}) and at 0.13 g kg^{-1} and 0.063 g kg^{-1}, were greater than 80%. The limit of quantification was confirmed as 0.063 g kg^{-1} in processed foods, which was sufficiently low for the purposes of monitoring BA. The accuracy of the proposed method is equivalent to the conventional method using steam-distillation extraction and high-performance liquid chromatography. The proposed method was both rapid and simple. Moreover, it provided International System of Units traceability without the need for authentic analyte standards. Therefore, the proposed method is a useful and practical tool for determining BA levels in processed foods.

1. Introduction

Benzoic acid (BA) occurs naturally in different foods, such as fruits, vegetables, spices and nuts, especially in the dairy products, at low concentrations [1–3]. In addition, BA and its sodium salt are commonly used as preservatives to prevent the alteration and degradation of foods by microorganisms, since they exhibit inhibitory activity against fungi, yeasts, molds, and bacteria [4–6]. However, some adverse effects, such as metabolic acidosis, convulsions, hyperpnoea, and allergic reactions, have been reported in experimental animals and in humans [7–9]. As a result, many countries regulate these compounds according to the specific legislation for food additives, based on the acceptable daily intake values (0–5 mg kg^{-1} of body mass) established by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives Expert Committee on Food Additives [10] and the maximum usage level of these preservatives in each type of food as determined by the Codex committee. Therefore, a reliable analytical method is required to determine their levels in processed foods and to ensure regulatory compliance.

Several analytical methods, including high-performance liquid chromatography (HPLC) [11–14], gas chromatography [15,16], and capillary electrophoresis [17], have been developed for the determination of BA in various processed foods. These methods require complicated and time-consuming pre-treatments to extract and/or clean-up BA from processed foods, such as steam distillation [14], solvent extraction [11,12,17], solid-phase extraction [13,16], and headspace solid-phase microextraction [15]. In addition, an authentic standard, such as a certified reference material (CRM), is required for accurate quantification, though this might be difficult to obtain or can be of questionable accuracy.

During the development of a quantification method for food additives in processed foods, we focused on quantitative proton nuclear magnetic resonance (qHNMR) spectroscopy for purity analysis in substances and quantification of complex mixtures using an International System of Units (SI)-traceable reference material as an internal standard (IS) [18]. In qHNMR, the content or concentration of the analyte is obtained using the ratio between the integral value of a specific signal of the analyte and that of an IS. The intensities of given NMR resonances of the analyte and the IS are directly proportional to "the number of nuclei of resonance line" times "the molar concentration" of the analyte and the IS. Therefore, its results become absolute. This method has additional advantages in terms of simple sample preparation, reduced sample consumption, rapid measurement, involved structural information, and non-destructive analysis [19,20].

Because of these factors, qHNMR has consequently been used to study the quantities of crude samples, such as metabolites in

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urine or serum [21,22], naturally occurring compounds in medicinal plants [23,24], and medicinal components in tablets [25]. qHNMR has also been used to analyze beverages, including the quantification of organic and amino acids in beer [26], (−)-epicatechin [27] and formic acid in apple cider [28], malic and citric acids in fruit juices [29], methanol in a traditional Cypriot spirit [30], organic compounds in vinegars [31] and wine [32], and saccharides in carrot (*Daucus carota* L.) root [33]. However, there have been few other reported applications of qHNMR in solid processed foods such as caviar, margarine, and fruit paste. We previously reported that qHNMR using an SI-traceable reference material combined with solvent extraction could be used to determine the absolute content of sorbic acid in processed foods [34]. Therefore, we anticipated the application of this method to quantify the absolute content of other food additives in processed foods.

Here, we developed and validated an absolute quantification method with SI-traceability for BA in processed foods using solvent extraction and qHNMR. We also compared the proposed method with the conventional method using steam distillation and HPLC.

2. Materials and methods

2.1. Processed food samples

Six processed foods not containing BA (caviar, margarine, avocado paste, soft drink, syrup, and soybean sauce) and four processed foods containing BA (margarine, soft drink, syrup, and soybean sauce) were purchased from markets in Tokyo, Japan.

2.2. Chemicals

All chemicals were of HPLC or analytical grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The water used was ultrapure, purified to 18 MΩ cm using a Millipore (Danvers, MA, USA) Milli-Q water purification system. BA (reagent grade) and 2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt (DSS-d_6) standard (Code No. 048-31071, Lot. EPL1095, purity: 92.2% ± 1.0%), the traceable reference material, were obtained from Wako Pure Chemical Industries, Ltd. DMSO-d_6 was purchased from Kanto Chemical Co., Inc.

2.3. Instruments

The qHNMR spectrum was measured on a JEOL JNM ECA 600 spectrometer (JEOL, Ltd., Tokyo, Japan). HPLC was performed on a Shimadzu HPLC system (LC-10 A) equipped with an SPD-M10Avp diode array detector (Shimadzu Corporation, Kyoto, Japan). The homogenization was performed using an ULTRA-TURRAX T 25 digital homogenizer (IKA Works, Wilmington, NC, USA). The ultra-microbalance used was XP2U (Mettler-Toledo AG, Greifensee, Switzerland). The semi-microbalance used was ME235S (Sartorius, Bloomington, MN, USA).

2.4. Pretreatment method for processed foods

2.4.1. Solvent extraction

Solvent extraction was examined using the modified method described by Toyoda et al. [35]. Briefly, portions (5 g) of the processed foods were accurately weighed in glass centrifuge tubes. Saturated sodium chloride solution (20 mL), 10% sulfuric acid (10 mL), and diethyl ether (20 mL) were added and subjected to high-speed homogenization for approximately 1 min. The homogenate was centrifuged at 1500 × g for 5 min, and the upper layer was transferred to a clean flask. The residue was homogenized with 20 mL diethyl ether and centrifuged at 1500 × g for 5 min. The upper-layer solution was added to the flask and then evaporated for 2 min to yield the extract for the qHNMR analysis.

For caviar, margarine, and avocado paste, 20 mL of methanol was added to their extracts. Subsequently, the methanol layer was evaporated to obtain the defatted extract for the qHNMR analysis.

For soybean sauce, extraction was performed as described above without the addition of 10% sulfuric acid solution and methanol.

2.4.2. Steam distillation

Portions (5 g) of the processed foods were accurately weighed into a 1-L distillation flask, and 100 mL of water, 10 mL of 15 w/v% tartaric acid solution and 60 g of sodium chloride were added. The mixture was distilled at a flow rate of 10 mL/min. When the volume in the flask reached approximately 490 mL, the distillate was transferred to a volumetric flask, and adjusted to 500 mL by the addition of water. The final solution was filtered with a 0.45-μm syringe filter and used for the HPLC analysis.

2.5. qHNMR analysis

2.5.1. qHNMR measurement parameters

qHNMR was carried out with the following optimized parameters [36]: irradiation frequency, 600 MHz; probe temperature, 25 °C; spinning, off; number of scans, 8; spectral width, 20 ppm; auto filter, on (eight times); acquisition time, 4 s; relaxation delay, 60 s; pulse angle, 90°; pulse width, 12.2 μs; and ^{13}C decoupling, multi-pulse decoupling with phase and frequency switching (MPF-8). The data were processed using the JEOL Alice 2 software for qNMR. The signal integral value calculated by using the software was used for the quantitative analysis. The chemical shift of all data was referenced to the DSS-d_6 resonance at 0 ppm.

2.5.2. Preparation of DSS-d_6 stock solution and determination of its concentration

DSS-d_6 standard (41.76 or 44.98 mg) was dissolved in 100 g of DMSO-d_6 as the stock solution. The concentrations of DSS-d_6 in the stock solution were calculated as 0.385 or 0.415 mg/g based on the purity of DSS-d_6 (92.2%).

2.5.3. qHNMR analysis of BA

The BA (15 mg) was accurately weighed, and dissolved in 1 g stock solution. The solution was then introduced into a 5-mm (outer diameter) NMR tube (Kanto Chemical Co., Inc.) with a height of 4 cm from the bottom of the tube and subjected to qHNMR analysis. The purity of the BA was calculated using the following equation:

$$\text{Purity}\ (\%) = \frac{I_{BA}/H_{BA}}{I_{DSS}/H_{DSS}} \times \frac{M_{BA}/C_{BA}}{M_{DSS}/C_{DSS}} \times 100 \quad (1)$$

where I_{BA} and I_{DSS} are the signal integral values of BA and DSS-d_6, H_{BA} and H_{DSS} are the number of protons of signal from BA and DSS-d_6, respectively, M_{BA} and M_{DSS} are the molecular weights of BA and DSS-d_6, respectively, C_{BA} is the BA concentration (15 mg g^{-1}), and C_{DSS} is the DSS-d_6 concentration in the stock solution (0.385 or 0.415 mg g^{-1}).

2.5.4. qHNMR analysis of BA in processed foods

The extract obtained from solvent extraction was dissolved in 1 g of stock solution and subjected to qHNMR analysis as described above. The BA content of the food sample was

calculated using the following equation:

$$\text{Content (g kg}^{-1}) = \frac{I_{BA}/H_{BA}}{I_{DSS}/H_{DSS}} \times \frac{M_{BA}/W_{FD}}{M_{DSS}/C_{DSS}} \quad (2)$$

where W_{FD} is the concentration of the food sample by weight ($g\,g^{-1}$; 5 g food sample/1 g stock solution).

2.6. HPLC analysis

The sample solution extracted by steam distillation was subjected to the HPLC analysis at 230 nm, with a L-column2 ODS (4.6 × 250 mm; Chemical Evaluation and Research Institute, Saitama, Japan) at 40 °C and a flow rate of 1.0 mL min^{-1} using MeOH–H_2O–200 mM phosphate buffer (pH 4.0) mixed solvents (36:59:5) as the mobile phase. The BA content of the food sample was calculated from the following equation:

$$\text{Content (g kg}^{-1}) = \frac{C \times V}{1000 \times W} \quad (3)$$

where C is the content of BA in the sample solution, V is the volume of sample extract solution, and W is the weight of the sample (g).

2.7. Neutralization titration analysis for BA purity

BA (250 mg) was accurately weighed and dissolved in 25 mL of neutralized 50% ethanol solution, which was prepared by adding 0.8 w/v% sodium hydroxide solution and a few drops of phenol red solution (0.1% in 47% ethanol). Subsequently, the obtained solution was titrated with 0.1 mol L^{-1} sodium hydroxide solution (factor: 1.003 at 20 °C) after the addition of a few drops of phenol red solution. BA purity was calculated using the following equation:

$$\text{Purity (\%)} = \frac{12.21 \times F \times V \times 100}{W} \quad (4)$$

where F is the factor of sodium hydroxide (1.003), V is the volume of sodium hydroxide solution added drop-wise into the sample solution, and W is the weight of BA (mg).

3. Results and discussion

3.1. qHNMR measurement of BA

The quantification of BA content in processed foods was performed in a two-step process, namely pretreatment and

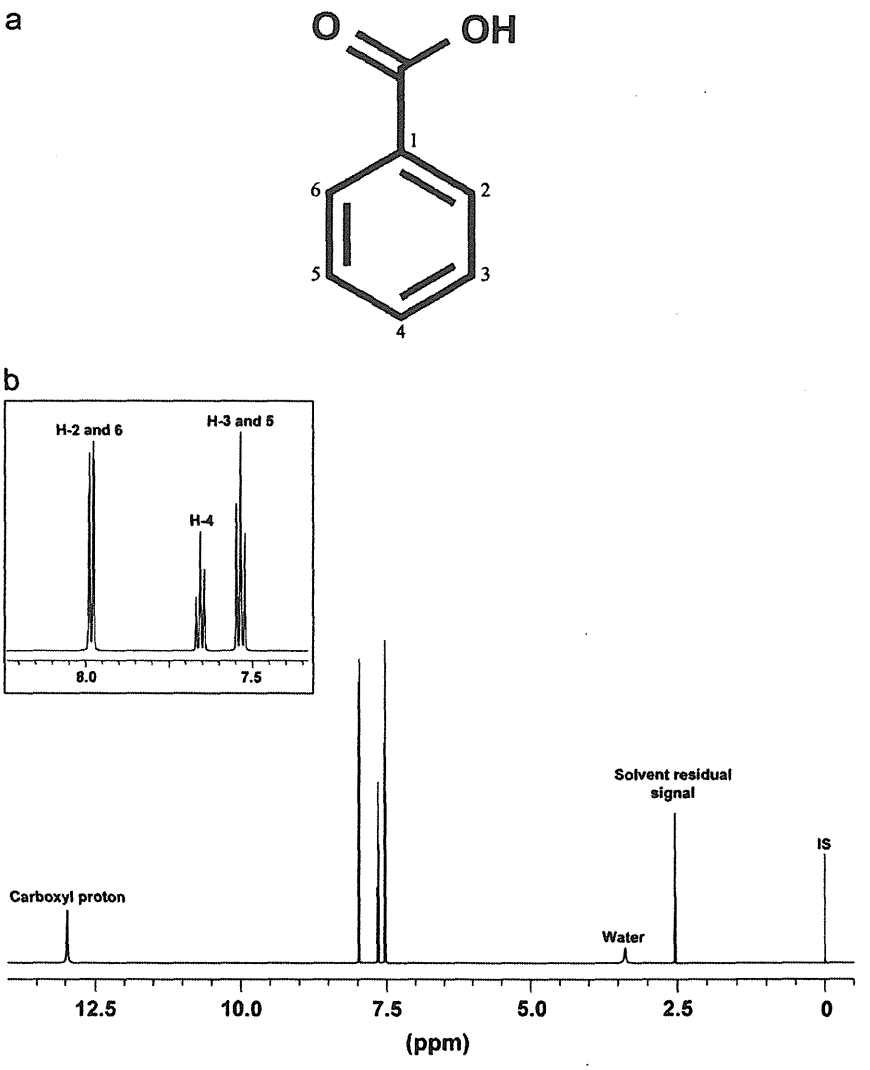

Fig. 1. Chemical structure of BA (a) and ^1H NMR spectrum of BA reagent in DMSO-d_6 containing DSS-d_6 (b). IS, internal standard (DSS-d_6).

quantification steps. In this study, we used the qHNMR analysis as a quantification step. To determine whether qHNMR could be applied for the quantification of the BA content in the processed foods, the BA was analyzed at the onset. As shown in Fig. 1, the ^{1}H NMR spectrum exhibited the characteristic signals due to five aromatic protons at δ_H 7.53 (H-3 and H-5, 2H), δ_H 7.65 (H-4, 1H), δ_H 7.98 (H-2 and H-6, 2H), and one carboxyl proton at δ_H 13.0 (1H). The purity of BA was calibrated from the ratio of the signal integral values (signal area values) of each signal to that of DSS-d_6 at δ_H 0. The calculated purities of BA are shown in Table 1. The three signals (δ_H 7.53, 7.65, and 7.98) were well resolved, and were therefore suitable for the qHNMR quantification. On the other hand, the signal at δ_H 13.0 deviated from these three signals and was deemed unsuitable for the BA quantification because this hydroxyl group is an exchangeable proton. From these data, three signals at δ_H 7.53, 7.65, and 7.98 were applied for the BA quantification by qHNMR. In particular, use of the signals at δ_H 7.53 and 7.98 produced more accurate quantification with a low BA content because the *S/N* ratios of these signals are higher than that of δ_H 7.65. To determine the linearity and the measuring range of the three signals, BA at eight different concentrations were prepared and the mean ratio of the integral value of each individual signal to that of DSS-d_6 versus the BA concentration obtained from three independent samples was plotted. As shown in Fig. 2, linear regressions with correlation coefficient of 0.9999 were obtained in the range of 0.16–50 mg g^{-1} for the signals at δ_H 7.53 and 7.98, and 0.32–50 mg g^{-1} for the signal at δ_H 7.65. In these concentration ranges, all relative errors between experimental value from qHNMR and gravimetric value of each signal was also less than 1% (Supplementary Table 1) and all *S/N* ratios of each signal were larger than 100.

3.2. Comparison of qHNMR and neutral titration methods on BA purity determination

We compared the purity of BA obtained by qHNMR and neutral titration methods to evaluate the precision of absolute quantification of qHNMR. As shown in Table 2, the purities of BA obtained from qHNMR and neutral titration methods were 99.6% ± 0.2% and 99.7% ± 0.1%, respectively. These results indicate that qHNMR and the neutral titration method have equivalent accuracy and precision for the absolute quantification of BA.

3.3. Pretreatment method for processed foods

Steam distillation is conventionally used as the pretreatment method for the BA quantification in processed foods. However, water of distillation solution remains as the largest peak in the ^{1}H NMR spectrum, resulting in lower intensity signal of sample, and an overlap of signals between water and sample. In addition, it takes time to completely evaporate the water from this solution *in vacuo*. To avoid these problems, we selected solvent extraction with diethyl ether. This pretreatment is unnecessary in multi-step purifications, because the BA content of processed foods can be readily determined if the BA signals on the ^{1}H NMR spectrum are sufficiently separated from interference signals. The proposed pretreatment is also rapid and gives low intensity of water signal interference following the qHNMR analysis.

3.4. Recovery test

The proposed method, combining solvent extraction and qHNMR, was applied to determine BA in processed foods. To assess its intra-day accuracy and precision, we performed the recovery test at 0.063 g kg^{-1}, 0.13 g kg^{-1}, and the maximum Japanese BA usage levels in six processed foods. Fig. 3 and Supplementary Fig. 1–6 show the ^{1}H NMR spectra of BA-spiked and blank sample extracts of caviar, margarine, avocado paste, soft drink, syrup, and soybean sauce.

Three signals at δ_H 7.53, 7.65, and 7.98 were well separated from the other groups. Moreover, signals of other ingredients of processed foods were minimal or noise level in this zone. On the basis of these data and the *S/N* ratio of each signal, the signals at δ_H 7.53 and 7.98 were applied for the quantification. As shown in Table 3, the

Table 1
Purity of BA determined by qHNMR.

Signal (δ, ppm)	Number of protons	Integral value[a]	Purity (%)[b]
7.53	2	139.0	99.6 ± 0.1
7.65	1	69.9	99.4 ± 0.3
7.98	2	139.3	99.7 ± 0.2
13.0	1	64.6	92.5 ± 1.3

[a] Values represent the mean of three independent experiments.
[b] Values represent the mean ± standard deviation of three independent experiments.

Table 2
Comparison of BA purities determined by qHNMR and neutralization titration methods.

	Purity (%)
qHNMR	99.6 ± 0.2[a]
Neutralization titration	99.7 ± 0.1

Values represent the mean ± standard deviation of three independent experiments.
[a] Values represent purities obtained from three signals (δ_H 7.53, 7.65, and 7.98).

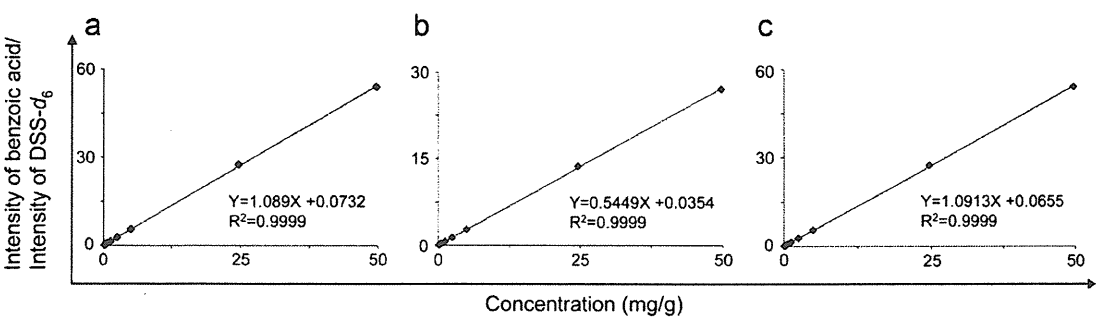

Fig. 2. Relationship between BA concentration and integral ratio of BA:DSS-d_6 signals. (a) δ_H 7.53, (b) δ_H 7.65, and (c) δ_H 7.98. The errors of analytical values in each concentration are under the data points.

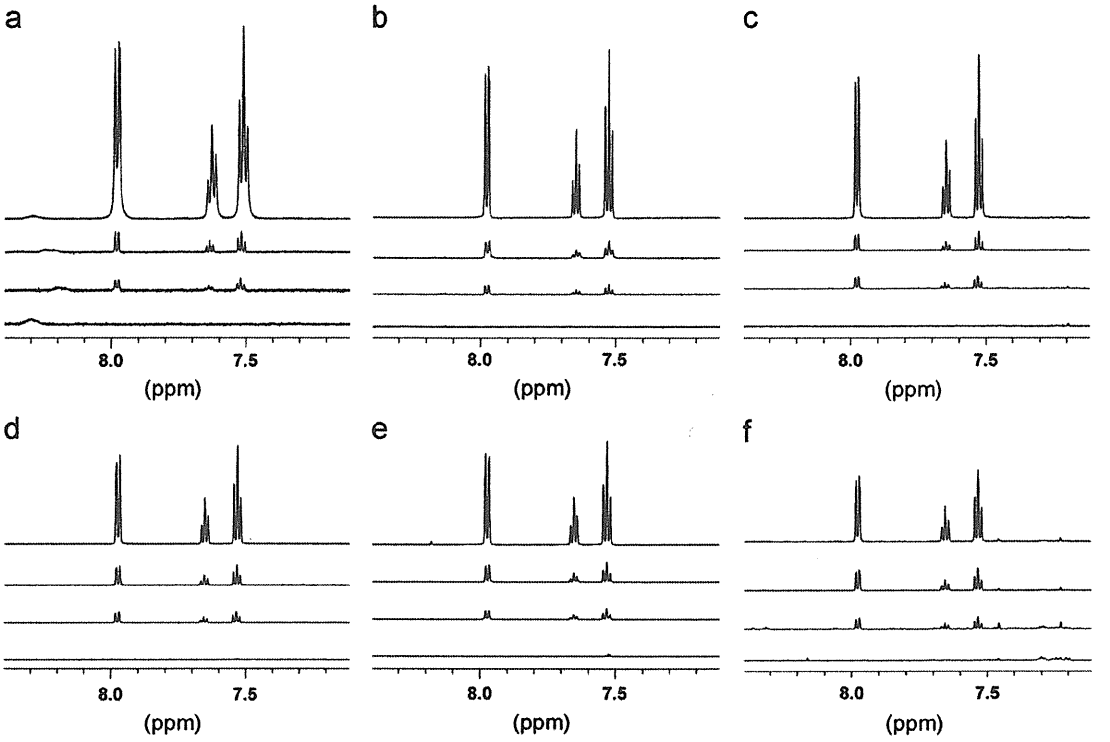

Fig. 3. ^{1}H NMR spectra in the range of δ_H 7.1–8.4 of each sample extract spiked with BA at the maximum usage level of each processed food (top), 0.13 g kg^{-1} (second), 0.063 g kg^{-1} (third), and blank (bottom): (a) caviar, (b) margarine, (c) avocado paste, (d) soft drink, (e) syrup, and (f) soybean sauce.

Table 3
Recovery of BA from processed foods.

Sample	Signal (δ, ppm)	0.063 g kg^{-1} spiked		0.13 g kg^{-1} spiked		Maximum usage level spiked		
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Level (g kg^{-1})	Recovery (%)	RSD (%)
Caviar	7.53	93.6	2.6	97.7	0.7	2.5	95.2	2.2
	7.98	96.5	1.5	98.7	0.4		95.1	2.0
Margarine	7.53	86.8	0.2	86.7	1.9	1.0	90.6	1.8
	7.98	84.5	0.2	86.5	2.1		90.7	2.0
Avocado paste	7.53	91.5	0.6	90.9	1.4	1.0	94.4	1.0
	7.98	89.6	0.2	89.9	2.6		93.7	1.2
Soft drink	7.53	80.5	2.0	89.4	2.6	0.60	91.9	0.8
	7.98	81.0	3.0	89.2	2.7		91.8	0.9
Syrup	7.53	81.9	3.9	92.3	1.9	0.60	96.5	1.3
	7.98	83.5	3.8	91.6	2.0		96.4	1.6
Soybean sauce	7.53	91.4	1.6	92.5	0.5	0.60	91.1	3.6
	7.98	88.5	0.6	92.2	0.4		91.1	3.6

Each recovery value represents the mean of three independent experiments performed on the same day.
RSD, intra-day relative standard deviation.

Table 4
Inter-day recoveries, repeatability, and intermediate precisions of BA in soft drink and margarine.

Sample	0.063 g kg^{-1}			Maximum usage level spiked		
	Recovery (%)	RSD$_r$ (%)	RSD$_{ip}$ (%)	Recovery (%)	RSD$_r$ (%)	RSD$_{ip}$ (%)
Soft drink	86.2	6.3	7.1	94.2	4.1	4.9
Margarine	84.6	2.8	5.4	87.1	3.6	5.5

Each recovery value represents the mean of analysis results in two independent experiments on five different days. RSD$_r$ and RSD$_{ip}$ are calculated by one-way analysis of variance of the recovery values obtained in duplicate on five different days.

recoveries of all samples spiked with BA at 0.063 g kg^{-1}, 0.13 g kg^{-1}, and the maximum usage levels obtained from the signal at δ_H 7.53 ranged from 80.5% to 97.7% and the relative standard deviation (RSD) values ranged from 0.2% to 3.9%. In soft drink and syrup spiked at 0.063 g kg^{-1}, the recoveries appeared to be slightly lower than those of other processed foods (80.5% and 81.9%). For use of the signal at δ_H 7.98, the recoveries and the RSDs were almost equivalent to those determined using the signal at δ_H 7.53.

Limit of quantification (LOQ) was determined based on the accuracy (recovery), precision (RSD), and the *S/N* ratio of signals for quantification in recovery test. Willetts and Wood [37] reported that the recovery in the case of content analyte in sample with larger than 0.5 mg kg^{-1} is required to be 80–110% in intralaboratory analytical method validation. They also reported that standard deviation of analysis in the case of average value of fraction of analyte in sample is 10^{-6}–10^{-5} must not exceed 10%. In the present data, the recoveries for all samples spiked at 0.063 g kg^{-1}, which is most low spiked concentration, were larger than 80% and their RSDs were less than 4%. In addition, the means of *S/N* ratios for all samples spiked at 0.063 g kg^{-1} were larger than 100 in all samples. On the basis of these results, this proposed method can efficiently determine BA in processed foods at concentrations of at least 0.063 g kg^{-1}. Therefore, we estimated that the LOQ of the proposed method was 0.063 g kg^{-1}. As the maximum usage levels of BA in processed foods are 0.60–2.5 g kg^{-1} in Japan, the proposed method is applicable to the monitoring of BA in processed foods at the inspection center, regulatory laboratory, and quarantine stages.

3.5. Validation

To validate the inter-day precision and accuracy of this method, recovery tests were performed on two levels of BA in two foods prepared on five different days. Among six foods used in the recovery tests, margarine and soft drink were selected as samples for the inter-day precision test, which were prepared using the extraction method with and without a degreasing step, respectively. BA was added at the maximum usage level (1.0 or 0.6 g kg^{-1}) and 0.063 g kg^{-1}. All data were calculated using the signals at δ_H 7.53. As shown in Table 4, the RSD_r (repeatability relative standard deviation) ranged from 2.8% to 6.3% and the RSD_{ip} (intermediate precision relative standard deviation) ranged from 5.4% to 7.1%. In addition, the recoveries ranged from 84.6% to 94.2% at all tests. These results clearly indicate that the proposed method has accuracy and acceptable precision. Therefore, the method is found to be reliable for the determination of BA levels.

3.6. Comparison of the proposed method and conventional method for commercial processed foods

The proposed method was applied to four commercial processed foods labeled with BA as a food additive, and compared with the conventional method using steam distillation extraction and HPLC. As shown in Fig. 4, the signals at δ_H 7.53 and 7.98 for margarine, soft drink, and syrup were clearly separated from the interference signals, with the exception of soybean sauce. For soybean sauce, the signal at δ_H 7.98 overlapped with the interference signals. Therefore, the BA content in processed food was determined using both the signals at δ_H 7.53 and 7.98 in margarine, soft drink, and syrup, and the signal at δ_H 7.98 in soybean sauce. In all food samples, no significant differences between the BA contents from the proposed method and those

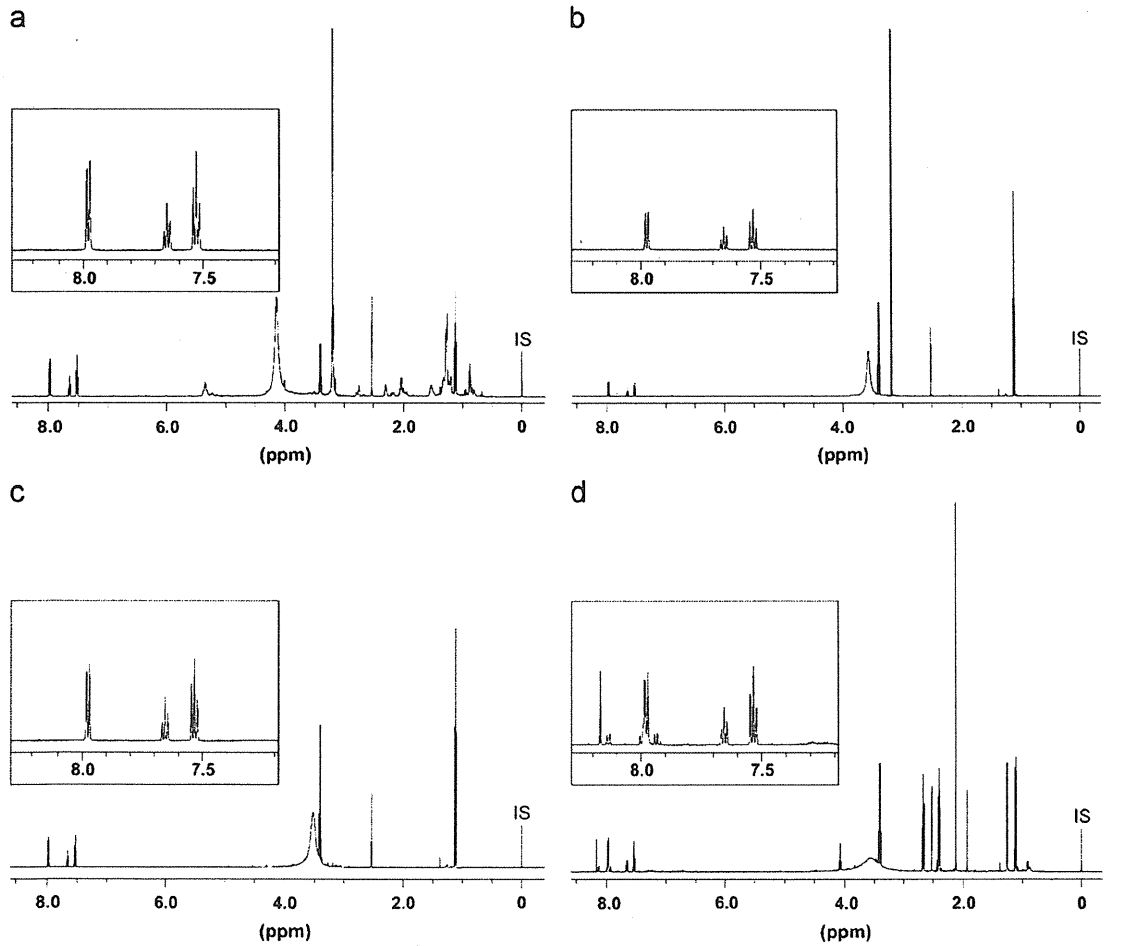

Fig. 4. ^1H NMR spectra of each sample solution from commercially produced food containing BA. The BA signals shown on the top is highlighted: (a) margarine, (b) soft drink, (c) syrup, and (d) soybean sauce. IS, internal standard (DSS-d_6).

Table 5
Comparison of BA contents in commercial foods determined by two methods.

Sample	Proposed method (solvent extraction/qHNMR)			Conventional method (steam distillation/HPLC)	
	Signal (δ, ppm)	Content (g kg^{-1})	RSD (%)	Content (g kg^{-1})	RSD (%)
Margarine	7.53	0.46	4.0	0.47	1.1
	7.98	0.46	4.0		
Soft drink	7.53	0.26	4.5	0.25	0.1
	7.98	0.26	5.0		
Syrup	7.53	0.48	2.1	0.45	0.9
	7.98	0.48	2.2		
Soybean sauce	7.53	0.45	4.9	0.47	0.5
	7.98	-	-		

Each value represents the mean and RSD of three independent experiments.
-, not quantifiable since the signal at δ_H 7.98 and that of the food ingredient overlapped.

from the conventional method were found by statistically evaluation ($P<0.05$) using Student's t-test (Table 5). These results indicated that the accuracy of the proposed method was comparable to that of the conventional method. With respect to precision, the RSDs of the proposed method are greater than the conventional method. However, since these are less than 5%, this precision allows for a reliable determination of BA in processed food. Therefore, the proposed method is available as an alternative method.

4. Conclusion

In this study, we developed and validated a method for determining BA levels in various processed foods using solvent extraction and qHNMR analysis. This is the first report of the successful determination of BA in processed foods using qHNMR. The proposed method has accuracy, precision, selectiveness, and linearity in the assessed concentration range. Moreover, it is an absolute quantification method with SI-traceability. This method is also more rapid and simple than the conventional method (the proposed method: 55 min, the conventional method: 125 min). In addition, the advantage of the method is that no authentic analyte standard is required for the determination of BA in processed foods. The LOQ is less than 10% of the maximum usage levels of all food regulated in Japan and by the Codex General Standard for Food Additives and also low enough for the purposes of monitoring BA. Therefore, the proposed method is a useful and practical tool to determine BA in processed foods.

BA is a naturally occurring component widely distributed in foods and plants and is also commonly used as a preservative in cosmetics, pharmaceuticals, and foods. The proposed method is applicable for the identification and quantification of BA in these samples. Moreover, this method is anticipated to play a predominant role for the determination of BA in complex matrices.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.062.

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Absolute Quantitation of Stevioside and Rebaudioside A in Commercial Standards by Quantitative NMR

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The extract prepared from the leaves of *Stevia rebaudiana* Bertoni (Asteraceae) contains sweet steviol glycosides, mainly stevioside and rebaudioside A. Highly purified stevia extracts have become popular worldwide as a natural, low-calorie sweetener. They contain various types of steviol glycosides, and their main components are stevioside and rebaudioside A. The content of each steviol glycoside is quantified by comparing the ratios of the molecular weights and the chromatographic peak areas of the samples to those of stevioside or rebaudioside A standards of the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) and other specifications. However, various commercial standard reagents of stevioside and rebaudioside A are available. Their purities are different and their exact purities are not indicated. Therefore, the measured values of stevioside and rebaudioside A contained in a sample vary according to the standard used for the quantification. In this study, we utilized an accurate method, quantitative NMR (qNMR), for determining the contents of stevioside and rebaudioside A in standards, with traceability to the International System of Units (SI units). The purities of several commercial standards were determined to confirm their actual values.

Key words stevioside; rebaudioside A; quantitative NMR; absolute quantitation

The extract produced from the leaves of *Stevia rebaudiana* Bertoni (Asteraceae) contains sweet steviol glycosides, mainly stevioside and rebaudioside A (Fig. 1), and has long been used as a sweetener in Japan. In 2008, the specifications for "steviol glycosides" were established by the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA), and "stevia extracts" were approved as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA). Thereafter, the purified stevia extracts specified in JECFA and FDA became popular worldwide.

According to the assay for steviol glycocides in the JECFA specifications,[1] the total content of nine types of steviol glycosides (Fig. 2) should not be less than 95%. In the assay method, rebaudioside A is quantified from the peak area on LC using the rebaudioside A standard, and each steviol glycoside except rebaudioside A is quantified from their respective peak areas and absorption coefficients using the stevioside standard as the quantification standard.

Various commercial standard reagents of stevioside and rebaudioside A are available, although their purities are different and their exact purities are not indicated in the product literatures. Therefore, if the claimed values are employed in the quantitative analysis of a sample, the quantitated contents of the stevioside and rebaudioside A in the identical sample will be assigned different measured values according to the standard used for the quantification.

Quantitative NMR (qNMR) using an internal standard with traceability to the International System of Units (SI units) has recently been used to determine the absolute purities of various chemical substances.[2–7] The qNMR method permits the absolute quantification of a target compound without the need for a standard of that compound. In addition, it is rapid and easy. The compound is quantified from the ratios of the integral values of the proton signals of the internal standard and the target compound. In addition, various compounds can be quantified using the same internal standard.

In this study, we utilized the qNMR method to determine the purity of stevioside and rebaudioside A in commercial standards. The knowledge of their absolute purities will increase the accuracy of other analytical methods that use standards, such as LC.

Experimental

Reagents for qNMR Pyridine-d_5 (99.5 atom% D) was purchased from Isotec (Ohio, U.S.A.). 1,4-Bis(trimethylsilyl)-benzene-d_4 (1,4-BTMSB-d_4) reference material (Wako Pure Chemical Industries, Ltd., Osaka, Japan; CAT No. 021-16441, Lot 081204; purity 99. 8±0.2%) was used as an internal standard for NMR measurements. Diethyl phthalate (DEP), a certified reference material (CRM) of the National Metrology Institute of Japan (NMIJ) (NMIJ CRM 4022-b), was purchased from Wako Pure Chemical Ind., Ltd.; its purity is certified to be 99.98±0.09%.

Stevioside and Rebaudioside A Standards Five stevioside standards were purchased: STD-I: Wako Pure Chemical Ind., Ltd., CAT No. 196-08131; STD-II: Wako Pure Chemical Ind., Ltd., CAT No. 193-15351, purity 99.0+% (LC, dried); STD-III: Kanto Chemical (Tokyo, Japan), CAT No. 37480-92, purity >98.0% (LC); STD-IV: USP (Maryland, U.S.A.), CAT No. 1622408: purity 0.97 mg/mg (not dry); and STD-V: ChromaDex (California, U.S.A.), CAT No. ASB-00019351-010: purity 98.4% (LC). Five rebaudioside A standards were also purchased: RBDA-I: Wako Pure Chemical Ind., Ltd., CAT No.

The authors declare no conflict of interest.

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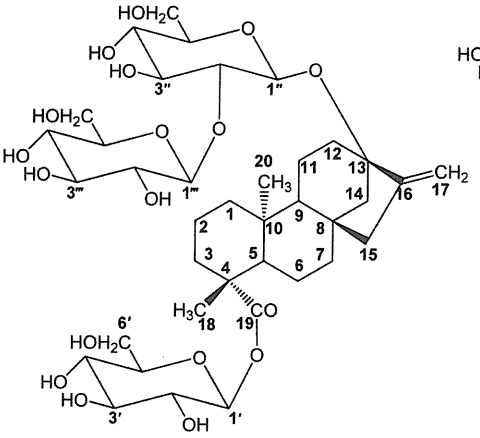
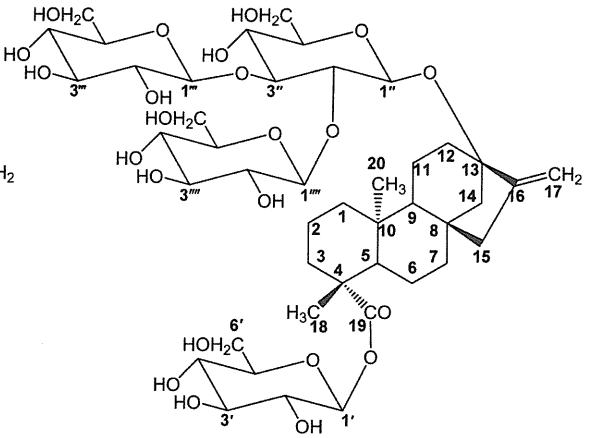
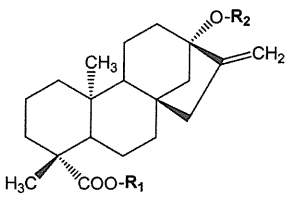


Stevioside



Rebaudioside A

Fig. 1. Structure of Stevioside and Rebaudioside A



Compound	R_1	R_2	MW
Stevioside	Glc(β)-	Glc(β1-2)Glc(β)-	804.9
Rebaudioside A	Glc(β)-	Glc(β1-2)Glc(β)-Glc(β1-3)┘	967.0
Rebaudioside B	H-	Glc(β1-2)Glc(β)-Glc(β1-3)┘	804.9
Rebaudioside C	Glc(β)-	Rha(α1-2)Glc(β)-Glc(β1-3)┘	951.0
Rebaudioside D	Glc(β1-2)Glc(β)-	Glc(β1-2)Glc(β)-Glc(β1-3)┘	1129.2
Rebaudioside F	Glc(β)-	Xyl(β1-2)Glc(β)-Glc(β1-3)┘	937.0
Steviolbioside	H-	Glc(β1-2)Glc(β)-	642.7
Dulcoside A	Glc(β)-	Rha(α1-2)Glc(β)-	788.9
Rubusoside	Glc(β)-	Glc(β)-	642.7

Glc: D-glucose, Rha: L-rhamnose, Xyl: D-xylose

Fig. 2. Structures and Molecular Weights of Steviol Glycosides

186-00651; RBDA-II: Wako Pure Chemical Ind., Ltd., CAT No. 183-02361, 99.0+% (LC, dried); RBDA-III: USP, CAT No. 1600121, purity 0.969 mg/mg (not dry); RBDA-IV: ChromaDex, CAT No. ASB-00018225-050; and RBDA-V: ChromaDex, CAT No. ASB-00018226-010, purity 96.8% (LC).

^{1}H-NMR Spectrometer and Parameters ^{1}H-NMR spectra were recorded on a JEOL JNM-ECA600 (600 MHz) spectrometer. NMR acquisition and processing were performed as previously described.[2-7] For each sample, 16 scans were recorded with a 90° pulse and a 60-s pulse delay, since high-precision qNMR spectra can be attained when the pulse angle is 90° and the pulse delay time is greater than the quintuple spin–lattice relaxation time ($>5\times T_1$). Chemical shifts are represented in ppm values relative to the proton signal of pyridine-d_5 (7.55 ppm). Integral values of the proton signals were obtained after Fourier transform of the free-induction decay (FID) data (window function: exponential function BF= 0.12 Hz, zero filling=1; trapezoidal function T1=T2=0, T3= 90, T4=100); and automatic phase correction.

Determination of 1,4-BTMSB-d_4 Concentration in the qNMR Reference Solution The qNMR reference solution was prepared as previously described.[3,4,6] The 1,4-BTMSB-d_4 reference material (20 mg) was dissolved in pyridine-d_5 (100 mL) to make the qNMR reference solution. The 1,4-BTMSB-d_4 concentration in the reference solution can be calculated from the accurate weight of 1,4-BTMSB-d_4 reference material and its purity certified with SI traceability (99.8±0.2%). In this study, the 1,4-BTMSB-d_4 concentration was accurately confirmed by qNMR using DEP as an internal standard. DEP (20.0 mg) was weighed and dissolved in 4.0 mL of the qNMR reference solution, and then 0.6 mL of the mixed solution was sealed in an NMR test tube (4.932–4.970 mm; diameter×8 in.; Wako Pure Chemical Ind., Ltd.) and subjected to ^{1}H-NMR measurement. The 1,4-BTMSB-d_4 concentration in the qNMR reference solution was calculated by using the ratio of the signal integral at 0.23 ppm (1,4-BTMSB-d_4) to that at 4.34 ppm (DEP):

$$C_{\mathrm{BTMSB}} = W_{\mathrm{DEP}} \times \frac{P_{\mathrm{DEP}}}{100} \times \left(\frac{M_{\mathrm{BTMSB}} \times I_{\mathrm{BTMSB}}}{H_{\mathrm{BTMSB}}} \Big/ \frac{M_{\mathrm{DEP}} \times I_{\mathrm{DEP}}}{H_{\mathrm{DEP}}} \right) \quad (1)$$

where C_{BTMSB} is the accurate concentration (mg/mL) of 1,4-BTMSB-d_4 in the qNMR reference solution; W_{DEP} is the concentration (mg/mL) of the reference material DEP in the mixed solution; P_{DEP} is the certified purity (99.98%) of DEP in the reference material DEP; M_{BTMSB} and M_{DEP} are the molecular weights of 1,4-BTMSB-d_4 and DEP (226.50, 222.24, respectively); I_{BTMSB} and I_{DEP} are the signal integrals at 0.23 ppm (1,4-BTMSB-d_4) and 4.34 ppm (DEP), respectively; and H_{BTMSB} and H_{DEP} are the number of protons producing the signal at 0.23 ppm (1,4-BTMSB-d_4, $CH_3\times6$) and 4.34 ppm (DEP, $CH_2\times2$).

Quantitative Determination of Stevioside and Rebaudioside A by qNMR Commercial standards of stevioside (STD-I through -V) and rebaudioside A (RBDA-I through -V) were dried at 105°C for 2 h, according to the preparation method of the standard described in the JECFA specifications,[1] and then 20 mg of each standard was accurately weighed and dissolved individually in 1.0 mL of the qNMR reference solution and subjected to ^{1}H-NMR measurement. The purities (%w/w) of stevioside and rebaudioside A in the standards were calculated from Eq. 2:

$$P_{STD\ (or\ RBDA)} = C_{BTMSB} \times \left(\frac{M_{STD\ (or\ RBDA)} \times I_{STD\ (or\ RBDA)}}{H_{STD\ (or\ RBDA)}} \Big/ \frac{M_{BTMSB} \times I_{BTMSB}}{H_{BTMSB}} \right) \times \frac{100}{W_{STD\ (or\ RBDA)}} \quad (2)$$

where $P_{STD\ (or\ RBDA)}$ is the purity (%w/w) of stevoside (or rebaudioside A) in the commercial standard; C_{BTMSB} is the accurate concentration (mg/mL) of 1,4-BTMSB-d_4 in the qNMR reference solution; $M_{STD\ (or\ RBDA)}$ and M_{BTMSB} are the molecular weights 804.9 (or 967.0) and 226.50, respectively; $I_{STD\ (or\ RBDA)}$ and I_{BTMSB} are the signal integrals at 5.68 or 2.71 ppm (stevioside) or 5.31 or 2.65 ppm (rebaudioside A) and 0.23 ppm (1,4-BTMSB-d_4), respectively; $H_{STD\ (or\ RBDA)}$ and H_{BTMSB} are the number of protons producing the signal at 5.68 or 2.71 ppm (stevioside, CH×1 each) [or at 5.31 or 2.65 ppm (rebaudioside A, CH×1 each)] and 0.23 ppm (1,4-BTMSB-d_4, $CH_3\times6$); and $W_{STD\ (or\ RBDA)}$ is the concentration (mg/mL) of stevioside (or rebaudioside A) in the commercial standard. In this study, an analytical software package for quantification, ALICE 2 for qNMR (JEOL RESONANCE, Tokyo), was employed to automatically calculate the purity of the stevioside and rebaudioside A in the samples from the qNMR FID data.

LC Analysis of Stevioside and Rebaudioside A Aliquots (40 μL) of the solutions prepared for qNMR were dried under N_2 gas and then dissolved in 1.6 mL of CH_3CN–water (30 : 70 v/v). The solutions (5 μL) were subjected to LC analysis, and the peak areas were measured. The LC system was an Alliance 2695 with a 2996 photodiode array detector (Waters Co., Massachusetts, U.S.A.), and the analysis conditions were as follows: LC column: Capcell Pak C18 MG (4.6 mm i.d.×250 mm, 5 μm, Shiseido Co., Ltd., Tokyo, Japan); column temperature, 40°C; flow rate, 1 mL/min; mobile phase, CH_3CN–ammonium formate (5 mM, pH 6.1, 32 : 68 v/v, isocratic elution); detection wavelength, 210 nm. Each solution was injected in duplicate.

Results and Discussion

Selection of ^{1}H-NMR Target Signals for Quantification by qNMR The ^{1}H-NMR spectra of stevioside, rebaudioside A, and six other steviol glycosides (rebaudiosides B, C, and D; steviolbioside; dulcoside A; and rubusoside) recorded in pyridine-d_5 are shown in Fig. 3. Proton signals of stevioside and rebaudioside A were assigned based on the chemical shifts in previous reports.[8,9] The 5.68 ppm (H-17α) and 2.71 ppm (H-14α) signals of stevioside and the 5.31 ppm (H-1'''') and 2.65 ppm (H-14α) signals of rebaudioside A could be distinguished. Rebaudiosides B and D showed signals at chemical shifts similar to the 5.68 ppm (H-17α) signal of stevioside, and rebaudioside D and rubusoside showed signals at chemical shifts similar to the 2.71 ppm (H-14α) signal of stevioside. Rebaudioside C and dulcoside A showed signals at chemical shifts similar to the 2.65 ppm (H-14α) signal of rebaudioside A. Steviolbioside showed signals at chemical shifts similar to the 5.31 ppm (H-1'''') signal of rebaudioside A. However, the contents of rebaudiosides B and D and rubusoside in the stevioside standards and of rebaudioside C, dulcoside A and steviolbioside in the rebaudioside A standards were very low by LC/UV analysis, less than 0.17 area% (data not shown). Therefore, each of the above two signals of stevioside (5.68, 2.71 ppm) and rebaudioside A (5.31, 2.65 ppm) was not affected by the signals arising from other steviol glycosides in the standards. Thus, the above four signals of stevioside and rebaudioside A were selected as target signals for qNMR.

In the present study, 1,4-BTMSB-d_4 (Fig. 3) was used as an internal standard for qNMR,[3,4,6] because the 1,4-BTMSB-d_4 signal (0.23 ppm) does not overlap with any signals arising from steviol glycosides and because we can accurately calculate the 1,4-BTMSB-d_4 concentration in the prepared qNMR reference stock solution from the certified purity (99.8±0.2%) with SI traceability. Thus, the qNMR method allows the absolute quantification of stevioside and rebaudioside A because the purity of 1,4-BTMSB-d_4 used as the internal standard is accurately known.

Confirmation of Linearity of qNMR-Quantified Values of Stevioside and Rebaudioside A In this study, 20 mg/mL standards were used in the tests to determine the purity of stevioside and rebaudioside A because weights less than 10 mg, as measured by a semi-microbalance, showed an uncertainty of greater than 0.2%. In the concentration range around 20 mg/mL (15–35 mg/mL), the linearity between the weight of the tested standards and the quantified levels of stevioside or rebaudioside A determined by qNMR could be investigated. As shown in Fig. 4a, using the ratios of the signal integrals at 5.68 ppm (H-17α) and 2.71 ppm (H-14α) of stevioside to that of 1,4-BTMSB-d_4, the amount of stevioside determined by qNMR correlated (R^2=0.9989) with the weights of the stevioside standard. The amount of rebaudioside A determined by qNMR also correlated (R^2=0.9996, 0.9985) with the weights of the rebaudioside A standard (Fig. 4b). These results showed the linearity between the weight of the tested standards and the quantified levels of stevioside or rebaudioside A by qNMR, suggesting that no calibration curves are necessary in the 15–35 mg/mL range. In Fig. 4, the slopes of the regression lines were 0.97–0.98, reflecting the purities of the stevioside or rebaudioside A in the standards. Using the two signals, quantitation levels due to the different protons of the compounds were comparable.

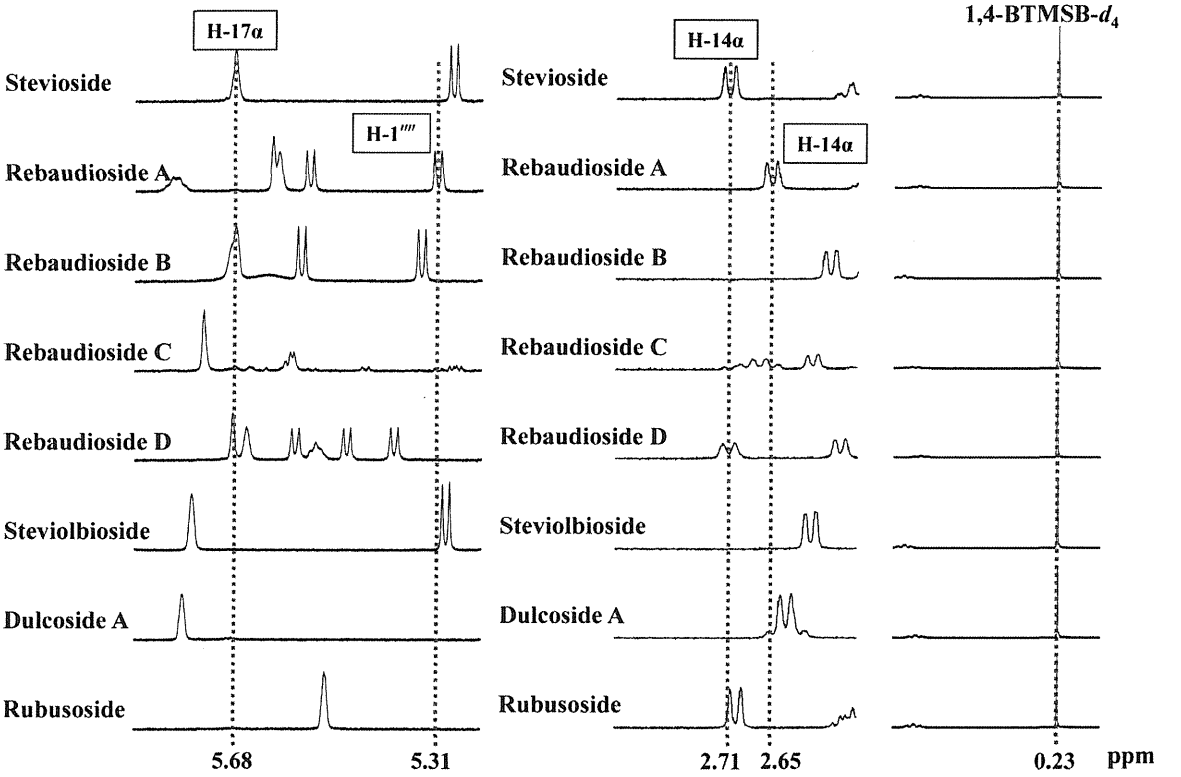


Fig. 3. qNMR Spectra of Steviol Glycosides

The spectra were obtained using a qNMR solution (pyridine-d_5) containing 1,4-BTMSB-d_4. Square labels indicate the proton positions of the signals of stevioside or rebaudioside A, as shown in Fig. 1.

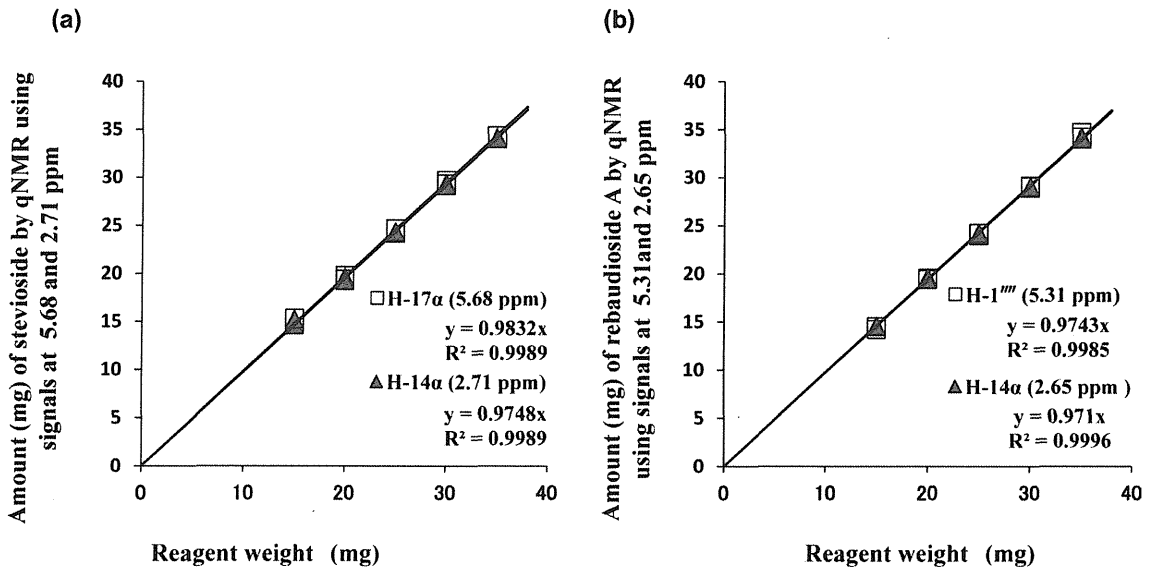


Fig. 4. Relationship between Reagent Weight and Amount of Stevioside or Rebaudioside A Determined by qNMR

The utility of qNMR for quantifying stevioside and rebaudioside A in the range of about 15–35 mg/mL of (a) STD-I and (b) RBDA-II was investigated.

Determination by qNMR of Stevioside and Rebaudioside A Purities in Various Commercial Standards The absolute purities of stevioside and rebaudioside A in five commercial standards after drying at 105°C for 2 h were determined by the qNMR method using the same internal standard. As shown in Table 1, the relative standard deviations of three trials were 0.2–1.1%. The values determined from the two signals, 5.68 ppm (H-17α) and 2.71 ppm (H-14α) for stevioside and 5.31 ppm (H-1'''') and 2.65 ppm (H-14α) for rebaudioside A, correlated well. Therefore, it appears that no remarkable signal arose from the overlap of the impurity to the signals for quantification. Thus, the averages of the purities were calculated from the two values derived from each of the two signals of stevioside and rebaudioside A, after confirming the similarity of the two values.

By qNMR, the purities of stevioside were 92.0–97.7% and

those of rebaudioside A were 94.6–96.6% in each of the five commercial standards (Fig. 5). The purities of STD-IV and STD-V were approximately 5% lower than that of STD-I, and the purities of RBDA-III, -IV, and -V were approximately 1–2% lower than that of RBDA-II. The qNMR-determined purity of stevioside and rebaudioside A in the standards was generally lower than that claimed in the product literature by manufacturers. This suggests that impurities or solvents that do not have UV absorbance might be present in the tested standards, and thus were not detected by LC/UV analysis. If the claimed purity of a commercial standard is different from the actual purity and if the claimed value is employed in the quantitative analysis of a sample, the quantitated contents of the stevioside and rebaudioside A of the sample will be incorrect. Thus, it is very important to confirm the accurate purity of stevioside and rebaudioside A in quantification standards.

Table 1. Purity Determination of Stevioside (a) and Rebaudioside A (b) in the Different Standards by qNMR and LC

(a)

Name	Purity (%) by qNMR[a)]	Purity (%) by LC[b)] using STD-I as standard	Purity (%) by LC[c)] using STD-IV as standard
Stevioside			
STD-I	97.7±1.0	—	98.0±1.5
STD-II	95.7±0.3	95.2±0.7	95.5±0.7
STD-III	95.9±0.4	96.1±0.3	96.4±0.3
STD-IV	92.0±0.5	91.7±0.9	—
STD-V	92.0±0.7	92.3±0.4	92.5±0.4

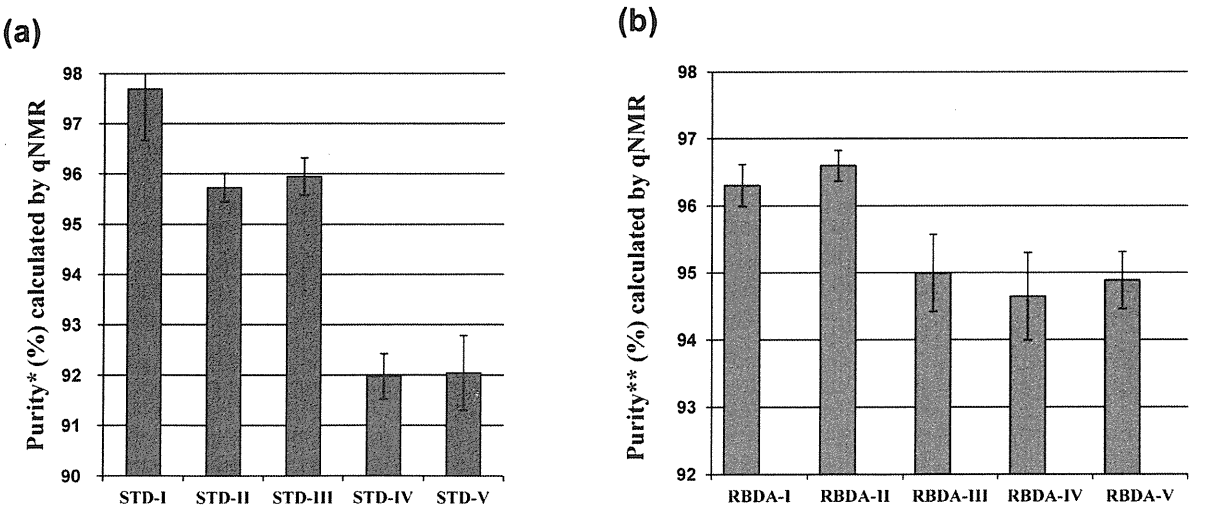
a) AV±S.D., n=3; the average of the values determined from the two signals, 5.68 ppm (H-17α) and 2.71 ppm (H-14α). *b*) AV±S.D., n=3; the purity value of STD-I determined by qNMR was used for calculation. *c*) AV±S.D., n=3; the purity value of STD-IV determined by qNMR was used for calculation.

(b)

Name	Purity (%) by qNMR[a)]	Purity (%) by LC[b)] using STD-I as standard	Purity (%) by LC[c)] using STD-IV as standard
Rebaudioside A			
RBDA-I	96.3±0.3	96.7±0.6	96.3±0.6
RBDA-II	96.6±0.2	—	96.2±1.0
RBDA-III	95.0±0.6	95.1±1.1	94.7±1.1
RBDA-IV	94.6±0.7	95.0±1.0	—
RBDA-V	94.9±0.4	95.3±1.0	94.9±1.0

a) AV±S.D., n=3; the average of the values determined from the two signals, 5.31 ppm (H-1$''''$) and 2.65 ppm (H-14α). *b*) AV±S.D., n=3; the purity value of RBDA-II determined by qNMR was used for calculation. *c*) AV±S.D., n=3; the purity value of RBDA-IV determined by qNMR was used for calculation.

Comparison of Results Obtained from qNMR and LC/UV Peak Areas Aliquots of solutions prepared for the qNMR of each of the five standards of stevioside and rebaudioside A (20 mg/mL, n=3) and those of STD-I and RBDA-II (15, 25 mg/mL, n=2) were used for LC analysis, and the peak areas were measured. Then, the purities of stevioside and rebaudioside A in commercial standards determined by qNMR were compared to their peak areas at 210 nm by LC/UV analysis. As shown in Fig. 6, the LC peak areas of stevioside and rebaudioside A correlated (stevioside: R^2=0.994, y=523.04x; rebaudioside A: R^2=0.994, y=437.25x) with the amounts determined by our qNMR method. Based on the ratio of the slopes of the regression lines for stevioside and rebaudioside A in Fig. 6, the accurate relative ratio of absorbance at 210 nm for stevioside to that for rebaudioside A in the same amounts was calculated as 1.20 (=523.04/437.25). These results suggest that the accurate relative ratio of absorbance between the same amounts of the different compounds can be calculated by a combination of qNMR and LC/UV analysis.



*Average purity calculated from the [17α] and [14α] signals

**Average purity calculated from the [1$''''$] and [14α] signals

Fig. 5. Purity Determination of (a) Stevioside and (b) Rebaudioside A in the Different Standards by qNMR

Recently, Pieri *et al.* reported[10] a quality control method for stevia extract products based on qNMR. Their method is very useful, but an NMR apparatus is necessary. In this study, an absolute quantification method employing qNMR was applied to determine the purities of stevioside and rebaudioside A in their commercial standards. By using standards with accurate purities determined by SI-traceable qNMR, the stevioside and rebaudioside A in various stevia extracts can be accurately quantified by various analytical methods such as LC.

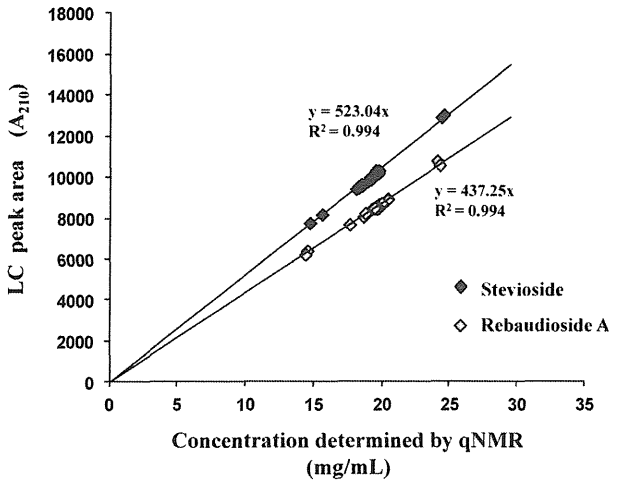


Fig. 6. Relationship between the Concentration of Stevioside or Rebaudioside A Determined by qNMR and Peak Areas at 210 nm

Signals at 2.71 and 5.68 ppm were used for the stevioside calculation, and signals at 2.65 and 5.31 ppm were used for the rebaudioside A calculation.

Table 1 shows the purities of stevioside and rebaudioside A in the different standards determined by qNMR and by LC using two of them as quantification standards. For the LC determination, the purities of each of the two quantification standards determined by qNMR were utilized for calculation. As shown in Table 1, the purities determined by qNMR and those determined by LC were similar, and the differences between then were 0.03–0.5%. These results suggest that the accurate quantification of a compound can be achieved by LC analysis if the purity of the quantification standard is determined by qNMR with SI traceability and is used for the LC/UV analysis.

Conclusion

Using 1,4-BTMSB-d_4 reference material with SI traceability as an internal standard, we utilized qNMR to examine the absolute purity of stevioside and rebaudioside A in commercial standards. The purities of stevioside and rebaudioside A in each of five commercial standards were precisely determined, and they correlated well with their peak areas obtained by LC/UV analysis. These results demonstrate that the qNMR method is useful for determining the purity of stevioside and rebaudioside A in commercial standards. In addition, these findings suggest that we can accurately quantify stevioside and rebaudioside A in various stevia extracts by LC using standard reagents whose purity has been accurately identified by qNMR.

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