intensity per proton at about 8.7 ppm (protoberberine alkaloid), and I_{HMD} is the signal intensity per proton at 0 ppm.

Quantitative determination of protoberberine alkaloids by qHNMR

Powdered crude drug sample (100.0 mg) was sonicated in 1 ml methanol for 30 min and centrifuged at 15000 rpm for 5 min. The extraction procedure was repeated three times. The supernatants were combined and concentrated to dryness in vacuo. The residue was dissolved in 1.00 ml of the qHNMR reference stock solution and subjected to ^{1}H-NMR measurement. The protoberberine alkaloid concentrations were calculated as chloride salts using Eq. 3:

$$C_{ALK} = \frac{I_{ALK}}{I_{HMD}} \times C_{HMD} \quad (3)$$

where C_{ALK} is the molar concentration of protoberberine alkaloid in the methanol extract, C_{HMD} is the molar concentration of HMD in the qHNMR reference stock solution, I_{ALK} is the signal intensity per proton of alkaloids (H-13), and I_{HMD} is the signal intensity per proton at 0 ppm (HMD).

For estimating the recovery rate, the crude drug sample spiked with 5.02 mg berberine chloride (Ber-1) was used for preparation of the extract, and subjected to qHNMR measurement.

HPLC analysis of protoberberine alkaloids

The protoberberine alkaloid (1.00 mg) was dissolved in 10.0 ml methanol, and 10 µl aliquots of the solution were subjected to HPLC analysis.

For HPLC determination of the alkaloid contents of Coptidis Rhizoma, powdered crude drug samples (100 mg) were sonicated in 1.0 ml methanol for 30 min at room temperature, and centrifuged for 5 min. The extraction was repeated two more times. The methanol extracts were combined and diluted with methanol to 20 ml. The resulting solution was subjected to HPLC analysis. Ber-1 for berberine, Pal-1 for palmatine, and Cop-1 for coptisine were used for construction of calibration curves. The concentration of the standard solution was corrected based on the qHNMR-determined purity of the reagent.

The chromatographic separation was performed using YMC-Pack ODS-AL (4.6 × 250 mm; YMC Co., Ltd., Kyoto, Japan). The mobile phase was composed of acetonitrile–water (1:1) containing NaH_2PO_4 (0.34%) and sodium dodecyl sulfate (0.17%). Flow rate was 1.0 ml/min and the elution was monitored at 345 nm.

Results and discussion

Selection of a target signal for quantification using ^{1}H-NMR

The ^{1}H-NMR spectrum of berberine chloride was recorded in methanol-d_4 as shown in Fig. 2. Signals from H-5 and H-6 protons were indistinguishable from those of water and solvent, but the other signals were clearly detected as separate signals in the spectrum. The ratio of the intensity of each signal per proton to the intensity of HMD was calculated. As shown in Table 1, the ratio was constant (37.0–37.7%) regardless of the signal used for calculation. The result indicates that any signal can be used as a target signal for qHNMR analysis. We then compared the ^{1}H-NMR spectra of berberine, palmatine, and coptisine. H-13 signals of berberine (8.60 ppm), palmatine (8.71 ppm), and coptisine (8.62 ppm) could be distinguished from each other, and were fully separated from other signals of the molecule (data not shown). The signals were also clearly identified when the methanol extract of Coptidis Rhizoma was subjected to NMR analysis (Fig. 3). Thus, H-13 was selected as a target signal for the quantitative ^{1}H-NMR.

Determination of purity of protoberberine alkaloid reagents

Through the qHNMR approach, absolute quantitative determination of organic molecules can be attained by comparing the signal intensity of a particular proton of the molecule to that of CRMs such as *p*-toluenesufonic acid, potassium hydrogen phthalate, dimethylsulfone, and benzoic acid. However, the signals from these compounds sometimes overlap the signals from the target compounds. We used hexamethyldisilane (HMD) as a qHNMR internal standard because the proton signal of HMD appears at 0 ppm, well separated from any proton of the target compounds [11]. HMD is less volatile and can be more readily

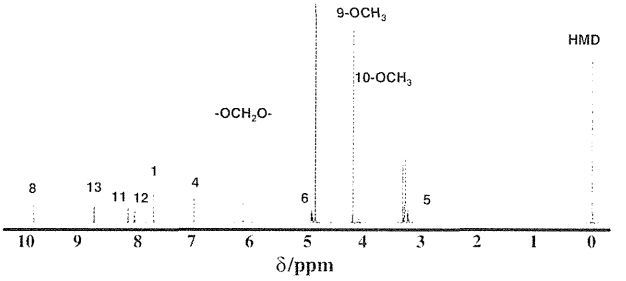

Fig. 2 A ^{1}H-NMR spectrum of berberine.The spectrum was recorded in methanol-d_4 containing hexamethyldisilane (HMD) as an internal standard. The position of the proton corresponding to each signal is shown in the spectrum

Table 1 Relative intensities of proton signals from berberine to that from HMD

Target signal (ppm)		Relative intensity[a] (% of HMD)	Target signal (ppm)		Relative intensity[a] (% of HMD)
H-1	7.62 s	37.5 ± 0.5	H-13	8.66 s	37.2 ± 0.4
H-4	6.92 s	37.0 ± 0.4	$-OCH_2O-$	6.06 s	37.0 ± 0.4
H-8	9.72 s	37.5 ± 0.4	$-OCH_3$	4.15 s	37.6 ± 0.4
H-11	8.07 d	37.5 ± 0.4	$-OCH_3$	4.06 s	37.7 ± 0.5
H-12	7.95 d	37.5 ± 0.5			

s Singlet, *d* doublet

[a] Average ± standard deviation from triplicate measurements

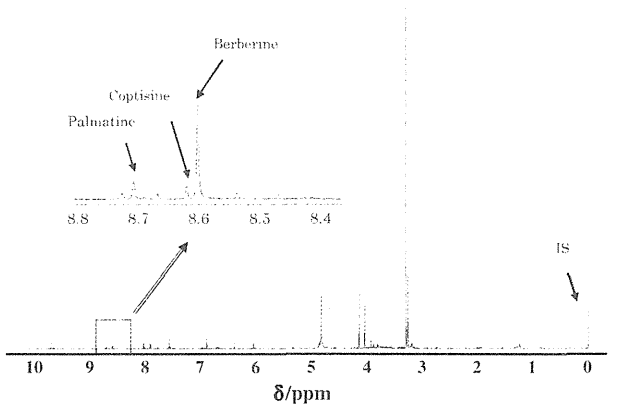

Fig. 3 A typical ^{1}H-NMR spectrum of the methanol extract of Coptidis Rhizoma. The *inset* shows H-13 signals from berberine, palmatine, and coptisine

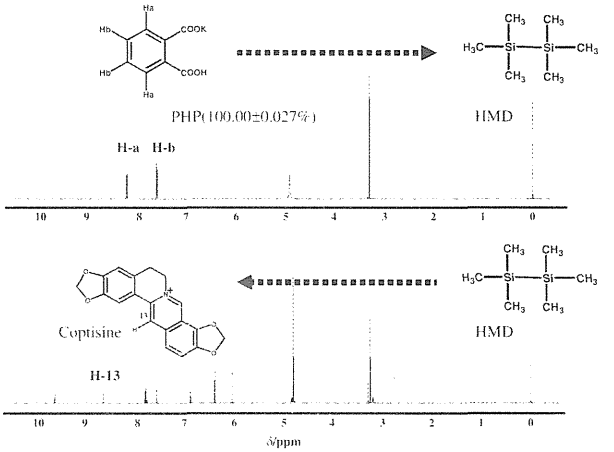

Fig. 4 A schematic diagram illustrating a basic principle of purity determination by ^{1}H-NMR spectroscopy. Purity of hexamethyldisilane (HMD) is first confirmed based on the signal intensity at 0 ppm relative to the signal intensity of potassium hydrogen phthalate (PHP) of certified reference material (CRM) grade at 8.20 or 7.53 ppm. Then the purity of the alkaloid (e.g., coptisine) is determined based on the ratio of signal intensity at 8.62 ppm (H-13) to that at 0 ppm

weighed than trimethylsilane (TMS), a commonly used ^{1}H-NMR standard.

Protoberberine alkaloid reagent purchased from various commercial sources was dissolved in 1.00 ml of the qHNMR standard solution containing HMD. The concentration of HMD was accurately determined by comparing the signal integral at 0 ppm of HMD with the intensities of aromatic hydrogen signals of PHP of CRM grade at 7.73 ppm and/or 8.20 ppm. The purity of CRM-grade PHP is 100.00 ± 0.027% and the value is traceable to SI units. Thus, the present qHNMR approach leads to absolute quantification of protoberberine alkaloid reagents with SI-traceability based on the CRM substrate (PHP), as shown schematically in Fig. 4. The qHNMR-determined purity of the reagent was not consistent with the purity described by the manufacturer except for Ber-3, the Japanese Pharmacopoeia Reference Standard (Table 2).

To confirm the qHNMR-determined purities of the reagents, the alkaloid solution was subjected to HPLC analysis and the peak area corresponding to each alkaloid was compared with the qHNMR-determined purity of the reagent (Fig. 5). The relative peak areas among three berberine alkaloid reagents and those between two palmatine reagents were quite consistent with the relative purities of these alkaloids determined by the qHNMR method, suggesting that the qHNMR-determined purity of the reagent reflects the true purity of the compound better than the purity claimed by the manufacturer.

It is interesting to note that Ber-2, distributed as a standard for crude drug evaluation, exhibited much lower purity than Ber-1, distributed as a reagent grade chemical. The purity of a palmatine reagent (Pal-1) was 15% less than that claimed by the manufacturer. In contrast, the purity described by the manufacturer of the berberine reagent (Ber-3) distributed as the Japanese Pharmacopoeia Reference Standard was identical with that determined by the qHNMR method. These results clearly indicate that protoberberine alkaloids contents in the crude drugs determined by HPLC may not be reliable

Table 2 Purity of the protoberberine alkaloid reagents determined by the qHNMR method

Alkaloid	Code	Description	Purity described by manufacturer (%)	Purity determined by qHNMR (%)[a]
Berberine chloride	Ber-1	Reagent grade	97	94.3 ± 3.8
	Ber-2	Standard for crude drug analysis	>99	90.6 ± 3.7
	Ber-3	Japanese Pharmacopoeia Reference Standard	90.6	90.7 ± 2.4
Palmatine chloride	Pal-1	Reagent grade	97	81.8 ± 1.5
	Pal-2	Standard for crude drug analysis	>99	98.7 ± 6.9
Coptisine chloride	Cop-1	Standard for crude drug analysis	[b]	91.0 ± 4.4

[a] Average ± standard deviation from triplicate measurements

[b] Described as "one spot by TLC"

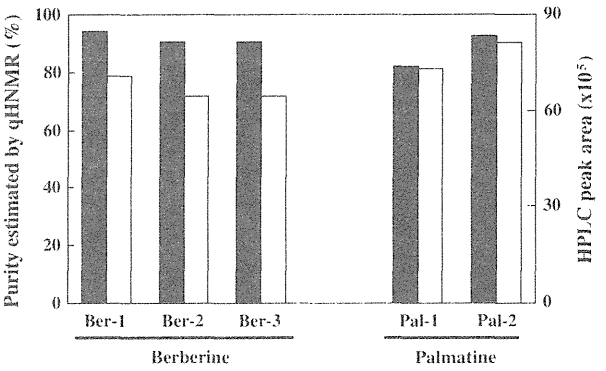

Fig. 5 Comparison of the purities of the reagents determined by qHNMR (*closed columns*) with the HPLC peak areas of the reagents (*open columns*). A 10 µl aliquot of the alkaloid solution (0.100 mg/ml in methanol) was injected into the HPLC column and elution was monitored at 345 nm. For information on the reagents, see Table 2

unless the purities of the standard compounds used for constructing calibration curves are strictly determined, and may be over-estimated because the real purity of the standard reagent is lower than that described by the manufacturer. Limited amounts of the Japanese Pharmacopoeia Reference Standard are available at present, and it is desirable that more natural product reagents of the Japanese Pharmacopoeia Reference Standard are provided to the market.

Determination of protoberberine alkaloid contents in Coptidis Rhizoma by qHNMR

The qHNMR method was applied to quantitative determination of berberine, palmatine, and coptisine in Coptidis Rhizoma. Five samples of Coptidis Rhizoma were obtained in markets, and their protoberberine alkaloid contents were estimated by comparing the intensities of H-13 signals of protoberberine alkaloids with the signal intensity of HMD. For all the Coptidis Rhizoma samples, the signals derived from H-13 of berberine, palmatine, and coptisine were clearly detected with no interfering signals, and can be distinguished each other (Fig. 3). The alkaloid contents varied in the ranges 5.11–6.58% for berberine, 1.32–1.69% for palmatine, and 0.95–1.63% for coptisine (Table 3). The recovery rate of the assay was determined to be 93.9% by spiking the berberine chloride (Ber-1) to the extract. We also determined the alkaloid contents of the crude drug samples by HPLC. The alkaloid contents by HPLC analysis were corrected based on the qHNMR-determined purities of the alkaloid reagents used for constructing the calibration lines. The alkaloid contents determined by the two methods were generally consistent each other. The coefficients of correlation were 0.888 for berberine and palmatine, and 0.808 for coptisine.

Quantitative determination of protoberberine alkaloids in Coptidis Rhizoma has been extensively investigated by using various methods including TLC densitometry [13], HPLC [14], UPLC [15], and capillary electrophoresis [16, 17]. The estimated alkaloid contents are in the ranges 5–8% for berberine and 0.5–2.5% for palmatine and coptisine. The results obtained in the present qHNMR method were comparable to those of previously described data.

Recently Li et al. reported the application of ^{1}H-NMR to determination of protoberberine alkaloids in Rhizoma Coptidis [18]. They used anthracene as an internal standard, and determined the alkaloid contents by comparing the signal intensities of H-13 from the alkaloids with that at 8.40 ppm from anthracene. However, they did not determine the purity of the anthracene reagent. In addition, the proton signal from anthracene is present in the signal-crowded region and may interfere with the H-13 signals from protoberberine alkaloids. These points make their results less reliable.

The present paper indicates that the purities of protoberberine alkaloid reagents can be accurately estimated based on the ratio of the signal integral corresponding to H-13 of protoberberine alkaloid to that of HMD. The HMD concentration is pre-determined based on the ratio of signal intensity to PHP of CRM grade to give SI-traceability to the qHNMR analysis. Although the present qHNMR approach can be applicable to direct measurement of protoberberine alkaloid contents in the crude drugs, the sensitivity of detection is relatively low (0.1 mg/ml). Therefore, it may be practical to apply HPLC determination in which the calibration curves of protoberberine

Table 3 Protoberberine alkaloid contents in Coptidis Rhizoma obtained in markets

Sample	Berberine content (%)[a]		Palmatine content (%)[a]		Coptisine content (%)[a]	
	by NMR	by HPLC	by NMR	by HPLC	by NMR	by HPLC
1	5.47 ± 0.33	5.19 ± 0.40	1.32 ± 0.11	1.16 ± 0.08	0.95 ± 0.12	0.77 ± 0.06
2	5.26 ± 0.12	5.45 ± 0.05	1.46 ± 0.04	1.31 ± 0.01	1.51 ± 0.05	1.37 ± 0.02
3	5.11 ± 0.62	5.26 ± 0.33	1.51 ± 0.20	1.36 ± 0.08	1.38 ± 0.19	1.28 ± 0.12
4	6.26 ± 0.07	5.84 ± 0.18	1.61 ± 0.03	1.34 ± 0.04	1.63 ± 0.02	1.23 ± 0.02
5	6.58 ± 0.06	6.91 ± 0.19	1.69 ± 0.02	1.67 ± 0.04	1.31 ± 0.06	1.34 ± 0.03

[a] Average ± standard deviation from triplicate measurements

alkaloids are constructed using the reagent whose purity is estimated by the qHNMR method.

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Scientific Research

Analysis of Residual Solvents in Annatto Extracts Using a Static Headspace Gas Chromatography Method

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ABSTRACT

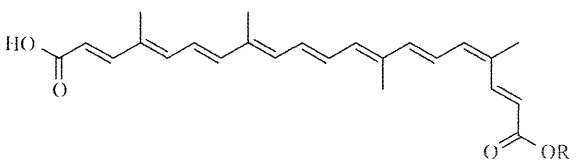
An analytical method for the quantification of residual solvents in annatto extracts, natural food colorants, was established using a static headspace gas chromatography (HSGC) coupled with a flame ionization detector (FID). As a sample diluent in a headspace sampling, dimethylformamide (DMF) was selected owing to its high capacity for dissolving both bixin-based and norbixin-based annatto extracts. The quantification of residual solvents was performed using the external standard method. The linearity of the calibration curves was assured with relative coefficients (R^2) that were greater than 0.999. The recoveries of all standard solvents spiked in the annatto extracts were in the range from 95.1% to 107.1% to verify the accuracy and the relative standard deviation (RSD%) values (n = 3) were in the range from 0.57% to 3.31%. The quantification limits (QL) were sufficiently lower than the limits specified by Joint FAO/WHO Expert Committee on Food Additives (JECFA). With the established HSGC method, six residual solvents (methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane) in 23 commercial annatto-extract products that consist of seven bixin-based and 16 norbixin-based products were quantified. The levels of residual ethyl acetate and hexane in all products were lower than the specified limits of JECFA. However, three samples of bixin-based products showed higher levels of residual 2-propanol (approximately 313.9 - 427.7 ppm) than the specified limit. Other bixin products also showed higher concentrations of residual methanol (approximately 166.6 - 394.7 ppm) and residual acetone (approximately 75.2 - 179.8 ppm) than the limits of JECFA. In the case of norbixin-based products, nine samples showed higher levels of residual acetone (approximately 42.6 - 139.5 ppm) than the limits of JECFA. This is the first survey of residual solvents in annatto extracts using the validated HSGC method.

Keywords: Annatto Extracts; Bixin; Norbixin; Headspace Gas Chromatography; Residual Solvents

1. Introduction

Annatto extracts are natural yellowish-orange colorants prepared from the seeds of the tropical tree *Bixa orellana* L. [1]. Annatto extracts have good heat stability during food processing and have been used in many countries to give a yellow-to-red color to foods, especially dairy products such as butter and cheese [1]. The principle pigments of annatto extracts are apocarotenoids, bixin, and norbixin [2] (**Figure 1**). Bixin is a major natural carotenoid contained in the outer layer of the seed and is a monomethylester of norbixin, a polyenedicarboxylic acid (**Figure 1**). Bixin is lipophilic in nature and therefore highly insoluble in water. Therefore, crude extracts containing bixin are often hydrolyzed with an alkali to prepare norbixin in order to increase the water solubility of the pigments [1,2]. The salts of norbixin obtained by alkali hydrolysis are soluble in water, however, the protonated form of norbixin formed after acid-precipitation purification becomes insoluble (**Figure 2**).

In 2007, the 39th Codex Committee on Food Additives (CCFA) divided annatto extracts into two classes on the basis of the principle pigments: bixin-based (INS No.160b (1)) and norbixin-based (INS No.160b (2)) [3]. In contrast, in the previous year, the 67th Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed to classify annatto extracts into five categories on the basis of the manufacturing process in addition to the principle



bixin: R = CH_3

norbixin: R = H

Figure 1. Structures of bixin and norbixin.

*Corresponding author.

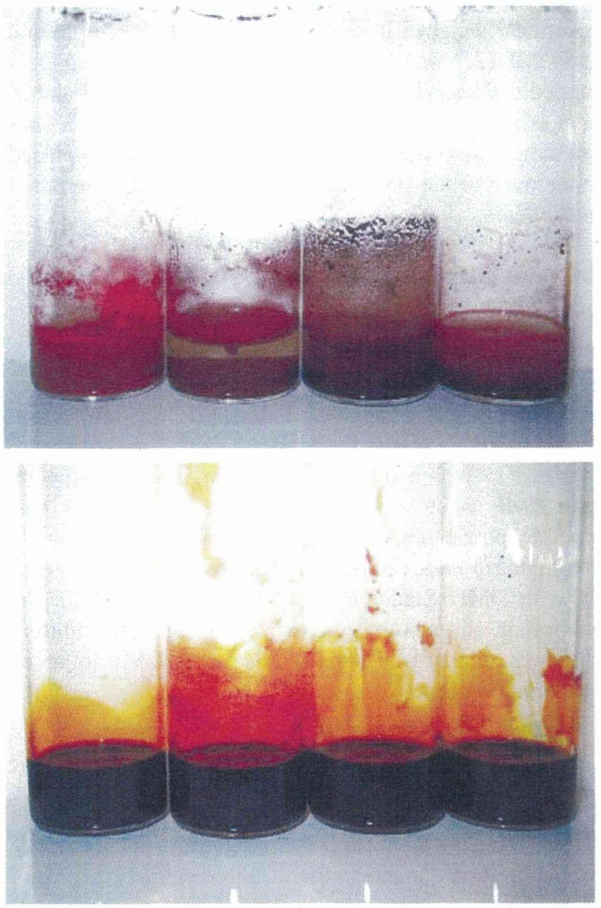


Figure 2. Solubility of annatto extracts in water (upper) and DMF (bottom). The two left bottles are bixin-based products (bix3 and bix4), and the two right bottles are norbixin-based products (nbx4 and nbx8).

pigments [4]: Annatto B (solvent-extracted bixin), Annatto E (aqueous-processed bixin), Annatto C (solvent-extracted norbixin), Annatto F (alkali-processed norbixin, acid precipitated), and Annatto G (alkali-processed norbixin, not acid precipitated). For the production of solvent-extracted bixin and norbixin, *i.e.* Annatto B and C, respectively, JECFA permitted the use of six food grade solvents as the extraction solvent: methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane, and specified residue limits for each solvent in the final products (**Table 1**) [4]. The European Food Safety Authority (EFSA) also specified limits for four residual solvents (methanol, acetone, hexane, and dichloromethane) against solvent-extracted bixin and norbixin (**Table 1**) [5]. The Code of Federal Regulations (CFR) of the United States does not classify annatto extracts, but states that annatto extracts should contain no more than six solvents (methanol, 2-propanol, acetone, hexane, dichloromethane, and trichloroethylene), residues of which are permitted in the corresponding spice oleoresins (**Table 1**) [6]. In Japan, annatto extracts are allowed to be used

Table 1. Specified limits (ppm) of residual solvents in JECFA, EU, and USA guidelines.

solvent	JECFA	EU	USA
methanol	50	*50	50
ethanol	*50	-	-
2-propanol	*50	-	50
acetone	30	*50	30
hexane	25	*50	25
ethyl acetate	*50	-	-
dichloromethane	-	10	*30
trichloroethylene	-	-	*30

*individually or in combination.

as one of the existing food additives, but have not yet been listed in the Japanese Standards of Food Additives. Therefore, specified limits of residual solvents are also not established.

JECFA designated static headspace gas chromatography (HSGC) with a flame ionization detector (FID) as the general analytical method for the determination of residual solvents in food additives [7]. The static headspace (HS) sampling method has more appropriate sensitivity than the direct injection method because it can clearly separate volatile analytes from the sample matrix and effectively concentrate them. Therefore, this method results in less complex sample preparation, decreased instrument contamination, and increased gas chromatography (GC) column life. The HS sampling process is based on thermostatic partitioning of volatile compounds between the sample diluent and the gas phase in a sealed vial. Therefore, the selection of the sample diluent is a critical factor affecting the precision of the HSGC analysis. A good sample diluent for HS samplings should have a high stability, high boiling point, and high capability for dissolving large amount of samples [8]. In the general HSGC analytical method of JECFA specification, two solvents are listed as sample diluents: one is water (Method I) and the other is methanol (Method II) [7]. Water is a good diluent for HSGC because it offers a very low partition coefficient for analytes and has a low vapor pressure. However, annatto extracts (bixin and the protonated form of norbixin) are insoluble in water as described above (**Figure 2**). Although annatto extracts are soluble in methanol, residual methanol in the sample cannot be determined if methanol is used as the diluent. Consequently, the general JECFA method is inapplicable for annatto extracts.

The US Pharmacopeia (USP) General Chapter Residual solvents (467), based on the European Pharmacopeia (EP) procedure [9], presented an HSGC method for testing procedures for residual solvents in pharmaceuticals. Interestingly, the procedures can be divided into two categories on the basis of the solubility of the samples: water-soluble materials and water-insoluble materials [10,11].

For the water-insoluble procedure, polar organic solvents, such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), are designated as the sample diluent. These polar organic solvents have a higher boiling point than water and a high capacity for dissolving a wide range of organic substances. The HSGC method using DMF as the sample diluent has a higher precision than when water is used in the quantification of residual solvents in drug substances [12]. In the JECFA specification of bixin-based products, DMF is designated as the solvent to dissolve and dilute samples in high-performance liquid chromatography (HPLC) analysis for impurities [9]. Therefore, we thought that DMF could become a suitable sample diluent for HSGC analysis of annatto extracts.

There are a few reports on the determination of residual solvents in annatto extracts [13-15]. In these reports, some samples showed high levels of residual methanol. However, most samples analyzed in these reports were not technical products (powder form) but liquid preparations. In addition, the principle pigments of the samples were not described. To our best knowledge, there is no information about residual solvents in annatto extracts after the specification was issued by the 67th JECFA. In order to secure the safety and assure good manufacture practices (GMP) of commercial food additives, a precise quantification of residual solvents is essential. In the present study, we first developed a reliable and analytical method using HSGC for quantification of residual solvents in annatto extracts, and then precisely determined the levels of six residual solvents specified by JECFA in 23 commercial annatto extracts, including both bixin-based and norbixin-based products.

2. Experimental

2.1. Samples

Twenty-three commercial products containing annatto extracts were collected from Japanese food additives manufacturers in 2011. The samples consisted of six bixin-based products (bix1-6) and 17 norbixin-based products (nbx1-17). All samples were red or reddish purple powder and were stored at −20°C until analyzed.

2.2. Chemical Reagents

Organic solvents (methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane) with ≥98% purity were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). DMF was used for residual solvent analyses and purchased from Kanto Chemical. Co., Inc. (Tokyo, Japan). Water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA).

2.3. Sample Preparation

A quantity (200 mg) of each annatto extract sample was accurately weighed in an Agilent 20 mL HS sample vial and DMF (2.5 mL) was added. The vial was immediately capped and sealed with a Teflon-lined septum and aluminum crimp cap, and then mixed thoroughly until the entire sample was dissolved. Then, the capped vial was placed in the oven of the HS sampler. All samples were prepared in triplicate.

2.4. Standard Solutions and Calibration Curves

A stock standard solution for each solvent was prepared as follows. Each 250 μL of methanol, ethanol, 2-propanol, and ethyl acetate, and each 150 μL of acetone and hexane was pipetted into a volumetric flask (20 mL) into which DMF (10 mL) had previously been added. The flasks were weighed to within 0.01 mg and then filled to capacity with DMF. A stock standard mixture solution was prepared by placing each stock standard solution (3.0 mL) in a volumetric flask (20 mL) and filling the flask to capacity with DMF. Standard mixture solutions used for the calibration curve were prepared by sequentially diluting the stock standard mixture solution with DMF to seven concentration levels.

For the HSGC analysis, the standard mixture solution (0.1 mL) was pipetted into an Agilent 20 mL headspace HS sample vial and DMF (2.4 mL) was added to the vial. The vial was immediately capped and sealed as mentioned above. The samples were prepared in triplicate. To establish calibration equations, the mean peak areas (n = 3) of standard solvents observed by HSGC analysis were plotted against concentration. External calibration curves were established over seven datapoints covering a concentration range of approximately 1.0 - 700 ppm for methanol, ethanol, 2-propanol, and ethyl acetate and approximately 0.5 - 350 ppm for acetone and hexane. All the solvent concentrations were calculated on the basis of the 200 mg annatto extracts being dissolved in 2.5 mL of DMF. The final concentration of each standard solution used for the calibration curve is shown in **Table 2**.

2.5. Headspace Gas Chromatography Procedure

An Agilent 6890 N GC equipped with an FID and a 7694

Table 2. Retention time and linearity of six standards solvents.

solvent	RT (min)	Range (ppm)	R^2
methanol	5.148	0.9 - 679.2	0.9997
ethanol	6.452	1.0 - 726.6	0.9992
2-propanol	7.542	1.0 - 740.5	0.9996
acetone	8.068	0.5 - 369.5	0.9998
hexane	9.608	0.4 - 327.8	0.9999
ethyl acetate	12.713	1.0 - 717.4	0.9998

K-special HS sampler was used for the experiments. The GC column was a GL Sciences AQUATIC-2 (25% phenyl/75% methyl polysiloxane)—fused silica capillary column: length, 60 m; internal diameter, 0.25 mm; film thickness, 1.40 mm (Part No. 123-1334, Serial No. US1613334-H). The initial temperature of the column oven was 40°C, and this was maintained for 5 min, then raised at a rate of 4°C/min to 92°C and maintained for 2 min, and then raised at a rate of 40°C/min to 230°C. The injection temperature was 250°C, and the FID detector temperature was 260°C. Helium at 205 kPa was used as the carrier gas (constant flow, 1.8 mL/min) and the split ratio was 25:1. The headspace HS was sampled as follows: the vial was maintained at 60°C for 20 min with continuous agitation. The size of injection loop was 3 mL. The needle temperature was 100°C and the transfer line temperature was 120°C.

2.6. Recovery

Recovery rates of standard solutions for three selected samples (bix1, bix4, and nbx8) were calculated using the standard addition method. The standard mixture solution spiked in the sample was individually prepared as follows. Bix1: stock standard solutions of methanol (0.2 mL), ethanol (0.2 mL), 2-propanol (1.4 mL), acetone (0.4 mL), ethyl acetate (0.2 mL), and hexane (0.2 mL) were pipetted into a volumetric flask (20 mL) and filled with DMF. Bix4: stock solutions of methanol (1.4 mL), ethanol (0.2 mL), 2-propanol (0.2 mL), acetone (1.4 mL), ethyl acetate (0.2 mL), and hexane (0.2 mL) were pipetted into a volumetric flask (20 mL) and filled with DMF. Nbx8: stock solutions of methanol (0.2 mL), ethanol (0.2 mL), 2-propanol (0.2 mL), acetone (0.8 mL), ethyl acetate (0.2 mL), and hexane (0.2 mL) were pipette in a volumetric flask (20 mL) and filled with DMF. The final concentration of each spiked standard solution was shown in **Table 3**. The samples (200 mg) were separately weighed in a HS vial (20 mL), dissolved in DMF (2.4 mL), and spiked with the standard mixture solution (0.1 mL) prepared for each sample. Quantitative analysis was performed by the HSGC procedure as described above. The recovery rate was calculated by comparing the amount of standard in the spiked sample with the amount in the non-spiked annatto extract sample (control). Each analysis was performed in triplicate.

Table 3. Quantification limit (QL) and detection limit (DL) and precision of six solvents.

solvent	QL (ppm)	DL (ppm)	Precision at WL* (RSD%, n = 3)	Precision at LL** (RSD%, n = 3)
methanol	12.93	3.53	2.79	8.98
ethanol	13.22	3.61	2.69	4.61
2-propanol	14.25	3.98	3.31	4.14
acetone	3.64	0.92	0.82	4.79
hexane	0.30	0.01	0.57	0.75
ethyl acetate	6.23	1.62	0.68	4.89

*working concentration level (20 - 50 ppm); **low concentration level (4 - 10 ppm).

3. Results and Discussion

3.1. Headspace Gas Chromatography Method

As the sample diluent for HSGC analysis, DMF was selected owing its high boiling point and high capacity for dissolving organic compounds. As expected, DMF was able to dissolve both bixin-based and norbixin-based products, while water, designated as a sample diluent by JECFA, was unable to dissolve either product (**Figure 2**). The equilibration temperature for HS sampling was set at 60°C, because this is the JECFA-recommended temperature for the general HSGC method, and it was reported that bixin gradually degrades to several products at temperatures above 70°C [16]. The equilibration time for HS sampling was determined as 20 min on the basis of the saturation of peak areas of standard solutions on the gas chromatogram (data not shown). The established HSGC procedure using a capillary column AQUATIC-2 gave a good separation of six standard solvent peaks (methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane) on the chromatogram (**Figure 3**). Retention time for each solvent is shown in **Table 2**. To assess linearity, calibration curves of six solvents were constructed over a range of seven concentrations using standard mixture solutions. Good linearity was achieved over the concentration ranges of approximately 1.0 - 700 ppm for methanol, ethanol, 2-propanol, and ethyl acetate, and approximately 0.5 - 350 ppm for acetone and hexane (**Table 2**). The regression coefficients (R^2) for the curves of six solvents range from 0.9992 to 0.9999 (**Table 2**). The sensitivity of the HSGC method is presented as QL with a signal-noise ratio of 10:1, and detection limit (DL) with a signal-noise ratio of 3:1. The QL values of methanol, ethanol, and 2-propanol are evaluated in the range from 12.93 to 14.25 ppm and the values of other solvents ranged from 0.30 to 6.23 ppm (**Table 3**). Because the QL values of all solvents are satisfactorily lower than the specified limits required by the JECFA guideline, the results demonstrate that the established HSGC method is sufficiently sensitive for the quantification of residues of six solvents in annatto extracts. To assess the accuracy of the method, recovery rates for six solvents were calculated using the spike of a standard solution mixture to three samples (bix1, bix4, and nbx8). Each concentration of the spiked solvent standard was selected on the basis of the specified limits of JECFA (**Table 4**). When the concentrations of the residual solvent measured using the HSGC method significantly exceeded the specified limits, the concentrations of spiked solvent

standards were selected on the basis of the measured concentrations, such as acetone (49.3 ppm) and 2-propanol (345.6 ppm) in bix1, methanol (317.0 ppm) and acetone (172.4 ppm) in bix4, and acetone (98.5 ppm) in nbx8 (**Table 4**). When the standard mixture solution was spiked at defined amounts in each sample prior to quantitative analysis, the recovery rates of the spiked standards in all samples were within the range of 95.0% - 109.7% during HSGC analysis (**Table 4**). Good recoveries clearly revealed that interference from the sample matrix should not have a significant impact on this HSGC method. Therefore, we considered that the external standard method was applied for the determination of residual solvents in annatto extracts. The precision of the method was tested by multiple injection (n = 3) of the standard mixture at both working-concentration level (WL, 20 - 50 ppm) on the basis of the specified limits of JECFA, and a lower concentration level (LL, 4 - 10 ppm) on the basis of the QL values. The RSD values were in the range 0.57% - 3.31% at the WL and 0.75% - 8.98% at the LL (**Table 3**).

These results clearly demonstrate that the developed HSGC method has acceptable linearity, accuracy, and precision, and is a reliable method for the accurate quantitative determination of residual solvents in annatto extracts.

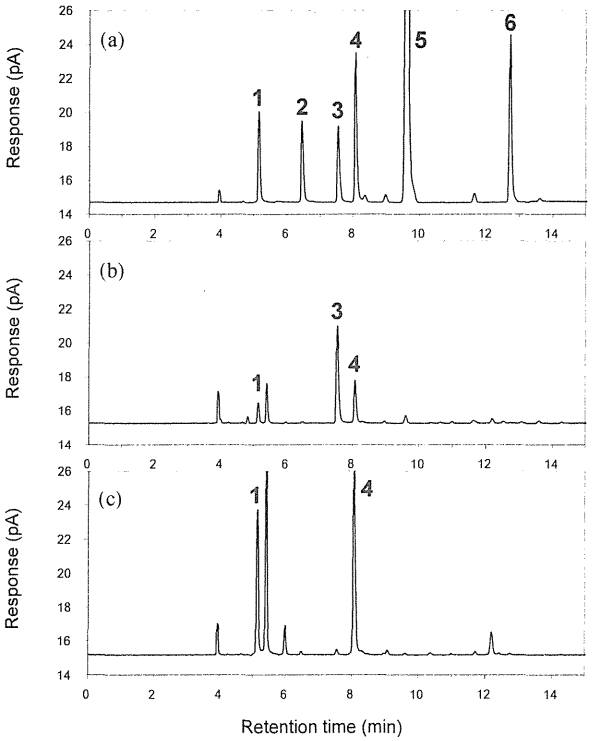


Figure 3. HSGC charts of (a) standard mixture; (b) bix1; and (c) bix4. Peak identities and concentrations in the standard mixture (a) are as follows: methanol (1, 270 ppm), ethanol (2, 290 ppm), 2-propanol (3, 300 ppm), acetone (4, 150 ppm), hexane (5, 130 ppm), ethyl acetate (6, 290 ppm).

3.2. Residual Solvents in Bixin-Based Products

Using the established HSGC method, the residual solvents in 23 annatto extract products were precisely quantified (**Table 5**). Representative chromatograms of bixin-based products (bix1 and bix4) are shown in **Figure 3**. In six bixin-based products, three samples (bix1-3) showed high concentrations of residual 2-propanol (approximately 313.9 - 427.7 ppm), which were much higher than the specified limit of JECFA (50 ppm). Because these samples were produced by the same manufacture, we presumed that the detected 2-propanol was likely to be a residue of the solvent used in the manufacturing process. Although other bixin-based samples (bix4-6) showed a lower concentration of residual 2-propanol, concentrations of residual methanol (approximately 112.8 - 383.5 ppm) and residual acetone (approximately 73.4 - 180.1 ppm) higher than the JECFA limits (50 and 30 ppm, respectively) were quantified. These may also be residue of solvents used in the manufacturing process, although the methanol might be generated by hydrolysis of the methylester in bixin during storage. Scotter *et al.* reported the powdered bixin is more unstable than oleoresin bixin and gradually degrades in complex reactions, even in dark and cold conditions [1]. In fact, bix4 was stored for a few years before used in this study. In the case of the sample containing 75% (w/w) bixin, we estimated that the degradation of only 0.16% (w/w) of bixin could generate 100 ppm of methanol in the sample. As a similar example, Sato *et al.* reported that a natural food colorant, gardenia blue, which

Table 4. Recoveries of six solvents spiked in three samples

sample	bix1		bix4		nbx8	
solvent	spiked (ppm)	recovery (%)	spiked (ppm)	recovery (%)	spiked (ppm)	recovery (%)
methanol	45.3	96.5	317.0	102.8	45.3	102.7
ethanol	48.4	95.0	48.4	100.2	48.4	101.2
2-propanol	345.6	101.6	49.4	100.2	49.4	102.6
acetone	49.3	101.9	172.4	104.1	98.5	98.4
hexane	21.9	97.3	21.9	98.2	21.9	100.1
ethyl acetate	47.8	99.3	47.8	105.2	47.8	103.0

Table 5. Concentrations of residual solvents in 23 commercial products.

type	No.	Concentrations of residual solvents (ppm) and RSD (%)											
		methanol		ethanol		2-propanol		acetone		hexane		ethyl acetate	
bixin	bix1	49.7	(0.4)	nd		369.3	(1.2)	44.0	(2.0)	0.6	(1.4)	nd	
	bix2	38.2	(5.0)	nd		316.2	(1.0)	28.2	(3.7)	0.7	(9.9)	nd	
	bix3	35.0	(2.9)	nd		438.4	(2.8)	27.5	(1.7)	nd		nd	
	bix4	394.5	(4.7)	nd		nd		179.8	(5.0)	nd		nd	
	bix5	119.4	(1.1)	nd		nd		95.7	(1.8)	nd		nd	
	bix6	166.0	(0.7)	nd		nd		75.2	(0.2)	nd		nd	
norbixin	nbx1	nd		nd		nd		27.1	(2.4)	nd		nd	
	nbx2	nd		nd		nd		23.4	(2.2)	nd		nd	
	nbx3	nd		nd		nd		26.8	(4.2)	nd		nd	
	nbx4	41.1	(1.5)	nd		nd		106.0	(0.8)	nd		nd	
	nbx5	nd		nd		nd		102.7	(1.8)	nd		nd	
	nbx6	nd		nd		nd		45.0	(3.1)	nd		nd	
	nbx7	432.9	(1.9)	4375	(1.0)	nd		36.0	(1.8)	nd		11.5	(2.5)
	nbx8	23.8	(2.0)	nd		nd		98.8	(2.0)	nd		nd	
	nbx9	25.6	(1.2)	nd		nd		24.8	(0.2)	nd		nd	
	nbx10	51.2	(0.5)	nd		nd		19.3	(2.1)	nd		nd	
	nbx11	nd		nd		nd		44.2	(4.4)	nd		nd	
	nbx12	nd		nd		nd		33.6	(0.8)	nd		nd	
	nbx13	nd		nd		nd		25.1	(2.6)	nd		nd	
	nbx14	nd		nd		nd		nd		nd		nd	
	nbx15	nd		nd		nd		nd		nd		nd	
	nbx16	nd		nd		nd		42.6	(1.4)	nd		nd	
	nbx17	nd		26.0	(0.8)	29.8	(1.6)	139.5	(3.6)	nd		nd	

Data are means for three trials; nd = not determined.

contains methylester structures, showed a high concentration of residual methanol, and suggested that methanol could be generated by spontaneous hydrolysis of the methylester [17]. Based on this knowledge, the concentration limit of residual methanol in gardenia blue is set as 1000 ppm in Japanese Standards of Food Additives. It might be necessary to investigate the generation of methanol by the degradation of bixin during storage. The residue levels of other solvents (ethanol, ethyl acetate, and hexane) were lower than QL in bixin-based samples.

3.3. Residual Solvents in Norbixin-Based Products

The represented chromatograms of norbixin-based products were shown in **Figure 4** (nbx4, nbx7, and nbx14). In the case of norbixin-based samples, the levels of residual 2-propanol, hexane, and ethyl acetate were lower than the limits of JECFA. However, residual acetone was detected at a higher concentration than the JECFA limit (30 ppm) in nine samples (nbx4, 5, 6, 7, 8, 11, 12, 16, and 17). Although the origin of acetone in the norbixin-based products is not as clear as in the bixin-based products, it should be noted that residual acetone was determined in 15 out of 17 samples examined. Residual methanol in the norbixin- based samples was at a lower level than the specified limit, with the exception of nbx7. In nbx7, ethanol was also detected at high concentration (4375 ppm) in addition to residual methanol, suggesting that the results may be caused by imperfect purification in the manufacturing process.

4. Conclusion

In this study, a reliable HSGC method using DMF as sample diluent is established for the determination of residual solvents in annatto extracts. With the established method, six residual solvents (methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane) specified by JECFA were precisely determined in 23 commercial bixin-based and norbixin-based products. The results revealed that some bixin-based products contained a higher concentration of residual methanol and 2-propanol than the JECFA-specified limit (50 ppm). Furthermore, 13 samples showed a higher concentration of residual acetone than the specified JECFA limit (30 ppm). We would like to note that

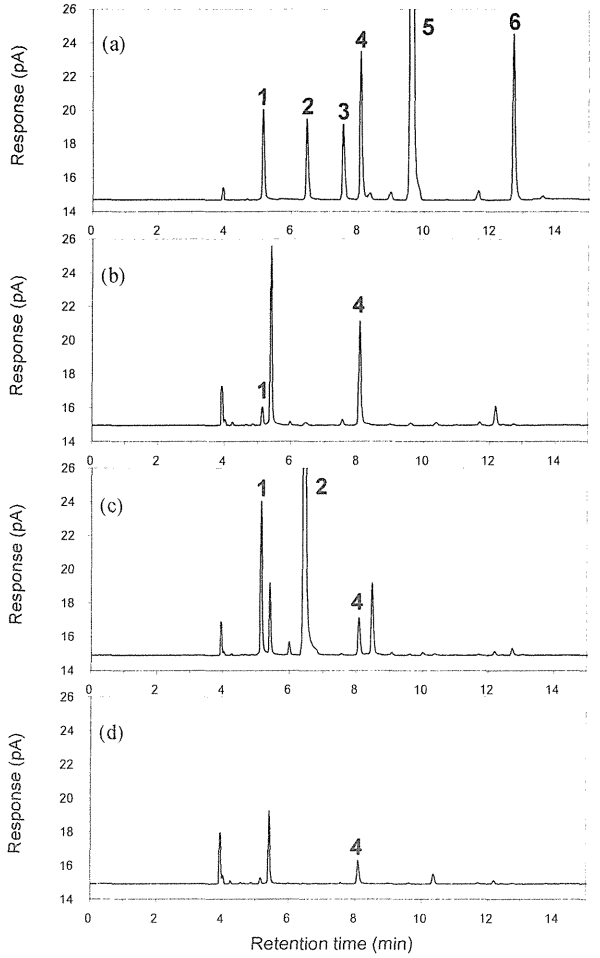


Figure 4. HSGC charts of (a) standard mixture; (b) nbx4; (c) nbx7; and (d) nbx14. Peak identities and concentrations in the standard mixture (a) are the same as in Figure 3.

all products used in this study were imported and were not processed by Japanese manufactures. In short, the findings of this study imply that annatto extracts distributed worldwide also contained a similar level of residual solvents as detected in this study. Based on the results, further investigation on worldwide products and a reevaluation of the current specified limits for residual solvents in annatto extracts is required.

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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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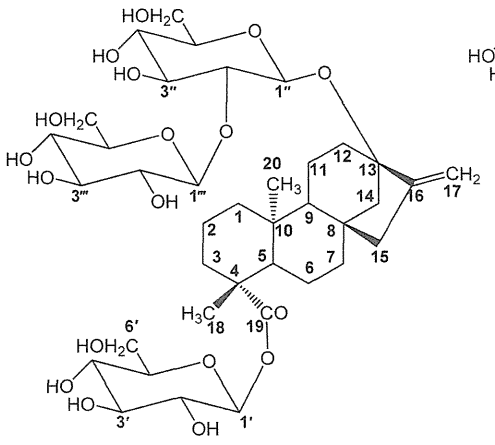
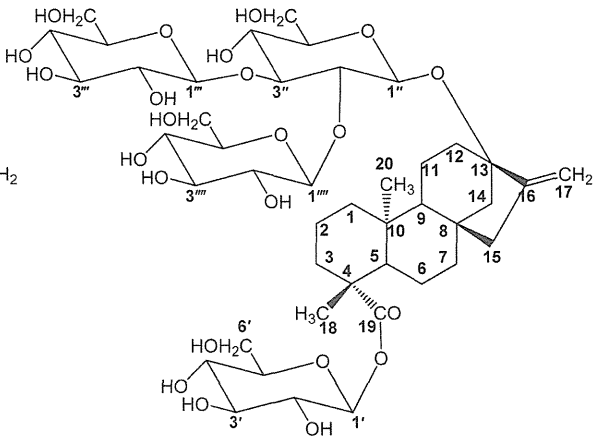
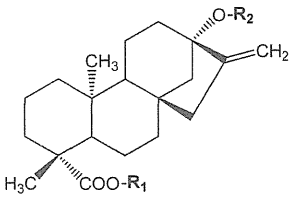


Stevioside



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_3×6) and 4.34 ppm (DEP, CH_2×2).

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_3×6); 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.

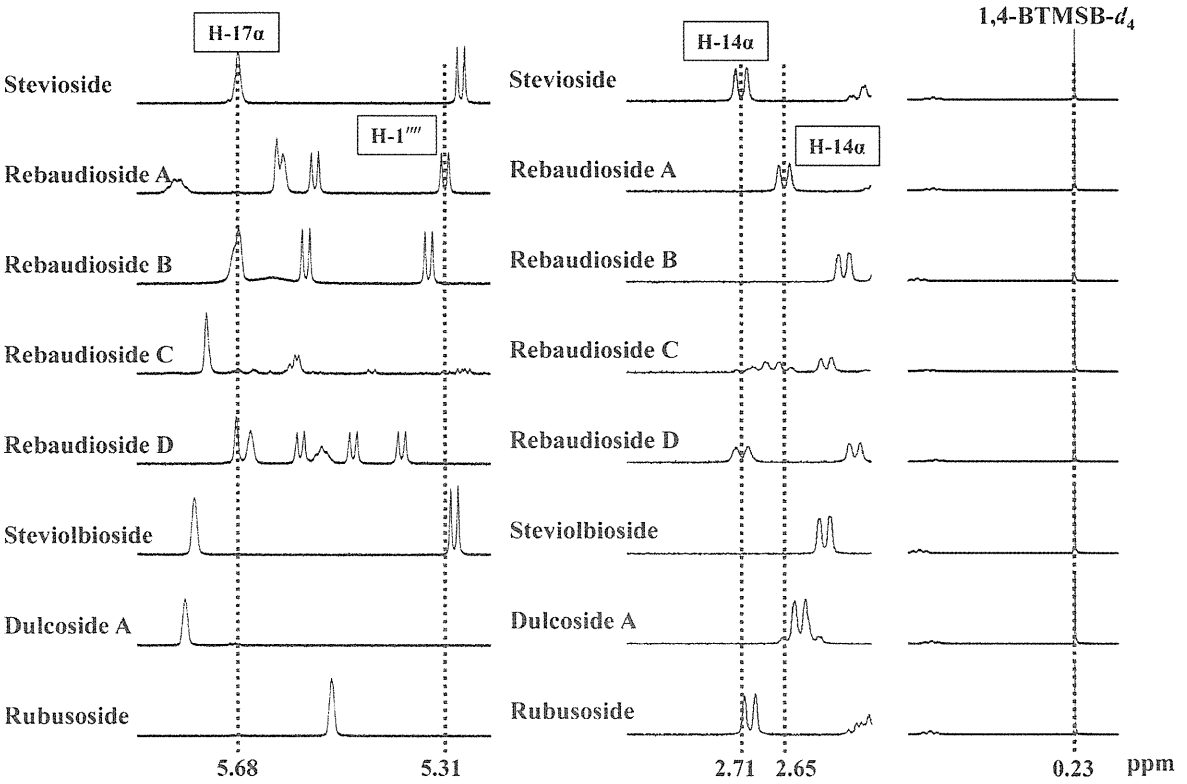


Fig. 3. qNMR Spectra of Steviol Glycosides

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.

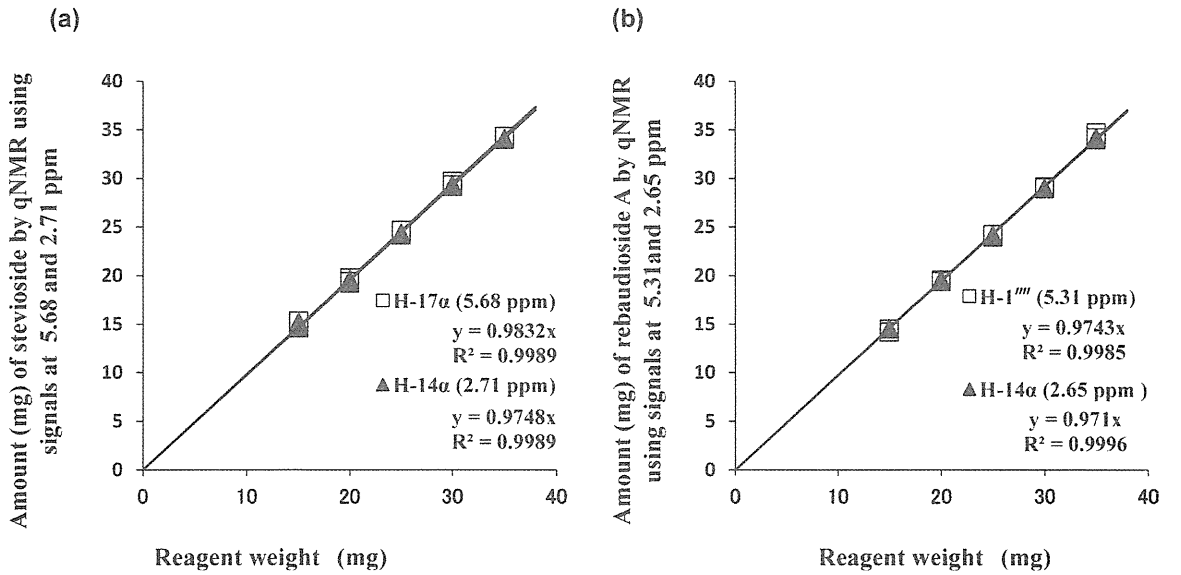


Fig. 4. Relationship between Reagent Weight and Amount of Stevioside or Rebaudioside A Determined by qNMR

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

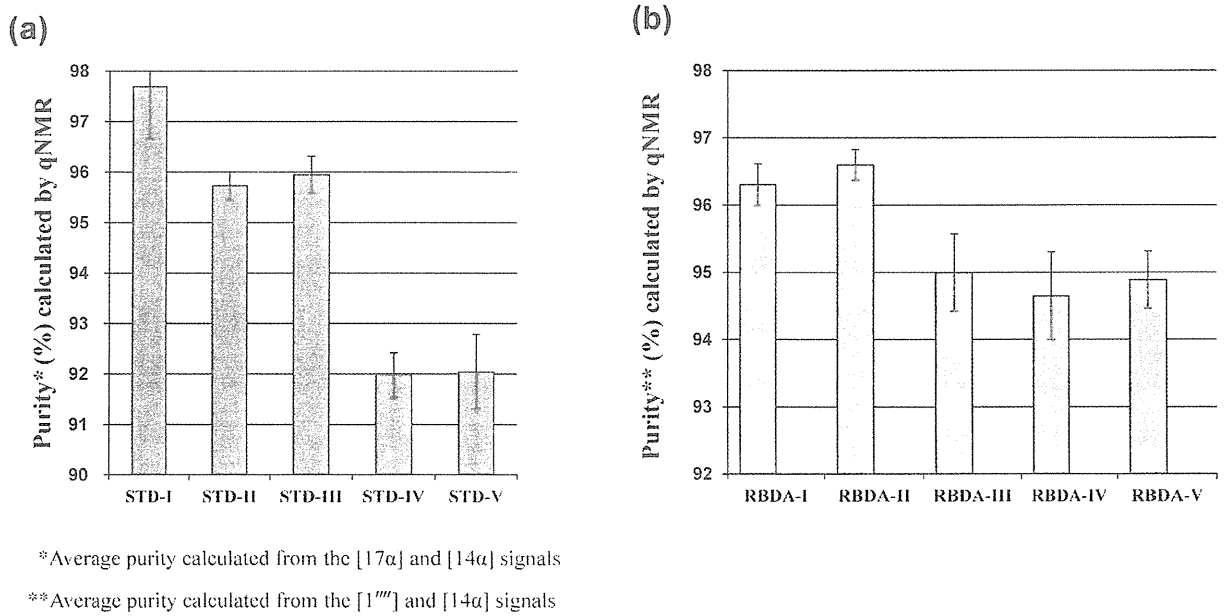
a) AV±S.D., n=3; the average of the values determined from the two signals, 5.68 ppm (H-17α) and 2.71 ppm (H-14α). *b*) AV±S.D., n=3; the purity value of STD-I determined by qNMR was used for calculation. *c*) AV±S.D., n=3; the purity value of STD-IV determined by qNMR was used for calculation.

(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

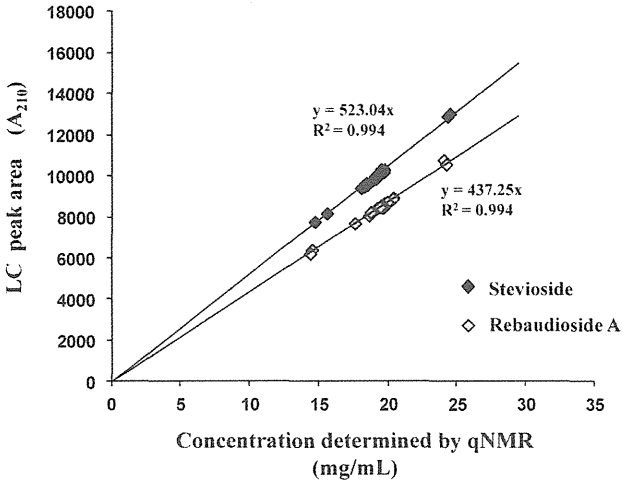


Fig. 6. Relationship between the Concentration of Stevioside or Rebaudioside A Determined by qNMR and Peak Areas at 210 nm

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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定量NMRによるトリコテセン系マイコトキシン類市販試薬の純度決定

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要　旨

定量NMR（qNMR）は測定対象化合物とは異なる基準物質との水素の原子数比から，あらゆる化合物の絶対量が算出可能である．計量学的に信頼性の高い純度が値付けられた1,4-BTMSB-d_4を基準物質とし，トリコテセン系マイコトキシン類の市販試薬5種7製品の純度を求めた結果，82.9-98.7%となり，製品表示値より10%前後下回るものが認められた．本研究より，マイコトキシン等の天然毒の定量用標品とされる希少な市販試薬の純度測定にqNMRが有効な手段となり得ることが示唆された．また，qNMRによる純度を試薬に標記することにより，定量値の国際整合性の確保が間接的に可能となると考えられる．

キーワード：トリコテセン，qNMR，純度，標準品

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緒　言

我が国は麦類の生育後期に降雨が多く，生産段階や貯蔵段階において赤かび病がまん延しやすい．麦類の赤かび病は麦類の品質低下や収穫量の減少の原因となるだけでなく，一部の赤かび病菌が産生するかび毒（マイコトキシン）により食品の安全性確保上の問題となっている．*Fusarium*属の一部の種より産生されるトリコテセン系マイコトキシンのデオキシニバレノール（Deoxynivalenol: DON）やニバレノール（Nivalenol: NIV）[1,2]は，食欲の減退，嘔吐，胃腸炎，下痢等の消化器系への症状や，免疫機能の抑制等の毒性を示す．日本ではNIVの産生菌の比率が高く，麦類の他，米やとうもろこし等の穀物の汚染が知られている．トリコテセン系マイコトキシンは，加工や調理工程においても完全に除去することは難しいため，農林水産省は，DONおよびNIVの実態調査やその低減技術に関する研究を進めており，食品の安全性向上のためのフードチェーンアプローチとして，生産段階における汚染防止・低減のための「麦類のデオキシニバレノール・ニバレノール汚染低減のための指針」をとりまとめている．さらに，DONについては，平成14年5月に小麦玄麦の暫定的な基準値が設定され，流通上の規制の強化が行われている[3]．

小麦等の穀類やそれらの加工品におけるトリコテセン系マイコトキシンの分析法としては，TLCをはじめ，HPLC/UV[4]，GC/ECD[5]，GC/MS[5]，ELISA[6]，LC/MS[7]，LC/MS/MS[8-12]等が報告されている．DONの公的な試験法（通知法）[13]には，「定性および定量試験として紫外分光光度型検出器付き高速液体クロマトグラフを用い，確認試験として液体クロマトグラフ・質量分析計又はガスクロ

マトグラフ・質量分析計を用いる」と規定されており，本試験法では，「DONを98%以上含むもの」を標準品として用いることとされている．しかし，国際単位系（International of Units: SI）に基づく計量トレーサビリティが確保された純度値を付与したDONの認証標準物質（certified reference material: CRM）等が流通していないため，現状では入手可能な市販試薬を便宜上用いることとなっている．一方，通常，市販試薬に表示された純度値は試薬メーカーが品質管理の目的で独自に値付けたHPLC等の面積百分率等の相対量を示しており，絶対量を示しているわけではない．したがって，厳密に言えば，クロマトグラフィーを利用した定量法では，DONの公的な試験法ですら，標準品の絶対量が不明である以上，得られた定量分析値が真値を示していない可能性があることを否定できない．このような背景から，科学的な根拠に基づいた有機化合物の標準品の純度決定法の開発が要求され続けてきた．

我々は，その基盤技術として核磁気共鳴（nuclear magnetic resonance: NMR）を用いた有機化合物の定量法（quantitative NMR: qNMR）の開発を行ってきた[14-22]．qNMRは，測定対象化合物の水素原子の数，言いかえれば物質量にトレーサブルな測定値を得ることが可能な方法であり，原理的にも「一次標準測定法」の中の「一次比率法」としての資格を有する分析法である．また，少量の測定対象の化合物の純度または含量の絶対量を非破壊で求められる方法であり，天然由来の貴重なサンプル等の純度決定等に関する問題を解消するものとして注目されている．本研究では，qNMRを応用し，かび毒のうち，DONおよびNIVを含め，トリコテセン系マイコトキシン市販試薬5種7製品の計量学的にトレーサブルな純度について検討したので報告する．

実験方法

1. 試料および試薬

15-アセチルデオキシニバレノール（15-acetyldeoxynivalenol ((3α,7α)-15-acetyloxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one): 15-Ac-DON）2製品（15-Ac-DON_Aおよび15-Ac-DON_B）はTrilogy Analytical Laboratory製およびBiopure製を，3-アセチルデオキシニバレノール（3-acetyldeoxynivalenol ((3α,7α)-3-acetyloxy-12,13-epoxy-7,15-dihydroxytrichothec-9-en-8-one): 3-Ac-DON）2製品（3-Ac-DON_Aおよび3-Ac-DON_B）は和光純薬工業株式会社製マイコトキシン試験用およびBiopure製を，デオキシニバレノール（deoxynivalenol ((3α,7α)-12,13-epoxy-3,7,15-trihydroxytrichothec-9-en-8-one): DON），ニバレノールn水和物（nivalenol n-hydrate ((3α,4β,7α)-12,13-epoxy-3,4,7,15-tetrahydroxytrichothec-9-en-8-one n-hydrate): NIV），T-2トキシン（T-2 toxin ((3α,4β,8α)-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol 4,15-diacetate 8-(3-methylbutanoate)): T-2）は和光純薬工業株式会社製マイコトキシン試験用を用いた．1,4-ビストリメチルシリルベンゼン-d_4（1,4-bis(trimethylsilyl)benzene-d_4: 1,4-BTMSB-d_4）は和光純薬工業株式会社製のSIトレーサビリティが証明された純度99.8%のqNMR用基準物質を，重アセトニトリル（acetonitrile-d_3）はIsotec製（99.8 atom % D）を用いた．

2. 装置

核磁気共鳴装置（NMR）はオートサンプラー付きJNM-ECA600（600 MHz）（日本電子株式会社（現：株式会社JEOL RESONANCE）製）を使用した．qNMRのケミカルシフト値は1,4-BTMSB-d_4を基準シグナル（0 ppm）とし，δ値をppm単位で表した．

ウルトラミクロ天秤はXP2U（メトラートレド製）を使用した．試料の秤量値は，最小目盛0.0001