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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

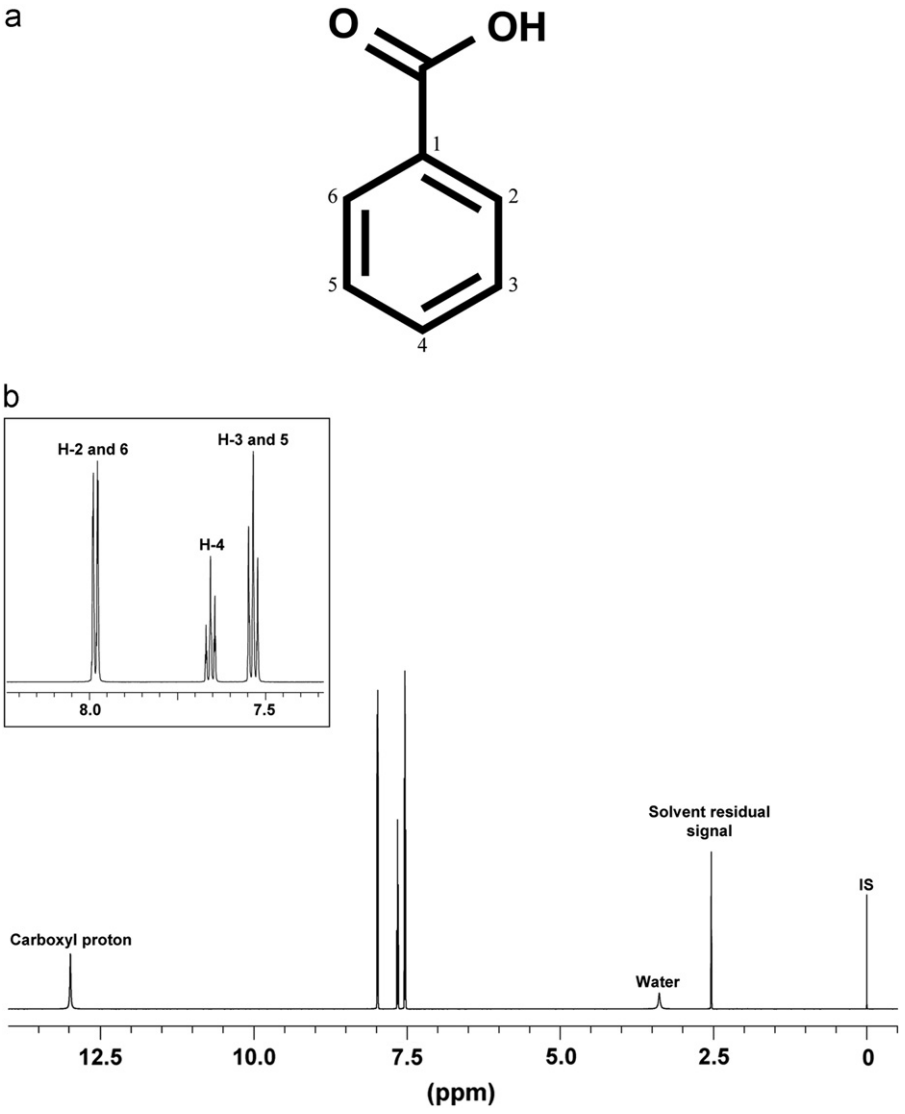

Fig. 1. Chemical structure of BA (a) and ^{1}H NMR spectrum of BA reagent in DMSO-d_6 containing DSS-d_6 (b). IS, internal standard (DSS-d_6).

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).

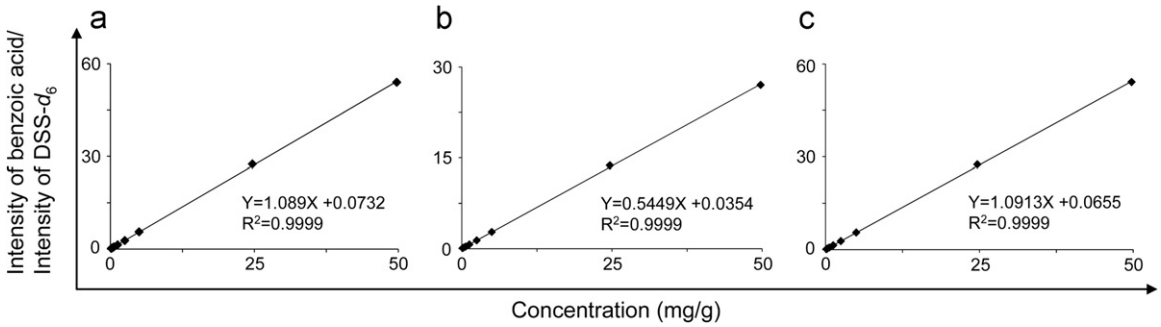

Fig. 2. Relationship between BA concentration and integral ratio of BA:DSS-d_6 signals. (a) δ_H 7.53, (b) δ_H 7.65, and (c) δ_H 7.98. The errors of analytical values in each concentration are under the data points.

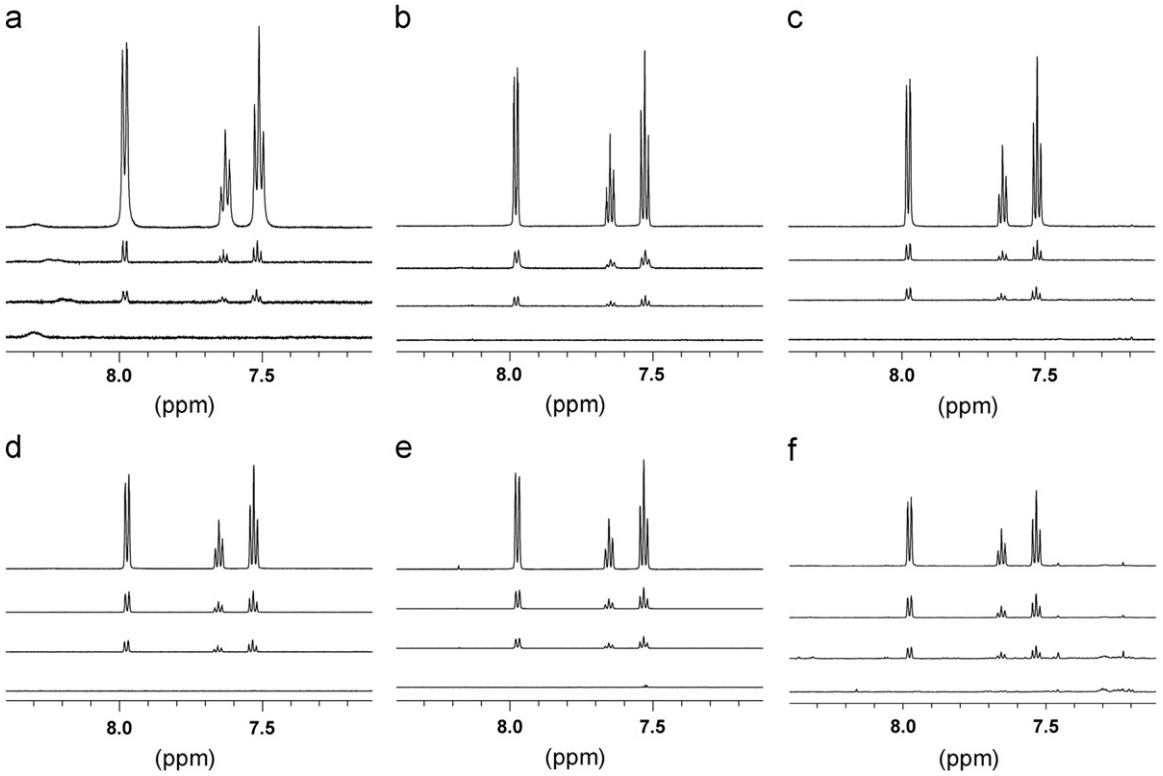

Fig. 3. ^{1}H NMR spectra in the range of δ_H 7.1–8.4 of each sample extract spiked with BA at the maximum usage level of each processed food (top), 0.13 g kg^{-1} (second), 0.063 g kg^{-1} (third), and blank (bottom): (a) caviar, (b) margarine, (c) avocado paste, (d) soft drink, (e) syrup, and (f) soybean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

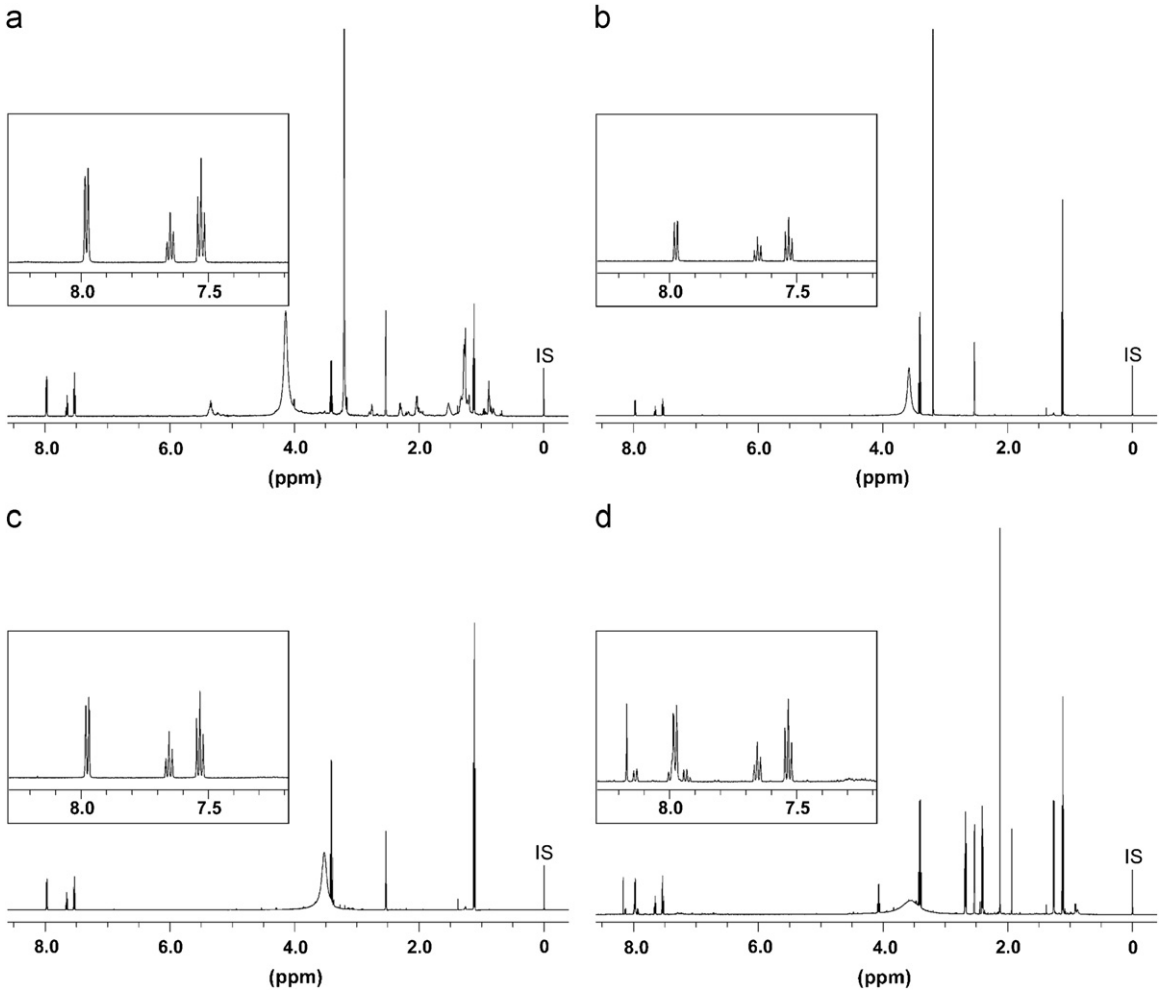

Fig. 4. ^1H NMR spectra of each sample solution from commercially produced food containing BA. The BA signals shown on the top is highlighted: (a) margarine, (b) soft drink, (c) syrup, and (d) soybean sauce. IS, internal standard (DSS-d_6).

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.

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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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