

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 monomethyl ester 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

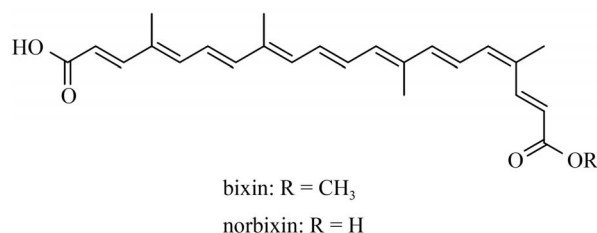


Figure 1. Structures of bixin and norbixin.

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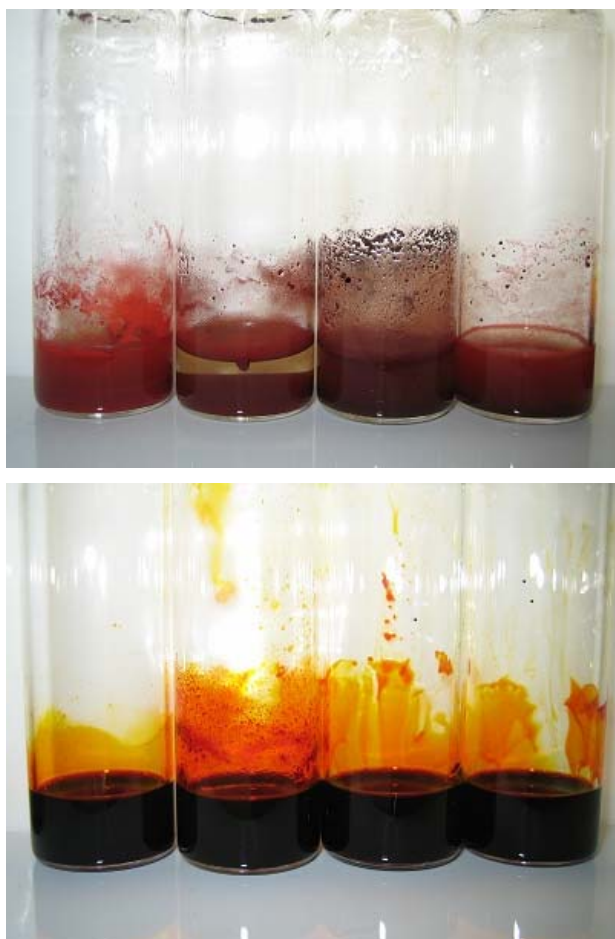


Figure 2. Solubility of annatto extracts in water (upper) and DMF (bottom). The two left bottles are bixin-based products (bix3 and bix4), and the two right bottles are norbixin-based products (nbx4 and nbx8).

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 thermodynamic 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 $\geq 98\%$ 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

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).

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.

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

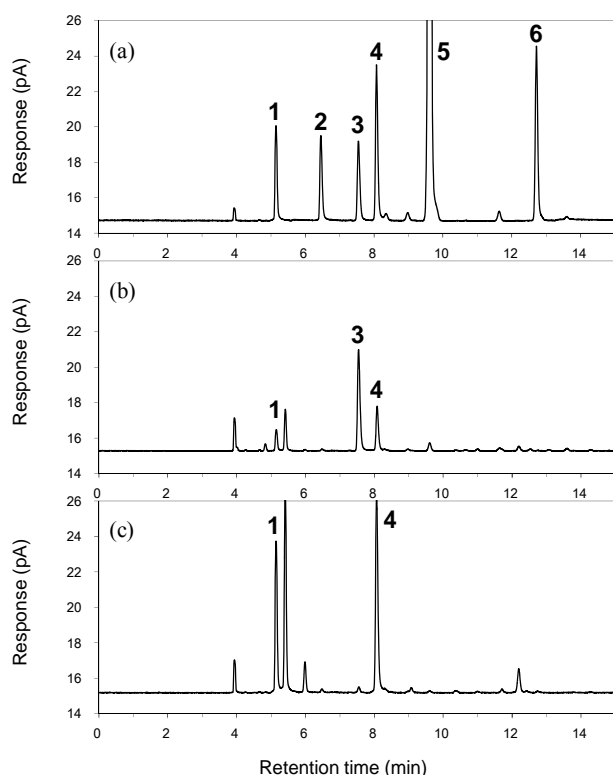


Figure 3. HSGC charts of (a) standard mixture; (b) bix1; and (c) bix4. Peak identities and concentrations in the standard mixture (a) are as follows: methanol (1, 270 ppm), ethanol (2, 290 ppm), 2-propanol (3, 300 ppm), acetone (4, 150 ppm), hexane (5, 130 ppm), ethyl acetate (6, 290 ppm).

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.

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.

Solvent	bix1		bix4		nbx8	
	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

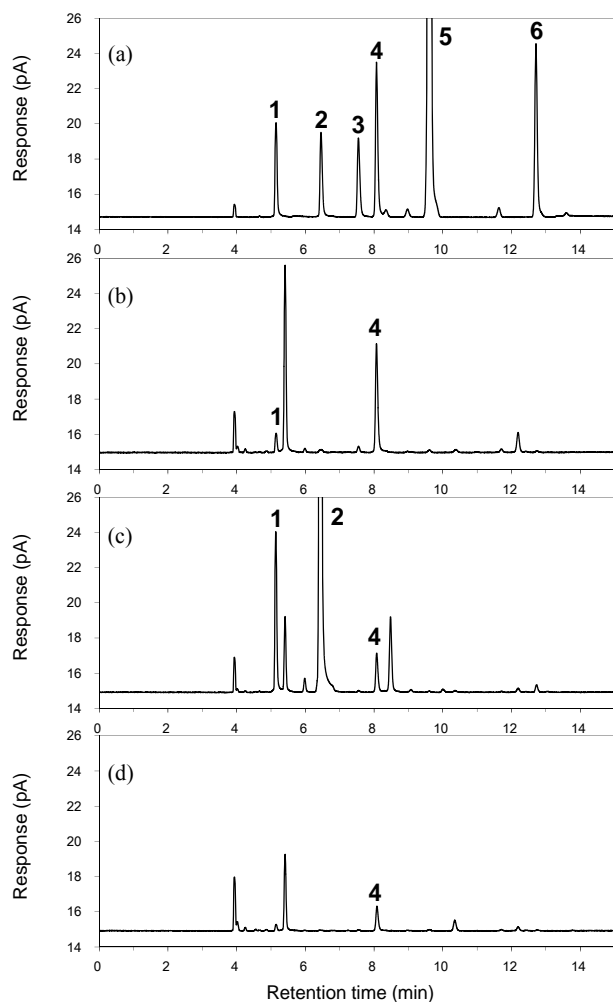


Figure 4. HSGC charts of (a) standard mixture; (b) nbx4; (c) nbx7; and (d) nbx14. Peak identities and concentrations in the standard mixture (a) are the same as in Figure 3.

all products used in this study were imported and were not processed by Japanese manufacturers. 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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