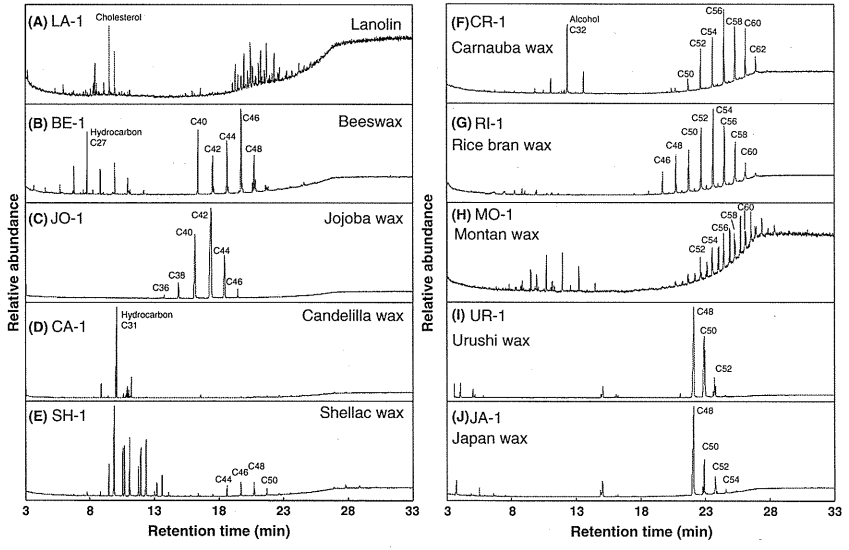

Figure 3. GC/MS TICs of 10 types of food additive gum bases. The number labels in the chromatograms indicate the number of carbons in the wax esters estimated from the retention times.

(Fig. 3A), wax esters with carbon numbers ranging from 48 to 52 were detected along with free cholesterols and sterol esters (Jover et al. 2002; Moldovan et al. 2002b), while in the chromatogram of beeswax (Fig. 3B), wax esters with carbon numbers ranging from 40 to 48 were detected along with hydrocarbons such as $C_{27}H_{56}$ (Bonaduce and Colombini 2004; Jiménez et al. 2004). For candelilla wax (Fig. 3D), saturated hydrocarbons such as $C_{31}H_{64}$ were the major constituents in accordance with a previous report (Lawrence et al. 1982; Tonogai et al. 1985). The shellac wax (Fig. 3E) contained wax esters with carbon numbers ranging from 44 to 50 along with free alcohols, free fatty acids, and hydrocarbons (Lawrence et al. 1982). As shown in Figure 3F–H, carnauba wax, rice bran wax, and montan wax contained similar wax esters in terms of their carbon numbers. However, these results suggest that the three gum bases may be distinguished by evaluating the TICs for the peaks of other characteristic constituents such as the alcohol with carbon numbers 32 in carnauba wax (Lawrence et al. 1982; Tonogai et al. 1985) and hydrocarbons in montan wax (Asano 1977; Lawrence et al. 1982). As previously reported (Tachibana et al. 1992; Jin et al. 2006), major components of urushi and Japan waxes were found to be triglycerides (Fig. 3I and J). As can be seen in the magnified chromatograms of urushi and Japan waxes (Fig. 4A and B, respectively). The ratio of glycerol 1,2-dipalmitate 3-oleate (PPO) to glycerol tripalmitate (PPP) (table in Fig. 4C for abbreviation definitions) for urushi wax (Fig. 4A) is higher than that for the Japan wax (Fig. 4B) (Tachibana et al. 1992; Jin et al. 2006). Accordingly, these two gum bases can be distinguished using this ratio.

These results thus demonstrate that food additive gum bases can be distinguished from the other based on the TIC patterns obtained using the established direct GC/MS analysis (without hydrolysis and derivatization) of the esters in these food additives.

Comparison of the MS chromatograms of carnauba wax, rice bran wax, and montan wax

As shown in Figure 3F–H, it was difficult to differentiate carnauba wax, rice bran wax, and montan wax using the TICs alone. Therefore, the MS chromatograms were analyzed. As shown in Figure 5, the MS spectrum of a standard of the saturated straight-chain ester, behenyl stearate), obtained using the established GC/MS method contained product ions derived from the fatty acid moiety of the ester ($[R^1COO]^+$, $[R^1CO]^+$, and $[R^1]^+$) and product ions derived from the alcohol moiety ($[R^2]^+$ and $[R^2OCO]^+$). It was observed that the product ions corresponding to the fatty acid and alcohol moieties of standard wax esters are generally observed in their MS spectra under these conditions. These results suggest that analysis of the MS spectra obtained using the established method can provide information on the constitutive fatty acids and alcohols of the esters.

Therefore, to more clearly distinguish between carnauba wax, rice bran wax, and montan wax, the MS chromatograms of the product ions derived from the constitutive fatty acids of the esters in these three waxes were compared. As can be seen in Figure 6, $[RCOO]^+$ product ions, of the constitutive fatty acids

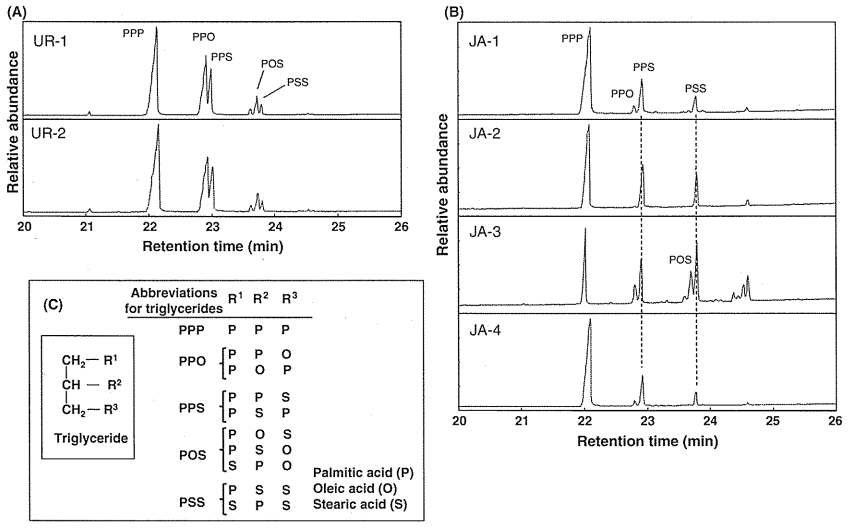

Figure 4. Magnified GC/MS TICs of (A) urushi waxes and (B) Japan waxes. (C) Abbreviations for the triglycerides are listed in the table (C). UR-1, UR-2, and JA-1 are food additive gum bases, while the other waxes are commercial samples.

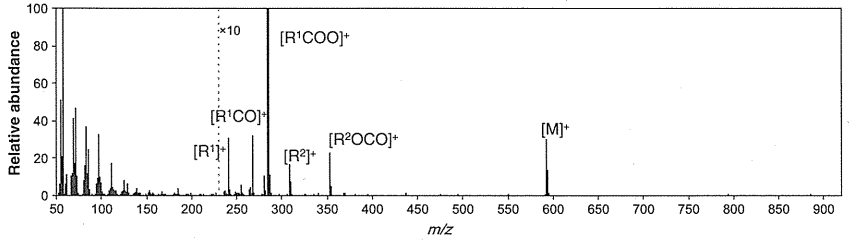

Figure 5. Mass spectra of C40 ester standards (C22:0 alcohol × C18:0 fatty acid, MW = 592) obtained using GC/MS method.

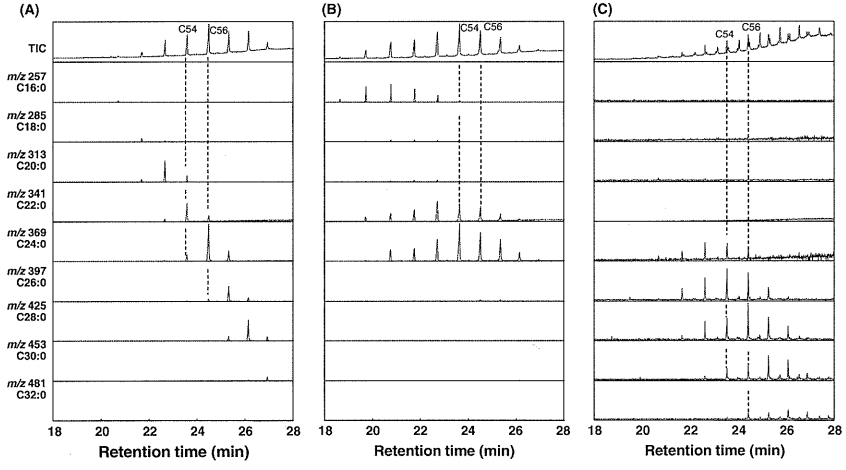

Figure 6. Mass chromatograms of the fragment ions derived from the constitutive fatty acids of the wax esters in (A) carnauba wax, (B) rice bran wax, and (C) montan wax via GC/MS analysis. For comparison, the TICs of the waxes are also shown (top of each figure). Number labels in the TICs indicate the carbon numbers of the wax esters.

Table 1. Estimated composition (%) of the constitutive fatty acids in the C54 and C56 wax esters detected in carnauba wax, rice bran wax, and montan wax based on the mass chromatograms of their fragment ions.

	Compositions (%)					
	C54 Wax ester			C56 Wax ester		
Constitutive fatty acids	Carnauba wax	Rice bran wax	Montan wax	Carnauba wax	Rice bran wax	Montan wax
C16:0	–	1	–	–	–	–
C18:0	–	1	–	–	–	–
C20:0	17	1	–	–	0	–
C22:0	61	28	–	11	29	–
C24:0	22	69	19	84	70	10
C26:0	–	–	39	5	–	29
C28:0	–	–	29	–	–	40
C30:0	–	–	13	–	–	15
C32:0	–	–	–	–	–	6

–: Not detected.

of the esters in the three waxes observed in the MS chromatograms, included C16:0 (m/z = 257), C18:0 (m/z = 285), C20:0 (m/z = 313), C22:0 (m/z = 341), C24:0 (m/z = 369), C26:0 (m/z = 397), C28:0 (m/z = 425), C30:0 (m/z = 453), and C32:0 (m/z = 481). These product ions were reanalyzed considering the data obtained using the GC/MS method established in this study. As can be seen in the TICs of the three gum bases (Fig. 6), wax esters with carbon numbers ranging from 50 to 56 were detected at retention times of 21–27 min. In the MS chromatogram of carnauba wax (Fig. 6A), carbon numbers of the major constitutive fatty acids of the esters increased with an increase in the carbon numbers of their corresponding esters. On the other hand, the esters detected in rice bran wax consisted of only three fatty acids C16:0, C22:0, and C24:0, regardless of the carbon number of the esters (Fig. 6B). In addition, as can be seen in Figure 6C, the esters detected in montan wax consisted of fatty acids with longer chains (C24:0–C32:0) than those of the esters detected in the other two gum bases. On the basis of these results, compositions of the constitutive fatty acids in the C54 and C56 esters in the three gum bases were then compared (Table 1). As shown in Table 1, among the three gum bases, composition of the constitutive fatty acids in the esters with identical carbon numbers were clearly different. These results indicate that these three gum bases can be distinguished by comparing the mass chromatograms and TICs obtained using the newly developed direct GC/MS method.

We previously reported (Tada et al. 2007) an analytical method for the determination of food additive gum bases using GC/MS after hydrolysis and derivatization, and identified the major constitutive fatty acids and alcohols of the gum bases. However, the method does not provide information on the constitutive fatty acid for each respective ester in the gum bases. With the present analytical method, the esters in the gum bases can be directly analyzed with simultaneous prediction of the constitutive fatty acids of the corresponding esters using the MS spectra of the ester peaks. In addition, this direct GC/MS method is simple, clear, and particularly useful for the rapid analysis of various types of food additive gum bases without the need for hydrolysis and derivatization of the esters in the gum bases.

Acknowledgments

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Conflict of Interest

None declared.

References

Asano, S. 1977. Characterization of Montan waxes by composition of fatty acids and hydrocarbons. Kanzei Chuou Bunsekisyo Hou 17:83–86 [in Japanese].

Bonaduce, I., and M. P. Colombini. 2004. Characterisation of beeswax in works of art by gas chromatography-mass spectrometry and pyrolysis-gas chromatography-mass spectrometry procedures. J. Chromatogr. A 1028:297–306.

Fitzgerald, M., and R. C. Murphy. 2007. Electrospray mass spectrometry of human hair wax esters. J. Lipid Res. 48:1231–1246.

Jiménez, J. J., J. L. Bernal, S. Aumente, M. J. del Nozal, M. T. Martín, and J. Bernal Jr. 2004. Quality assurance of commercial beeswax Part I. Gas chromatography-electron impact ionization mass spectrometry of hydrocarbons and monoesters. J. Chromatogr. A 1024:147–154.

Jin, Z.-L., A. Tada, N. Sugimoto, K. Sato, A. Masuda, K. Yamagata, et al. 2006. Analysis of constituents in urushi wax, a natural food additive. J. Food Hyg. Soc. Japan 47:167–172 [in Japanese].

Jover, E., Z. Moldovan, and J. M. Bayona. 2002. Complete characterization of lanolin steryl esters by sub-ambient pressure gas chromatography-mass spectrometry in the electron impact and chemical ionization modes. J. Chromatogr. A 970:249–258.

Lawrence, J. F., J. R. Iyengar, B. D. Page, and H. B. S. Conacher. 1982. Characterization of commercial waxes by high-temperature gas chromatography. J. Chromatogr. 236:403–419.

Moldovan, Z., E. Jover, and J. M. Bayona. 2002a. Systematic characterisation of long-chain aliphatic esters of wool wax by gas chromatography–electron impact ionisation mass spectrometry. J. Chromatogr. A 952:193–204.

Moldovan, Z., E. Jover, and J. M. Bayona. 2002b. Gas chromatographic and mass spectrometric methods for the characterization of long-chain fatty acids. Application to wool wax extracts. Anal. Chim. Acta 465:359–378.

Regert, M., J. Langlois, E. Laval, A.-S. Le Hô, and S. Pagés-Camagna. 2006. Elucidation of molecular and elementary composition of organic and inorganic substances involved in 19th century wax sculptures. Anal. Chim. Acta 577:140–152.

Stránskzý, K., M. Zarevúcka, I. Valterová, and Z. Wimmer. 2006. Gas chromatographic retention data of wax esters. J. Chromatogr. A 1128:208–219.

Tachibana, S., K. Itoh, K. Ohkubo, T. Oki, and M. Sumimoto. 1992. Chemical conversion of extractives for the production of chemicals and fuels IX, chemical compositions and physical properties in haze-wax from 13 cultivars grown in Ehime prefecture. Mokuzai Gakkaishi 38:1151–1158 [in Japanese].

Tada, A., A. Masuda, N. Sugimoto, K. Yamagata, T. Yamazaki, and K. Tanamoto. 2007. Analysis of constituents of ester-type gum bases used as natural food additives. J. Food Hyg. Soc. Japan 48:179–185 [in Japanese].

Tonogai, Y., S. Tsuji, Y. Ito, and M. Harada. 1985. Fundamental studies on a method for the analysis of natural waxes in foods by gas chromatography (Systematic determination method of natural waxes in foods. I). J. Food Hyg. Soc. Japan 26:160–168 [in Japanese].

Vrkoslav, V., K. Urbanová, and J. Cvačka. 2010. Analysis of wax ester molecular species by high performance liquid

chromatography/atmospheric pressure chemical ionisation mass spectrometry. J. Chromatogr. A 1217:4184–4194.

Vrkoslav, V., M. Háková, K. Pecková, K. Urbanová, and J. Cvačka. 2011. Localization of double bonds in wax esters by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry utilizing the fragmentation of acetonitrile-related adducts. Anal. Chem. 83:2978–2986.

Zhang, L., Y. Yun, Y. Liang, and D. Cao. 2010. Discovery of mass spectral characteristics and automatic identification of wax esters from gas chromatography mass spectrometry data. J. Chromatogr. A 1217:3695–3701.

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Research Article

Evaluation of gardenia yellow using crocetin from alkaline hydrolysis based on ultra high performance liquid chromatography and high-speed countercurrent chromatography

Gardenia yellow is globally the most valuable spice and food color. It is generally a mixture of water-soluble carotenoid glycosyl esters which consist of crocetin bis(gentiobiosyl) ester as the main component. Crocetin is a natural carotenoid dicarboxylic acid that may be a candidate drug for pharmaceutical development, however, it is either present in trace amounts or is absent in natural gardenia yellow products. We here propose that crocetin produced by alkaline hydrolysis can be used to qualitatively evaluate gardenia yellow products using an ultra high performance liquid chromatographic assay. A useful and efficient isolation technique for isolating high-purity crocetin from gardenia yellow using high-speed countercurrent chromatography is described. High-speed countercurrent chromatographic fractionation followed by an ultra high performance liquid chromatographic assay showed that *trans*-crocetin is easily converted to about 15% *cis*-crocetin (85% *trans*-crocetin). Crocetin in gardenia yellow was quantitatively evaluated. Our approach is based on the hydrolysis process for converting crocetin glycosyl esters to crocetin before evaluation and isolation using the ultra high performance liquid chromatographic and high-speed countercurrent chromatographic methods. The combination of hydrolysis and chromatographic methods allows evaluation of the purity and quantity of crocetin in gardenia yellow.

Keywords: Crocetin / Gardenia Yellow / High-speed countercurrent chromatography
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1 Introduction

Gardenia yellow, obtained from the fruits of *Gardenia augusta* Merrill and *Gardenia jasminoides* Ellis, is used as a natural food additive in Asia. The yellow pigment mainly comprises the derivatives of water-soluble carotenoid glycosyl esters, all-*trans*-crocetin bis(gentiobiosyl) ester (8,8'-diapo-ψ,ψ-carotenedioic acid bis(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester), called crocin (Fig. 1). Four types of crocetin glycosyl esters differing in the number of glucose units have been identified in gardenia materials [1]. Moreover, eight *trans*/*cis*-crocetin glycosyl esters, including all-*trans* crocin, in gardenia yellow were evaluated by LC–MS [2]. Recently, many researchers are paying attention to these crocetin components for their biological characteristics [3, 4]. Specifically, crocetin has the potential effects that inhibit amyloid-β fibril formation related to Alzheimer's disease [5, 6], and it significantly induces cell cycle arrest through p53-independent mechanisms accompanied by P21 induction [7]. Therefore, it is needed to evaluate crocetin components from gardenia yellow based on useful and effective methods. However, trace levels of crocetin exist in commonly available gardenia yellow products. In addition, commercial gardenia yellow contains geniposide, an iridoide glycoside that may cause negative bioactive effects [8]. Therefore, we have not been able to evaluate the drug potential of crocetin due to a lack of highly purified crocetin.

In Japan, two types of gardenia yellow colorant products, differing in their crocin and crocetin composition, are available as food additives for processed foods. The Japanese government has set a specification for gardenia yellow in its current regulation for food additives [9] and stipulates the purity test for checking the level of geniposide by HPLC at 238 nm and the identification test for the characteristics by TLC. TLC detection requires alkaline hydrolysis of the various crocetin glycosyl esters, including crocin and crocetin. By the alkaline hydrolysis process, a wide variety of the crocetin glycosyl esters would be completely converted to crocetin in gardenia yellow products. This process shows that high-purity, useful quantities of crocetin in gardenia yellow products can be efficiently isolated, and that crocetin in gardenia yellow can be quantified and used as the analytical standard in QC.

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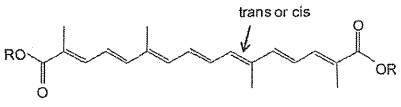
Abbreviations: HSCCC, high-speed countercurrent chromatography; **PDA**, photodiode array detection



R = H: Crocetin
R = Gentiobiosyl: Crocin

Figure 1. Structures of *trans*- and *cis*-crocin.

HPLC methods have been used to quantify crocetin in biological samples for pharmacokinetic studies [10, 11], and LC with photodiode array detection (PDA) and ESI-MS have been used to characterize and quantify crocetin in gardenia yellow [12].

The preparative isolation of gardenia yellow components, such as crocin isomers and other glycosyl esters, impurities and geniposide, have been reported using aqueous two-phase extraction, column chromatography, macroporous resin LC, molecularly imprinted polymer SPE, centrifugal partition chromatography, and high-speed countercurrent chromatography (HSCCC) [13–20]. However, the isolation of crocetin from gardenia yellow products has not been reported due to the complete lack or trace levels of crocetin in the products. Thus, the specific isolation and quantification of crocetin from gardenia yellow products has not been reported. On the other hand, the HSCCC techniques are reported for the purification of natural chemicals in raw materials [21–25]. Thus, we innovate a way to use the HSCCC technique for the purification of crocetin from gardenia yellow.

In this study, we evaluated gardenia yellow using chromatographic techniques based on the hydrolysis conversion of total crocetin glycosyl esters to crocetin, as outlined in the Japanese specifications and standards for food additives. Crocetin was isolated by HSCCC and quantified by UHPLC. The usefulness of the methodology for standardizing crocetin quantification is demonstrated.

2 Materials and methods

2.1 Chemicals and reagents

Gardenia yellow colorant products were obtained from San-Ei Gen F.F.I. (Osaka, Japan; Gardenia Yellow No. 1) and RIKEN VITAMIN (Tokyo, Japan, Gardenia Yellow No. 2, 3, 4 for Japanese types). HPLC-grade water and methanol were obtained from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH), tetrahydrofuran (THF), acetonitrile, DMSO, oxalic acid, and formic acid were obtained from Sigma–Aldrich (St. Louis, MO) and Wako Chemical (Osaka, Japan). Purified water was obtained from an Aquarius PWU200 automatic water distillation apparatus (Advantec, Tokyo, Japan).

2.2 Sample preparation

Gardenia yellow solution (0.1 g/5 mL) was prepared by diluting an aliquot of the solution in water/methanol (50:50, v/v). For alkaline hydrolysis of gardenia yellow, the product (0.1 g) was added to 0.02 mol/L NaOH (10 mL), incubated at 50°C for 30 min, diluting 10 times with DMSO and loading into the UHPLC system.

2.3 UHPLC instrument and conditions

RP analysis was performed using a Waters Acquity H Class LC system (Waters, Milford, MA) and an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 100 mm) at 40°C. The injection volume was 5 μL. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) delivered at a flow rate of 0.3 mL/min. The gradient elution was as follows: 0.0 min [A/B: 80:20], 15 min [A/B: 10:90], 17 min [A/B: 10:90], and 17.1 min [A/B: 80:20]. Elution of the analytes was monitored between 210 and 500 nm.

2.4 UHPLC–TOF-MS instrumentation and conditions

The separated analytes were detected by a LCT Premier XE TOF-MS (Waters, Milford, MA). In separation, the UHPLC conditions and instrument described above were employed. ESI (positive ionization mode) conditions were as follows: capillary voltage, 3.0 kV; sample cone, 50 V; source temperature, 120°C; desolvation temperature, 350°C. The cone and desolvation gas flows were 50 and 650 L/h, respectively, and were obtained using a nitrogen source. The analytical mode and dynamic range were the V mode and normal. The aperture 1 voltage was 15 V. The scan mode was used from m/z 100 to 1000.

2.5 HSCCC isolation

Following hydrolysis, the sample solution was prepared using SPE (OASIS-HLB, 200 mg/6 mL, Waters, Milford, MA). Methanol and water (5 mL each) were used to condition the cartridge. Then, double-diluted sample solution (5 mL) with water (5 mL) was loaded into the cartridge. After washing with pure water (1 mL), the yellow components were eluted using methanol (5 mL) and the crude extract in the test tube was evaporated to dryness at 30°C. These residues were added to the two mutually equilibrated solvent phases (0.5 mL each; see Table 1) in a test tube andde mixed to equilibrate. After settling, equal volumes of the upper and lower phases (100 μL each) were transferred into separate test tubes and diluted by 10% with methanol. Then, the samples were assayed by UHPLC. The peak area at 14 min (crocetin-1) and 16 min (crocetin-2) at 420 nm in each phase was assessed and the K value of each component was determined. The K

Table 1. The partition coefficient (K) and separation factor (α) values of crocetins

Two phase solvent system	Ratio (v/v)	Partition coefficient *trans*-Crocetin	$K \pm$ SD ($n = 3$) *cis*-Crocetin	α
Hexane/ethyl acetate/methanol/water	9:1:5:5	0.01 ± 0.01	0.03 ± 0.01	4.2
	8:2:5:5	0.09 ± 0.01	0.18 ± 0.02	2.1
	7:3:5:5	0.36 ± 0.11	0.49 ± 0.02	1.4
	6:4:5:5	0.74 ± 0.11	1.10 ± 0.05	1.5
	5:5:5:5	1.74 ± 0.09	1.77 ± 0.14	1.0
	4:5:4:5	7.52 ± 0.11	7.69 ± 0.24	1.0
Hexane/ethyl acetate/methanol/0.1% acetic acid in water	9:1:5:5	0.13 ± 0.03	0.36 ± 0.04	2.8
	8:2:5:5	0.66 ± 0.08	0.74 ± 0.14	1.1
	7:3:5:5	1.52 ± 0.32	1.23 ± 0.19	1.2
	6:4:5:5	3.35 ± 0.20	2.69 ± 0.06	1.2
	5:5:5:5	4.74 ± 0.14	3.11 ± 0.17	1.5
	4:5:4:5	7.86 ± 1.90	9.41 ± 1.80	1.2
Hexane/ethyl acetate/methanol/0.1% formic acid in water	9:1:5:5	058 ± 0.03	0.28 ± 0.04	2.1
	8:2:5:5	1.33 ± 0.08	0.61 ± 0.14	2.2
	7:3:5:5	2.51 ± 0.32	1.11 ± 0.19	2.3
	6:4:5:5	3.36 ± 0.20	1.63 ± 0.06	2.1
	5:5:5:5	5.57 ± 0.14	1.88 ± 0.17	3.0
	4:5:4:5	14.9 ± 1.90	3.04 ± 1.80	4.9

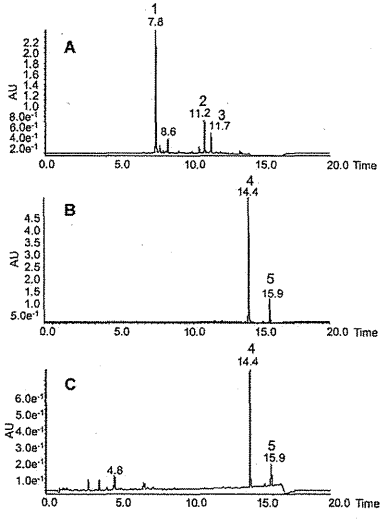


Figure 2. UHPLC chromatograms of gardenia yellow. (A) Chromatogram of gardenia yellow at 420 nm. (B) Chromatogram of gardenia yellow at 420 nm after alkaline hydrolysis. (C) Chromatogram of gardenia yellow at 250 nm after alkaline hydrolysis.

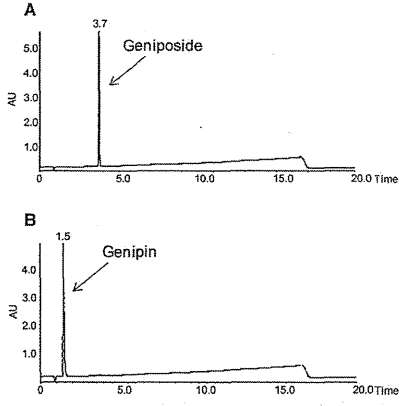


Figure 3. UHPLC chromatograms of geniposide and genipin. (A) Chromatogram of geniposide (reaction time, 0 min; Fig. 4B). (B) Chromatogram of genipin (reaction time, 180 min, Fig. 4B).

value was calculated as $K =$ (peak response of crocetin-1 or -2 in the upper phase solvent) / (peak response of crocetin-1 or -2 in the lower phase solvent). The α value was calculated as $\alpha = K_m / K_n$, $K_m > K_n$ for crocetin-1 and -2.

HSCCC was performed using an Easy-Prep CCC (multilayer coil planet centrifuge, Kutsuwa, Hiroshima, Japan) with a 7.6 cm orbital radius that produces a synchronous type-J planetary motion with a maximum speed of 1500 rpm. This centrifuge was equipped with three column holders and three multilayer coiled columns. Each multilayer coiled column on the holder consists of nine coiled layers of 1.6 mm i. d. polytetrafluoroethylene tubing with a capacity of about 120 mL. All three columns are connected in series to provide a total capacity of about 350 mL. The beta values of the coil range from 0.5 at the internal terminal to 0.75 at the external terminal. The separated effluent from the tail outlet of the coil-column was transferred and divided using a splitter valve (Low-pressure Micro Splitter Valve, GL Science, Tokyo, Japan). Part of each sample was delivered to fraction collection and part was used for PDA monitoring. A micro splitter valve was used to obtain a flow rate of 0.2 mL/min prior to PDA monitoring.

The volatile phase of the two-phase system composed of hexane/ethyl acetate/methanol/0.1% formic acid in water (7:3:5:5) at room temperature was thoroughly equilibrated. The phases were separated before use. First, the coiled column was entirely filled with the upper stationary phase. Second, 8.4 mg of dried crude extract was dissolved in 1.0 mL of each phase. Finally, these supernatants were then loaded into the column. The column was rotated at 1000 rpm, while the lower mobile phase was pumped into the head of the column at a flow rate of 1.2 mL/min using an HPLC pump (PU-2080 pump, JASCO, Tokyo, Japan). Then, each fraction (Fractions

A and B) were manually recovered, evaporated to dryness, and weighted. The purity was evaluated by the above-described UHPLC assay that the analytes was monitored between 210 and 500 nm.

3 Results and discussion

3.1 Analysis of gardenia yellow using the UHPLC assay

Conditions for quantifying the components of gardenia yellow were optimized and included, reaction time, separation, and detection using the UHPLC conditions described in Section 2. Crocin, crocetin glycosyl esters and crocetin are bright yellow, have a maximum absorbance at about 420 nm, and the wavelength is commonly used for the analysis of food additives. Separation of these compounds was optimized using 0.1% formic acid in water/methanol; a chromatogram demonstrating clear separation and detection by UHPLC is shown in Fig. 2A. Crocetin glycosyl esters were converted to crocetin by alkaline hydrolysis, as shown by the identical chromatogram in Fig. 2B. Many impurities with retention times between 1 and 10 min generated after the hydrolysis process were detected at 250 nm (Fig. 2C). Geniposide and

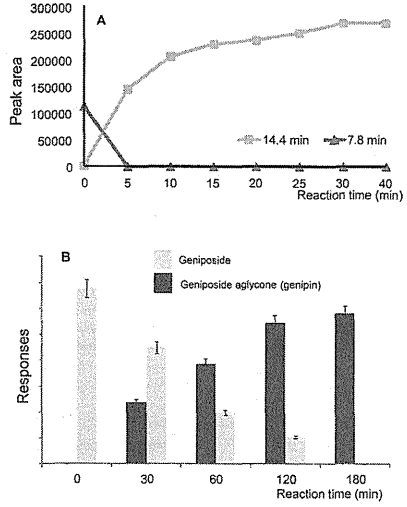


Figure 4. Reaction-time-dependent change of gardenia yellow components based on alkaline hydrolysis. (A) Peak response of 1 (retention time, 7.8 min) and 4 (retention time, 14.4 min) following alkaline hydrolysis. (B) Peak response of geniposide and genipin following alkaline hydrolysis.

genipin, the bioactive components in gardenia yellow, were analyzed under identical conditions at 240 nm (Fig. 3). Thus, the UHPLC assay can be used to simultaneously evaluate the major and minor components in gardenia yellow. TOF-MS was used to identify the crocetin and the glycosyl ester peaks on the UHPLC chromatogram. Three main components were identified at 420 nm: *trans*-crocetin bis(β-D-gentiobiosyl) ester (*trans*-crocin) (1; maximum absorption: 442 nm, retention time: 7.8 min), *trans*-crocetin β-D-gentiobiosyl ester (2; maximum absorption: 434 nm, retention time: 11.2 min) and *cis*-crocetin bis(β-D-gentiobiosyl) ester (*cis*-crocin) (3; maximum absorption: 442 nm, retention time: 11.7 min) (Fig. 2). These peaks were analyzed by TOF-MS; the m/z values were m/z 999.283 $[M + Na]^+$ for peak 1, m/z 837.522 $[M + Na]^+$ for peak 2 and m/z 999.282 $[M + Na]^+$ for peak 3. Previous study estimated the ionization and retention time patterns of several *trans*/*cis*-crocetin gentiobiosyl and/or glucosyl esters using similar chromatographic technique and detection at 430 nm with TOF-MS [2]. Two main contents of *trans*- and *cis*-crocetin chromatographic peaks were detected at 420 nm following hydrolysis: *trans*-crocetin (peak 4, maximum absorption: 422 nm, 14.4 min) and *cis*-crocetin (peak 5, maximum absorption: 422 nm, 15.9 min) (Fig. 2). TOF/MS analysis of both peaks showed m/z 329.441 $[M+H]^+$ ions [2].

3.2 Alkaline hydrolysis of gardenia yellow

Investigation of the conversion from crocetin glycosyl esters to crocetin showed that sodium hydroxide (NaOH) hydrolyzes crocetin glycosyl esters at 50°C and plateaus after 30 min, judging from the peaks at 7.8 (*trans*-crocin: 1) and 14.4 min (*trans*-crocetin: 4) (Fig. 4A). PDA was used to monitor impurity levels, such as geniposide, in these samples. UHPLC was used to investigate the stability of geniposide under alkaline conditions at 50°C. The degradation levels of geniposide in 0.02 mol/L NaOH observed at 0, 30, 60, 120, and 180 min, and shown in Fig. 4B, decreased in a time-dependent manner and a new peak absorbing at 240 nm appeared at 1.5 min (Fig. 3B). This new peak was a geniposide hydrolysis product, genipin (maximum absorption: 238 nm); TOF-MS, provided an m/z 226.929 $[M+H]^+$ in positive mode, suggesting that the presence of geniposide, an impurity in gardenia yellow, should not be evaluated in alkaline solution, and that hydrolysis is more compatible with evaluating genipin rather than geniposide [26].

3.3 Isolation of crocetin from crude extract

A pure crocetin standard was required to quantitatively evaluate the components of gardenia yellow. Crocetin was isolated following the alkaline hydrolysis of gardenia yellow. Following hydrolysis, the NaOH in the crocetin solution should be removed prior to HSCCC isolation and calculation of the K

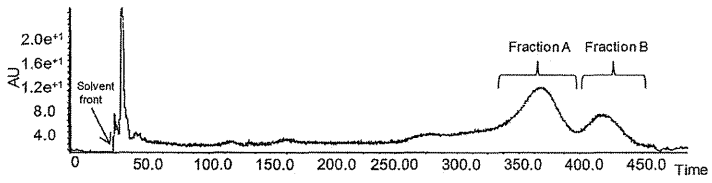


Figure 5. HSCCC chromatogram of the crude extract of gardenia yellow. Fraction A from 340 to 390 min and Fraction B from 400 to 450 min.

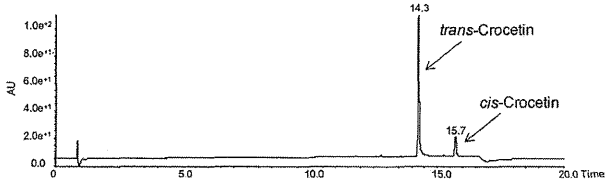


Figure 6. UHPLC chromatogram of Fraction A from HSCCC isolation. PDA detection range from 210 to 500 nm.

value from the two-phase solvent system. In this process, SPE was used for the extraction of hydrolyzed gardenia yellow. These data indicated that hydrolyzing 0.1 g of food additive sample (60% for gardenia yellow; 40% for dextrin from San-Ei Gen F.F.I.) provides 8.4 mg of crude extract using the SPE procedure. The samples were neutralized and analyzed by HSCCC.

Optimal K and α values were evaluated using the two-phase solvent system and the UHPLC assay. The results are shown in Table 1. Based on these results, we selected the hexane/ethyl acetate/methanol/0.1% formic acid in water (7:3:5:5) two-phase solvent system for further experiments. In the absence of formic acid, the α values remains incomplete (< 1.5); the addition of acetic acids did not significantly improves the α values of *trans* and *cis*-crocetins whereas formic acid caused a dramatic change of the α values of *trans* and *cis*-crocetins. For HSCCC isolation, the two-phase solvent system of hexane/ethyl acetate/methanol/0.1% formic acid in water (7:3:5:5) provided useful K (>1.0) and α (>1.5) values. Using this two-phase solvent system, the retention of the stationary phase was 88% in HSCCC column. The total separation time was about 8 h, and the total elution volume was 600 mL for the HSCCC system. The HSCCC chromatogram (Fig. 5) showed two fractionated effluent peaks (fractions A and B); the amounts of peaks fractions A and B are 2.0 and 1.2 mg, respectively. The A and B fractions were analyzed by UHPLC; the chromatogram of fraction A is shown in Fig. 6. Under natural conditions (noncontrol of temperature and protection from light), *trans*-crocetin is easily converted to about 15% *cis*-crocetin. We investigated the conversion of crocetin isomers in this purified fraction over a 10 h period and found 85% *trans*-crocetin and 15% *cis*-crocetin using UHPLC fraction. No impurities such as geniposide, genipin, and others were detected between 210 and 350 nm.

3.4 Quantitative evaluation of gardenia yellow

We attempted the chromatographic evaluation of gardenia yellow using UHPLC assay with HSCCC for isolation of crocetin standard. The approach was based on the hydrolysis conversion of crocetin glycosyl esters to crocetin used in the Japanese specifications for food additives [9]. Gardenia yellow products available in Japan were evaluated using our experimental assay. Using the *trans*-crocetin calibration curve $y = 549.7x + 7374.6$ with $r^2 = 0.998$. The concentrations of *trans*-crocetin in five gardenia yellow products obtained from general Japanese markets were determined to be 67.6 (No. 1), 45.5 (No. 2), 57.9 (No. 3), and 60.3% (No. 4) based on the novel hydrolyzed crocetin values. In addition, the crocetin was not detected in gardenia yellow (No.1) before hydrolysis (Fig. 2A).

4 Conclusions

Previous studies on crocetin in gardenia yellow focused on its biological characteristics as a drug candidate. In this study, we propose that crocetin produced by alkaline hydrolysis can be used together with UHPLC and HSCCC methods to evaluate and purify crocetin from products. A useful and efficient technique for isolating high-purity crocetin from gardenia yellow using HSCCC was described, and crocetin in gardenia yellow was accurately evaluated by using the described UHPLC assay. Our approach is based on the accepted hydrolysis process for converting crocetin glycosyl esters to crocetin, followed by evaluation and isolation using UHPLC and HSCCC methods. *trans*-Crocetin is easily converted to about 15% *cis*-crocetin under general conditions. Except for this, the above results provide a preliminarily experimental design for the quantitative evaluation of various gardenia yellow products.

We are grateful to San-Ei Gen F.F.I. and RIKEN VITAMIN for providing us with gardenia yellow colorant products. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan and by a Grant from the Japan Food Chemical Research Foundation.

The authors have declared no conflict of interest.

5 References

[1] Chen, Y., Zhang, H., Li, Y. X., Cai, L., Huang, J., Zhao, C., Jia, L., Buchanan, R., Yang, T., Jiang, L. J., *Fitoterapia* 2010, *81*, 269–273.

[2] Lech, K., Witowska-Jarosz, J., Jarosz, M., *J. Mass Spectrom.* 2009, *44*, 1661–1667.

[3] Gutheil, W. G., Reed, G., Ray, A., Anant, S., Dhar, A., *Curr. Pharm. Biotechnol.* 2012, *13*, 173–179.

[4] Alavizadeh, S. H., Hosseinzadeh, H., *Food Chem. Toxicol.* 2014, *64*, 65–80.

[5] Ahn, J. H., Hu, Y., Hernandez, M., Kim, J. R., *Biochem. Biophys. Res. Commun.* 2011, *414*, 79–83.

[6] Kong, Y., Kong, L. P., Luo, T., Jiang, W., Li, S., Zhou, Y., Wang, H. Q., *CNS Neurol. Disord. Drug Targets* 2014, in press, doi: 10.2174/1871527313666140806125410.

[7] Li, C. Y., Huang, W. F., Wang, Q. L., Wang, F., Cai, E., Hu, B., Du, J. C., Wang, J., Chen, R., Cai, X. J., Feng, J., Li, H. H., *Asian Pac. J. Cancer Prev.* 2012, *13*, 3757–3761.

[8] Ozaki, A., Kitano, M., Furusawa, N., Yamaguchi, H., Kuroda, K., Endo, G., *Food Chem. Toxicol.*, 2002, *40*, 1603–1610.

[9] The Ministry of Health, Labour and Welfare of Japan, *Japan's Specifications and Standards for Food Additives*, 2010, pp. D479–D482.

[10] Mohammadpour, A. H., Ramezani, M., Tavakoli Anaraki, N., Malaekeh-Nikouei, B., Amel Farzad, S., Hosseinzadeh, H., *Iran. J. Basic. Med. Sci.* 2013, *16*, 47–55.

[11] Chryssanthi, D. G., Lamari, F. N., Georgakopoulos, C. D., Cordopatis, P., *J. Pharm. Biomed. Anal.* 2011, *55*, 563–568.

[12] Carmona, M., Zalacain, A., Sánchez, A. M., Novella, J. L., Alonso, G. L., *J. Agric. Food Chem.* 2006, *54*, 973–979.

[13] Feng, J., He, X., Zhou, S., Peng, F., Liu, J., Hao, L., Li, H., Ao, G., Yang, S., *J. Sep. Sci.* 2014, *37*, 314–322.

[14] Chen, J. F., Fu, G. M., Wan, Y., Liu, C. M., Chai, J. X., Li, H. G., Wang, J. T., Hu, M., Zhang, L.N., *J. Chromatogr. B* 2012, 893–894, 43–48.

[15] Mohajeri, S. A., Hosseinzadeh, H., Keyhanfar, F., Aghamohammadian, J., *J. Sep. Sci.* 2010, *33*, 2302–2309.

[16] Zhang, M., Hu, P., Liang, Q., Yang, H., Liu, Q., Wang, Y., Luo, G., *J. Chromatogr. B* 2007, *858*, 220–226.

[17] Kim, C. Y., Kim, J., *Phytochem. Anal.* 2007, *18*, 115–117.

[18] Zhou, T., Fan, G., Hong, Z., Chai, Y., Wu, Y., *J. Chromatogr. A* 2005, *1100*, 76–80.

[19] Oka, H., Ikai, Y., Yamada, S., Hayakawa, J., Harada, K. I., Suzuki, M., Nakazawa, H., Ito, Y., *Modern Countercurrent Chromatography (ACS Symposium)*, ACS Publications, Washington, DC 1995, Chapter 8, pp. 92–106.

[20] Pan, I. H., Chiu, H. H., Lu, C. H., Lee, L. T., Li, Y. K., *J. Chromatogr. A* 2002, *977*, 239–246.

[21] Sun, Y., Gu, S., Guo, L., Xia, X., Zhang, H., Wang, J., *J. Sep. Sci.* 2014, *37*, 1703–1709.

[22] Chen, P., Wu, D., Pan, Y., *J. Sep. Sci.* 2013, *36*, 3660–3666.

[23] Skalicka-Woźniak, K., Walasek, M., Ludwiczuk, A., Głowniak, K., *J. Sep. Sci.* 2013, *36*, 2611–2614.

[24] Fang, L., Liu, Y., Yang, B., Wang, X., Huang, L., *J. Sep. Sci.* 2011, *34*, 2545–2558.

[25] Costa, F. D., Leitão, G. G., *J. Sep. Sci.* 2010, *33*, 336–3347.

[26] Hou, Y. C., Tsai, S. Y., Lai, P. Y., Chen, Y. S., Chao, P. D., *Food Chem. Toxicol.* 2008, *46*, 2764–2769.

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ドイツ製ブラッドオレンジジュースに含まれていたコチニール色素によるアナフィラキシーの1例

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ドイツ製ブラッドオレンジジュースに含まれていたコチニール色素によるアナフィラキシーの1例

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要約

29歳，女性。ドイツ滞在中にブラッドオレンジジュースなどを摂取後に，全身性膨疹，眼瞼浮腫，咳嗽，呼吸困難などのアナフィラキシー症状が出現。プリックテストなどの結果より，ブラッドオレンジジュース中に含まれたコチニール色素によるアナフィラキシーと診断した。コチニール色素を含む食品の経口摂取による即時型アレルギーの近年の報告はすべて日本人症例であり，日本人ではフランス製赤色マカロンやブラッドオレンジジュースなどを原因食物としたコチニールアレルギーを発症しやすい要因が潜在している可能性が疑われる。そのため特に本邦では今後コチニールアレルギーの発症に留意する必要がある。

Key words：コチニール，カルミン，アレルギー，ブラッドオレンジジュース，アナフィラキシー

Ⅰ．はじめに

天然の赤色色素であるコチニールを含む食品の経口摂取後に生じる即時型アレルギーは，もっぱら女性に発症し，化粧品の使用による経皮感作の機序が考えられている[1)2)]。以前はカンパリ摂取後に生じた報告が最多であった[3)]が，2007年以降カンパリ原液にコチニール色素は使用されなくなった。

今回われわれは，ドイツ製ブラッドオレンジジュース摂取後にアナフィラキシー症状を発症した女性症例を経験したため，若干の文献的考察とともに報告する。

Ⅱ．症　例

症　例　29歳，女性

主　訴　食後に生じたアナフィラキシー発作

初　診　2012年11月

既往歴　レボフロキサシン（クラビット®）服用後に息苦しさを感じた既往あり。

現病歴　当院初診直前の3週間はヨーロッパに滞在しており，初診の前日に帰国。ドイツ滞在中の11月上旬，昼食にドイツソーセージ，緑キャベツ，ポテト，ブラッドオレンジジュースを食べたところ，約1時間後より咳嗽，咽喉の閉塞感，軽度呼吸困難が出現し，引き続き，眼瞼浮腫，耳の詰まった感じ，全身性膨疹も生じ

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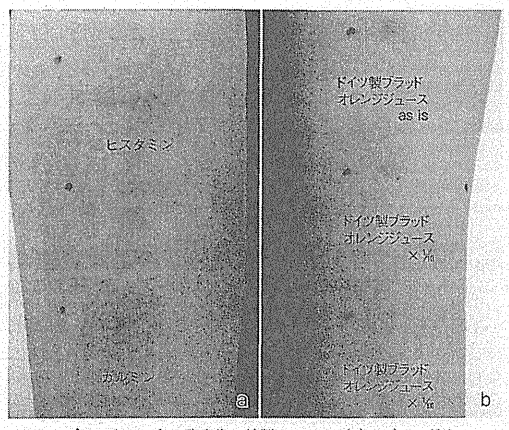

図1 ブリックテストの臨床像：精製カルミン（a），ブラッドオレンジジュース as is で（3+）陽性，ブラッドオレンジジュース 10 倍希釈，100 倍希釈で（2+）陽性（b）を認めた。

てきた。手持ちの抗アレルギー剤を服用し，何とか症状は緩和した。

原因検索を希望して当院を受診。ただし，これまでに化粧品や口紅などの使用後に皮疹や違和感が生じた既往はない。

初診時臨床検査所見 総 IgE 値は 195 IU/*ml* と軽度上昇。CAP FEIA 法による特異的 IgE は，牛肉，豚肉，鶏肉，オレンジ，ジャガイモではすべて<0.34 UA/m*l* と陰性であったが，サーモフィッシャーサイエンティフィックファディア社に測定を依頼したコチニールでは，0.929 UA/m*l* とクラス 2 陽性であった。

ブリックテスト結果（図 1） 陽性コントロールの二塩酸ヒスタミン（10 mg/m*l*）では 6×6 mm/12×12 mm（膨疹径/紅斑径），精製カルミンで 6×6 mm/15×17 mm。症状発現時に患者が実際に摂取していたブラッドオレンジジュース as is で 6×6 mm/とともに（3+）陽性。ブラッドオレンジジュース 10 倍希釈で 5×4 mm/，100 倍希釈で 3×3 mm/とともに（2+）陽性。その他，コチニール抽出物，持参のドイツ製ソーセージ（食べたものとは別の種類），陰性コントロールの蒸留水では，すべて陰性であった。

表1 ブラッドオレンジジュース中のカルミン酸の定量結果

	カルミン酸濃度（mg/g）	平均値（mg/g）
検体 1	0.0355	0.0364
検体 2	0.0375	
検体 3	0.0362	

ブラッドオレンジジュース中のカルミン酸の定量結果（表 1） 患者が持参したドイツ製ブラッドオレンジジュースの成分欄には Karmin と明記されていた。同ジュース中のカルミン酸の定量を試みたところ，3 個のボトルで検討した結果，平均 0.0364 mg/g のカルミン酸を含有していることが判明した。

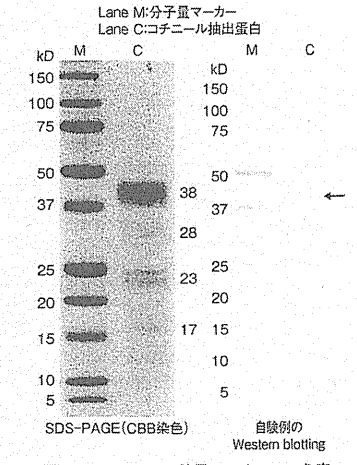

図2 Immunoblot 結果：コチニール色素中の虫体遺残蛋白由来抗原に合致する約 38 kDa の分子量の領域に陽性バンドを検出した。

Immunoblot 結果（図 2） エンジムシ虫体から抽出した蛋白の転写膜に，患者血清を供して IgE 反応性を検討したところ，約 38 kDa の分子量の領域に陽性バンドを検出した。

自験例の診断 臨床経過，プリックテストなどの結果より，自験例をドイツ製ブラッドオレンジジュース中に含まれたコチニール色素によるアナフィラキシーと診断した。また，immunoblot で約 38 kDa の分子量領域に陽性バンドを検出したことより，自験例の原因抗原はコチニール色素中の虫体遺残蛋白であると考えた。さらに，近年コチニールアレルギーの感作経路として，化粧品などによる経皮感作が考えられているが，自験例では化粧品や口紅などの使用後に症状発現の既往は認められず，感作経路は明らかになしえなかった。

Ⅲ．考　案

コチニールとは，サボテン科のベニコイチジクなどに寄生するカイガラムシ科エンジムシの雌の乾燥虫体を，水ないしエタノールで抽出して得られる天然の赤色色素である[1)5)]。コチニールの主色素はアントラキノン系カルミン酸であるが，コチニール中には，不純物として 1.15～2.2%程度の虫体由来遺残蛋白を含んでいる。現在なお多くの国で，食品（ジャム，ハム，ソーセージ，炭酸飲料水，イチゴ牛乳，漬け物，キャンディー，羊羹，蒲鉾，氷菓など），化粧品（口紅，頬紅，マニキュア，ヘアダイ，アイシャドーなど），医薬品（錠剤，粉末剤）などにコチニールは含まれている[3)5)]。

一方，カルミン酸を水酸化アルミニウムとカルシウムイオンを用いて沈澱させ，沈澱物から得られたカルミン酸のアルミニウムレーキをカルミンとよぶ。コチニール色素中のカルミン酸は約 2～4%であるのに対して，カルミンは約 20～50%ものカルミン酸を含んでいる[6)]。本邦では，医薬品・化粧品に対するカルミンの使用は認められているが，食品添加物としての使用は認められておらず，欧米でのみ許可されている。

コチニールアレルギーの原因抗原に関しては，① コチニール色素中の虫体遺残蛋白由来，② 主成分であるカルミン酸自体，と 2 つの見解が存在している。前者については，コチニール色素は不純物として約 1.15～2.2%の蛋白質を含んでいるが，この不純蛋白中のエンジムシの分泌性リパーゼ様蛋白が原因抗原であり，約 39～45 kDa の分子量を有していると考えられている[7)～9)]。一方，後者に関しては，分子量が 492 kDa しかないカルミン酸に対するアレルギーの発症は考えにくいが，カルミン酸がハプテンとして血清蛋白と結合することにより抗原として認識され，これに対するアレルギーを起こすと

表2　コチニール色素の経口摂取による即時型アレルギーの報告例（2011年以降）

症例	報告者	報告年	年齢/性別	発症した症状	原因食物
1	豊永ら[3]	2011	31/女	全身膨疹，嘔吐，顔面腫眼，咽頭違和感，下痢，血圧低下，意識レベル低下	フランス製赤色マカロン
2	秋山ら[10]	2011	26/女	顔面，特に眼周囲の腫眼，呼吸困難	カンパリオレンジ
3	田中ら[11]	2012	30代/女	咽喉の痒み，全身膨疹，下痢	市販の栄養ドリンク
4	佐伯ら[12]	2012	40/女	全身膨疹，嘔吐	赤色チョコマカロン，ハム
5	野村ら[13]	2012	42/女	顔面腫眼，胸内苦悶	アセロラドリンク，カンパリ，赤いオレンジジュース，魚肉ソーセージ
6	大澤ら[14]	2012	26/女	全身膨疹，血圧低下	コチニール含有ワイン（ブラッドオレンジミモザ）
7	北林ら[15]	2012	39/女	アナフィラキシーショック	イチゴのマカロン，赤色ミルクセーキ
8	原田ら[16]	2012	49/女	胃部不快感，呼吸困難，全身性膨疹，嘔吐	フランス製赤色マカロン
9	原田ら[16]	2012	33/女	咽喉の灼熱感，眼周囲の腫眼，全身性膨疹	フランス製赤色マカロン
10	石川ら[17]	2013	52/女	顔面腫眼，全身紅斑，下痢	フランス製赤色マカロン
11	安藤ら[18]	2013	48/女	全身の蕁麻疹，アナフィラキシー	魚肉ソーセージ，赤ワインゼリー，赤いノンアルコールカクテル，イチゴ牛乳
12	自験例		29/女	咳嗽，咽喉の閉塞感，軽度呼吸困難，眼瞼浮腫，耳の詰まった感じ，全身性膨疹	ドイツ製ブラッドオレンジジュース

の可能性も論じられている[10]。ただし，自験例では約38 kDaの分子量領域に陽性バンドを検出したことより，カルミン酸自体が抗原ではなく，不純物中に抗原性が存在していると診断した。

2011年に豊永らは，コチニール色素の経口摂取による即時型アレルギー症例の，2008年までの既報告18例に自験例を加えた19例を集計している[3]が，患者は23～52歳の全例が女性症例であった。発症が女性に限定される理由として，コチニール色素を含有した化粧品の使用による経皮感作の可能性が疑われている[1)2)]。ただし，自験例では化粧品や口紅使用後に症状発現の既往は認められなかった。豊永らが集計した2008年までの報告例18例中12例は海外からの報告であり，本邦からの報告は6例のみであった。また，原因食物はカンパリ摂取後に生じた報告が最多であり，18例中11例を占めていた。しかし，2007年にコチニール色素から合成色素に変更されたため，同年以降カンパリ原液にコチニール色素は含まれなくなっている。

表2に豊永らの集計以降に発症した，コチニール色素の経口摂取による即時型アレルギー症例の一覧を示す。2011年以降の報告例の原因食物は，12例中6例がフランス製赤色マカロン，3例がブラッドオレンジジュースであった。ただし極めて興味深いことに，これらの症例はすべて本邦からの報告であり，赤色マカロンの原産国であるフランスや，自験例のブラッドオレンジジュースの原産国であったドイツを含めて，海外からの報告は1例も認められていない。このような傾向をきたす原因として，本邦では高濃度のカルミン酸を含むカルミンの食品中への使用が認可されていないために経口的な免疫寛容が生じにくいとの逆説的な仮説も考えうるが，人種差を含めて，日本人には赤色マカロンやブラッドオレンジジュースによるコチニールアレルギーを発症しやすい何らかの要因が潜在

しているとの可能性も疑われる。そのため今後特に本邦では，フランス製赤色マカロンやブラッドオレンジジュースによるコチニールアレルギーの発症に留意する必要がある。

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自験例の要旨は日本皮膚アレルギー・接触皮膚炎学会第43回総会学術大会において報告した。

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文　献

1) 扇谷陽子ほか：アレルギーの臨，29：677-682，2009
2) Greenhawt MJ et al：Ann Allergy Asthma Immunol, 103：73-75, 2009
3) 豊永三恵子ほか：皮膚病診療，33：515-518，2011
4) 寿　順久ほか：アレルギー，56：1510-1514，2007
5) 竹尾直子ほか：日皮会誌，118：1085-1093，2008
6) Ferrer A et al：Int Arch Allergy Immunol, 138：243-250, 2005
7) Yamakawa Y et al：J Dermatol, 36：72-74, 2009
8) 山川有子ほか：臨皮，66：8-13，2012
9) Obgiya Y et al：J Allergy Clin Immunol, 123：1157-1162, 2009
10) 秋山美知子ほか：皮膚臨床，53：701-704，2011
11) 田中祐輔ほか：アレルギーの臨，432：768，2012
12) 佐伯葉子ほか：日皮会誌，122：2352，2012
13) 野村和夫：日皮会誌，122：2509，2012
14) 大澤研子ほか：日皮会誌，122：2914，2012
15) 北林　耐ほか：アレルギー，61：1537，2012
16) 原田　晉ほか：アレルギー，61：1538，2012
17) 石川照子ほか：アレルギー，62：441，2013
18) 安藤亜希ほか：日皮会誌，123：1818，2013

特集◆蕁麻疹・痒疹

フランス製赤色マカロンに含まれるコチニール色素が原因と思われるアナフィラキシーの1例
――最近のコチニールアレルギーについて――

山川　有子*1,*2　山野　朋子*1,*2　相原　道子*2
穐山　　浩*3　　池澤　善郎*4

要　約

30歳，女性。フランス製赤色マカロンを摂食中から，即時型アレルギー反応が出現。赤色マカロンにはカルミンあるいはコチニール色素が含有されており，皮膚プリックテストにてカルミンおよびコチニール色素に陽性を示し，コチニール色素によるⅠ型アレルギーと診断した。近年，コチニール色素のアレルギーの原因物質としてフランス製赤色マカロンが数例報告され，これらが経皮感作後に発症している症例もあり，注意が必要である。

Key words：コチニール色素，カルミン，経皮感作，フランス製赤色マカロン

Ⅰ．はじめに

コチニール色素は，エンジムシの一種であるコチニールの雌を乾燥化したものから抽出される，天然赤色色素である[1]。日本では食品衛生法の既存添加物名簿に収載され，赤色飲料，蒲鉾，漬物，菓子などの食品に含まれている。コチニール色素の主成分はカルミン酸で，カルミン酸のアルミニウムレーキ色素であるカルミンは，日本において既存添加物名簿所収でないため食品に使用できない。一方，海外では，コチニール色素，カルミンとも食品添加物としての使用が認められている。今回われわれは，フランス滞在中に，フランス製赤色マカロンに含まれていたカルミンあるいはコチニール色素が原因と思われる即時型アレルギーを起こした患者を経験した。患者からは文書にて，諸検査および臨床研究の同意を得た。

Ⅱ．症　　例

患　者　30歳，女性

主　訴　アナフィラキシーの原因精査目的

家族歴・既往歴　特になし。

現病歴　2012年6月，フランスにて赤いマカロンを摂食中に，舌の違和感，5分後には顔の腫脹，眼球結膜充血，眼瞼浮腫と鼻閉感が出現した。フェキソフェナジン塩酸塩（アレグラ®）を内服したが，全身に蕁麻疹が出現した。翌日現地の病院を受診し，ステロイド剤の点滴，内服処方により諸症状は改善した。6月末，日本に帰国直後，当院を初診した。フランスで摂食した赤いマカロンにはカルミンが含有されており，カルミンあるいはコチニール色素を原因とするアナフィラキシーを疑った。患者は，それまでフランス製の口紅や頬紅を使用したことがあったが，当時は特に皮膚症状などはなかったとのことであった。

検査所見　①皮膚プリックテスト（skin prick test，以下SPT）：ヒスタミン（10 mg/ml），生理食塩水，コチニール色素入りカンパリ，コチニール色素抽出液（旧カンパリと同一色価），コチニール色素抽出物から不純物を取り除いた精製品（旧カンパリと同一色価），フランス製赤色マカロン，日本製赤色マカロン，フランス製カルミン含有口紅・頬紅，日本製カルミン非含有口紅を用意した。患者の前腕にそれ

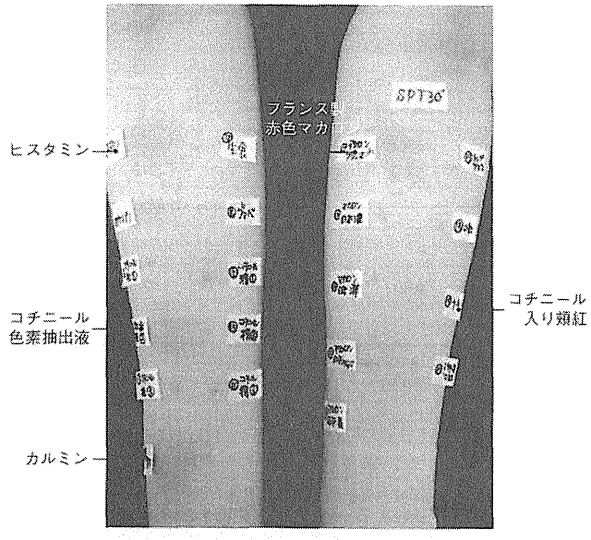

図1　SPT：コチニール色素抽出液3+，カルミン3+，コチニール色素精製品+，フランス製赤色マカロン2+，フランス製カルミン含有頬紅+，日本製カルミン非含有口紅－であった。

表1　SPTの結果

SPT試薬	15分後結果	30分後結果
コチニール色素抽出液	3+	3+
コチニール色素精製品	－	+
カルミン	3+	3+
カンパリ（コチニール色素入り）	+	2+
フランス製赤色マカロン	2+	2+
日本製赤色マカロン	－	－
フランス製口紅（コチニール色素入り）	+	+
日本製口紅（コチニール色素非含有）	－	－

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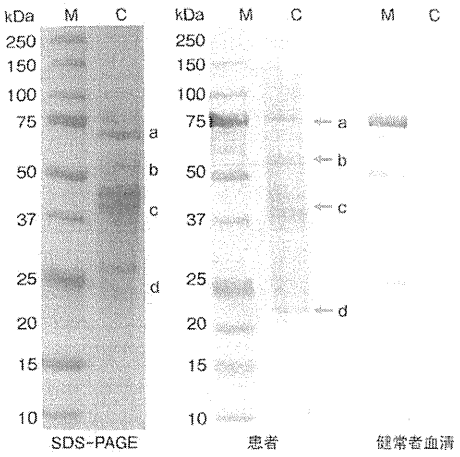

図2　ウエスタンブロッティング結果：患者血清においてコチニール抽出蛋白に対し，顕著なIgE反応が現れたバンドは38～45 kDa（バンドC）であった。また，75～70 kDa（バンドA），50～55 kDa（バンドB），23～26 kDa（バンドD）においてもIgE反応がみられた。なお健常者血清では，IgE反応はみられなかった。

ぞれの液体を一滴ずつたらし，Lancet針を各々に刺してから残液を拭き取った。15分後，30分後，それぞれの膨疹の直径がヒスタミンの膨疹の直径と同程度を3+，2倍のものは4+，1/2のものは2+，1/4のものは+と判定した[2]。その結果，コチニール色素抽出液3+，カルミン3+，コチニール色素精製品+，フランス製赤色マカロン2+，フランス製カルミン含有頬紅+，日本製カルミン非含有口紅−であった（図1，表1）。

② ウエスタンブロッティング：SDS-PAGE法によりコチニール色素を抽出し，その蛋白をpolyvinylidene difluoride membrane（PVDF膜）に転写し，患者血清で1次抗体処理した後，抗ヒトIgE抗体を2次抗体処理し，抗原性を示す蛋白の分子量を推定した。その結果，患者には38～45 kDaの強いバンドと，75～70 kDa，50～55 kDa，23～26 kDaに弱い反応がみられた。したがって，患者は各々の蛋白質に対するIgEを有することが判明した。なお，健常者にはこれらのバンドは検出されず，IgEを有さないことが証明された（図2）。

診断および経過　初診時までの経過と検査結果から，フランス製赤色マカロンに含有されているカルミン，コチニール色素によるアナフィラキシーと診断した。その後患者は，コチニール色素やカルミン含有の食物，飲料物の摂取およびカルミン含有の化粧品の使用を控えており，特に問題は生じていない。

Ⅲ．考　察

コチニールはヒラウチワサボテンの寄生生物であるカイガラムシ科エンジムシの一種であり，コチニール色素はコチニールの雌を乾燥化したものから抽出される赤色色素である。コチニール色素は，天然系食用着色料のなかでは安全性がもっとも高く，日本の食品衛生法の既存添加物名簿に収載され，紅色の飲料水，漬物，蒲鉾，菓子類などに含まれている。

コチニール色素の主成分はカルミン酸で，カルミン酸にアルミニウムなどの金属を結合させて不溶性にしたアルミニウムレーキ色素がカルミンである。カルミンは，日本では医薬品添加物や化粧品では使用が認められているが，既存添加物名簿所収でないため，食品添加物としての使用が認められていない。しかし欧州連合（EU）では，コチニール色素と同様にカルミンもE120として食品ごとに使用が許可されている。

自験例はSPTにてコチニール色素抽出液3+，カルミン3+，フランス製赤色マカロン2+だったことや，ウエスタンブロッティング法にてコチニール色素の38～45 kDaの蛋白質に対するIgEが認められたことから，フランス製マカロン中のカルミン，コチニール色素によるアレルギーと診断した。また，不純蛋白を除いたコチニール色素精製品のSPTは+と減弱していたことからは，コチニール色素のアレルゲンは供給源であるカイガラムシ由来の不純蛋白質であると考えられた。これらの結果は，以前われわれが報告した症例と同様であった[3)～5)]。

日本では1997～2013年までに，学会発表を含めコチニール色素のアレルギーは，表2のように18例報告されている[3)～11)]。報告数は2008年までは7例であったが，2010年以降は11例と増加している。コチニール色素は古来インカ帝国の時代から広く使用されてきた色素であるが，これによるアレルギーの報告は最近2，3年で急激に増加している。その理由として，コチニール色素アレルギーの発表以前は診断がつかずにいた患者も，その原因の可能性を認識するようになったことがあげられる。また原因物質は，2008年までは飲料物が5例と多かったが，2010年からは自験例を含め赤色マカロンが6例と多い。マカロンはフランスの代表的菓子で，種々の色どりが豊富で人気があり，赤色マカロンでは鮮明な赤色を出すためにカルミンが使用されている。自験例はフランス滞在中に摂食しているが，日本にても近年，フランス製輸入マカロンが販売されていることも報告増加の一因

表2　本邦におけるコチニール色素アレルギー報告例（1997～2012年）

症例	報告者	報告年	年齢/性別	原因物質	接触皮膚炎の有無
1	Kume ら[6)]	1997	28/女	カンパリ，イチゴ牛乳	なし
2	Yamamoto ら[7)]	1998	26/女	口紅	あり（口紅）
3	山川ら[4)]	2004	35/女	カンパリ	なし
4	山川ら[4)]	2004	44/女	カンパリ	なし
5	山川ら[4)]	2004	52/女	イチゴジュース	なし
6	寿ら[8)]	2005	28/女	赤色食品	なし
7	竹尾ら[9)]	2007	23/女	カンパリ	あり（化粧品）
8	豊永ら[10)]	2010	31/女	赤色マカロン	あり（口紅アイシャドー）
9	秋山ら[11)]	2011	26/女	カンパリ	なし
10	山川ら[5)]	2011	49/女	グアバジュース	なし
11	田中	2012	30/女	栄養ドリンク	なし
12	佐伯	2012	40/女	チョコマカロン	なし
13	野村	2012	42/女	カンパリ，ジュース，ソーセージ	なし
14	原田	2012	49/女	赤色マカロン	あり（口紅）
15	原田	2012	33/女	赤色マカロン	あり（口紅）
16	大澤	2012	26/女	ワイン	なし
17	石川	2012	52/女	赤色マカロン	なし
18	自験例	2013	30/女	赤色マカロン	はっきりせず

であろう。

前述のように，カルミンは日本において食品添加物としての使用は認められていない。しかし化粧品への使用は可能であり，さらに輸入された化粧品の赤色色素としては繁用されている。日本における報告全例が女性であり，年齢が23～52歳まで（平均年齢36歳）であることは，注目すべき点である。現在までの18例のなかには，口紅や頬紅での接触炎の既往があるものが5例ある。自験例はカルミン含有の口紅や頬紅を使用していた経験があり，SPTにてもこれらのカルミン含有化粧品で陽性となっている。2008年，経皮的接触はアレルゲンの感作を惹起促進するという，食物アレルギー獲得機序仮説が提唱された[12]。自然界にある蛋白質を含有した自然成分化粧品を使用することにより，経皮感作が成立し，その関連食品のアレルギーを獲得する可能性がある。コチニール色素のアレルギーについても，すでに1994年に口紅で感作された後に喘息を発症した報告がある[13]。化粧品の口紅，頬紅，アイシャドーなどに含まれるコチニール色素あるいはカルミンを経皮吸収し，その感作成立後，これらを含む食物の摂取によるアナフィラキシーが誘発されたという経皮感作経路が想定される。一方，経皮曝露経験のない症例は，コチニール色素を経口摂取し感作成立後，再び摂取して生じる古典的経口感作も考えられる。

現在コチニール色素からアレルゲン蛋白質が除去され，より純度の高いコチニール色素精製品が食品添加物として使用されつつある。このため経口感作によるコチニール色素のアレルギーの獲得は減少することが期待される。しかし輸入品のなかには，コチニール色素を含有することが把握されていない食品もある[5]。またコチニール色素と明記されていても，そのコチニール色素には不純タンパク質が含まれている外国製品もある。さらに，日本では食料品に入れることができないカルミンが輸入品に含有されている可能性があり，注意が必要である。化粧品の経皮感作の問題からは，化粧品においても，現在使用されているカルミンから純度の高い精製コチニール色素が使用されることが望ましい。経口感作とともに経皮感作を減らすことにより，コチニール色素のアレルギー獲得機会が減少し，新たな患者発症が予防されることが望まれる。

2012年5月11日，消費者庁はコチニール色素に関する注意喚起を発表した。これにより新たにコチニール色素によるアレルギーの報告が増え，疫学，実態，感作経路などの把握がより明確となることを期待したい。

謝　辞　コチニール色素抽出物，精製品，カルミンのご提供を賜り，ご助言をいただきました三栄源エフ・エフ・アイ株式会社に厚く御礼申し上げます。

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文　献

1) Lloyd AG：Food Chem. 5：91, 1980
2) Alas K et al：Acta Allergol. 29：238, 1974
3) 山川有子：日本食品化学研究振興財団第10回研究成果報告書：40-44，2004
4) Yamakawa Y et al：J Dermatol. 36：72-74, 2009
5) 山川有子ほか：臨皮，66：8-13，2012
6) Kume A et al：Environ Dermatol. 6：148-152, 1999
7) Yamamoto A et al：Environ Dermatol. 6：185-189, 1999
8) 寿　順久ほか：アレルギー，56：1510-1514，2007
9) 竹尾直子ほか：日皮会誌，118：1085-1093，2008
10) 豊永三恵子ほか：皮膚病診療，33：515-518，2011
11) 秋山美知子ほか：皮膚臨床，53：701-704，2011
12) Lack G：J Allergy Clin Immunol. 121：1331-1336, 2008
13) Kägi MK et al：Lancet, 344：60-61, 1994

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Spectrophotometric Sequential Injection Analysis System for Estimating the Concentration of Lipid Hydroperoxides in Edible Oils

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Abstract

A sequential injection analysis (SIA) system with spectrophotometric detection was developed as an alternative method for estimating the concentration of lipid hydroperoxides, which are the primary products of the lipid peroxidation process. The lipid hydroperoxide quantification was based on a ferric thiocyanate method. Benzoyl peroxide was used to produce a standard calibration curve for the estimating the lipid hydroperoxide concentrations. The linear range was up to 0.5 mmol/L, for benzoyl peroxide standard, and the limit of detection (3S/N) was 0.015 mmol/L. The relative standard deviation, at 0.3 mmol/L, was 1.3% for 11 injections, and the recoveries were found to be in the range of 97.2–99.5%. The lipid hydroperoxide concentrations in eight edible oils determined using the proposed SIA system were significantly correlated with peroxide values obtained using a conventional American Oil Chemists' Society's method ($r = 0.987$, $n = 8$, $p < 0.01$).

Keywords Sequential injection analysis, lipid hydroperoxide, ferric thiocyanate method, peroxide value

1. Introduction

The quality of a fat or oil is defined and controlled by several physical and chemical parameters, such as its viscosity, color, turbidity, iodine value, *p*-anisidine value, saponification value. These parameters depend on the source of the oil (e.g., for a plant oil, the geographic, climatic, and agronomic growth variables affect the parameters), oil's processing and storage conditions. The fatty acid composition and the oxidative stability of a fat or oil are clearly of utmost importance to its qualities [1].

A fat or oil may be oxidized in different ways, including through autoxidation, photo-oxidation, thermal oxidation, or hydrolytic processes. All of these processes lead to the production of undesirable flavors and of products that are harmful to health.

The oxidation mechanism for an unsaturated lipid comprises three steps; initiation, propagation, and termination. Each step leads to the formation of intermediate and/or final products. Lipid hydroperoxides, which are the fundamental primary products of the lipid peroxidation process, can be changed into secondary products, such as peroxides, aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones [2–3]. These lipid peroxidation products are not only poisonous but also accelerate nutritional damage, increasing the rate at which fats and oils become rancid during the production, storage, and marketing processes [3–5].

Some parameters, such as the peroxide value (POV), the acid value and carbonyl value, can be used to evaluate the degree of lipid peroxidation in fats and oils. POV is one of the most important parameter, affecting the quality of fats and oils, for determining the amount of lipid hydroperoxides. It is often expressed in milliequivalents peroxide/kilogram of a fat and oil.

The iodometric titration method is an official method and is the most widely used method for determining POV [6–7]. It is based on the redox reaction between hydroperoxides in a fat or oil sample and potassium iodide (KI) under the acidic medium to form released iodine (I_2). The released iodine forms a complex with soluble starch, which acts as an indicator. The iodine is quantified by titrating with sodium thiosulfate. This method is simple and sensitive, but its accuracy depends on many parameters, such as reaction time, light, presence of oxygen, and temperature. Moreover, it is a batchwise method that requires rather large amounts of analytical sample and organic solvents (up to 5 g of a fat and oil sample and 50 mL of solvent per analysis).

To minimize the deleterious organic solvent, a Fourier transform infrared (FTIR) spectroscopic method has been developed to quantify peroxide in various samples, such as palm olein oil, crude palm oil, crude palm kernel oil, sunflower oil, olive oil, and rapeseed oil [8–12]. The FTIR method has significant advantages over the standard American Oil Chemists' Society's (AOCS) iodometric titration method in terms of the consumption of solvent and reagents and its rapidity. A chemometric approach based on partial least-squares analysis has been often used with the FTIR technique to construct a calibration model for predicting the POV in the samples. The disadvantages of this method are as follows: can be cumbersome, requires a skillful operator to construct the model, and has a relatively low degree of specificity.

Automatic systems that offer precise and accurate results, rapid yielding (giving high sample throughput) based on flow injection analysis, and several detection methods including spectrophotometry [2, 4, 5, 13], fluorometry [3], potentiometry with a triiodine electrode [14] and chemiluminescence [15–16], have been developed for determining the lipid hydroperoxides in oils and fats. A stable and continuous baseline is necessary when using a basic flow injection system. Thus, the reagents are consumed continuously, causing large amounts of waste generation.

In this study, a sequential injection analysis (SIA) system was developed for determining the lipid hydroperoxides in edible oils. This system used the well-established reaction; the oxidation of Fe(II) to Fe(III) by hydroperoxide and/or peroxide in the oil or fat samples. Subsequently, the colorimetric measurement of thiocyanate complex was used as the principle reaction [17–22]. In this system, benzoyl peroxide was selected as the standard peroxide for building the calibration curve that was used to determine the lipid hydroperoxide, because benzoyl peroxide is more stable, is easier to handle and reacts more rapidly than hydrogen peroxide [2].

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2. Experimental

2.1 Chemicals

All of the chemical and reagent used in this study were of analytical grade, and they were used without further purification. Milli-Q water was purified using Auto Pure WQ501 (Yamato Scientific Co., Ltd., Tokyo, Japan) was used throughout the experiments.

A stock solution of 20% (w/v) SCN^- was prepared by dissolving 2 g of ammonium thiocyanate (Wako Pure Chemical Industries, Osaka, Japan) in 10 mL of MilliQ water. The working solution for each experiment was prepared by diluting the stock solution with MilliQ water to indicated concentration.

A stock solution of 12.5 mmol/L Fe(II) solution was prepared by dissolving 24.9 mg of iron(II) chloride tetrahydrate (Wako Pure Chemical Industries) in 10 mL of MilliQ water. The working Fe(II) solution for each experiment was prepared by diluting the stock solution with 3.5% HCl in 75% ethanol in proper concentration.

A standard benzoyl peroxide stock solution (10 mmol/L) was prepared by dissolving 16.5 mg of benzoyl peroxide (Sigma-Aldrich, St. Louis, MO, USA) in 99.7% isopropanol in the 10 mL volumetric flask. The working benzoyl peroxide solution for each experiment was prepared by diluting the stock solution with 5 mL of isopropanol to indicated concentrations.

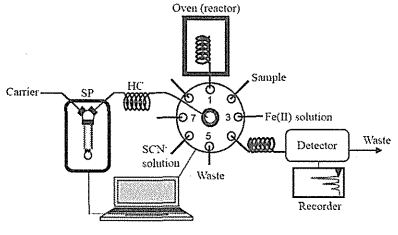

Figure 1. Proposed SIA system for determining the lipid hydroperoxides. Carrier, 80% isopropanol; SP, syringe pump; HC, holding coil.

2.2 Edible oil samples

Edible oil samples were purchased in local supermarkets and kept under refrigeration until they were analyzed. Before the analysis on the developed SIA system, each sample was diluted to reduce viscosity: 0.2 g of an oil sample was dissolved in 5 mL of isopropanol, and this solution was then diluted in four times its volume in isopropanol. The lipid hydroperoxide concentration in this solution was then determined.

2.3 AOCS official method Cd 8b-90 for the determination of lipid hydroperoxides

The POV (milliequivalents peroxide/kg: meq/kg) of each edible oil was determined according to the AOCS official method Cd 8b-90 [6] in order to compare the results obtained using our SIA system.

2.4 SIA system for determining the lipid hydroperoxides

A schematic of the proposed SIA system is shown in Figure 1. The system (MGC JAPAN Co., Ltd., Japan) consisted of a 2500 µL syringe pump, a holding coil (Teflon tube; 1.5 mm i.d., 1.5 m long), an eight ports selection valve, a reactor tube (Teflon tube; 0.8 mm i.d. , 4 m long), a mixing coil (Teflon tube; 0.8 mm i.d., 1 m long), a visible spectrophotometer S-3250 (Soma Optics, Tokyo, Japan), a recorder (Unicorder U-228; Pantos Nippon Denshi Kagaku, Kyoto, Japan), a reactor (Reactor 522; Flom, Tokyo, Japan) and a personal computer. An SIA MPV lite ever 2.55 for auto-pret system (MGC JAPAN Co., Ltd.) was used to provide the automatic control of this system.

2.5 Analytical process

Protocol sequence for determining the lipid hydroperoxides in oil samples are listed in Table 1. The analysis was started by operation of syringe pump to aspirate 100 µL of air via valve position 1 to a holding coil, followed by aspirating 40 µL of SCN^- solution via valve position 6, 30 µL of Fe(II) solution via valve position 3, and 100 µL of standard benzoyl peroxide solution or oil sample via valve position 2, 30 µL of Fe(II) solution, and 40 µL of SCN^- solution to the holding coil. After that, the aspirated solutions were dispensed through valve position 1 to a reactor by reversing a flow of the syringe pump. Three rounds of flow reversal were used to improve mixing of between analyte and reagents. The mixed solution was incubated at 60°C for 40 sec at the reactor. A 150 µL of air and mixed solution that was held in the line but outside the reactor was aspirated into a holding coil and dispensed to waste. After that, 150 µL of mixed solution was aspirated into the holding coil and dispensed toward a detector in order to detect the Fe(III)-thiocyanate complex at 500 nm. The residue of mixed solution in the line was sucked into the holding coil, followed by dispensing to waste. As a final step, it was necessary to clean the reactor to remove the remained mixed solution to prevent the contamination of reaction in a next cycle.

3. Results and Discussion

3.1 Optimization of the SIA system for determining the lipid hydroperoxides

Lipid hydroperoxide readily oxidizes Fe(II) to Fe(III), and this reaction was used as the principle to qualify the lipid hydroperoxide. It could be measured colorimetrically as the thiocyanate complex which has an absorption at 500 nm. Using the proposed SIA system (Figure 1), the effects of

Table 1. Protocol sequence of the proposed SIA system for estimating the concentration of lipid hydroperoxides.

Step	Operation	Valve position	Volume (μL)	Flow rate (μL/sec)
1	Aspiration of air	1	100	50
2	Aspiration of SCN^- to holding coil	6	40	50
3	Aspiration of Fe(II) to holding coil	3	30	50
4	Aspiration of standard or sample	2	100	50
5	Aspiration of Fe(II) to holding coil	3	30	50
6	Aspiration of SCN^- to holding coil	6	40	50
7	Dispensing aspirated zones to reactor and flow reversal at three times	1	-	-
8	Incubation of mixed solution 40 second	1	-	-
9*	Aspiration of mixed solution to holding coil	1	150	50
10	Dispensing solution to waste	5	Empty	500
11	Aspiration of mixed solution to holding coil	1	150	50
12	Aspiration of carrier to syringe	-	2350	500
13	Dispensing solution to detector	4	Empty	50
14**	Aspiration of solution to holding coil	1	500	100
15	Dispensing solution to waste	5	Empty	500
Cleaning step				
16	Aspiration of carrier to syringe	-	2000	500
17	Dispensing carrier to reactor	1	1,000	100
18**	Aspiration of solution to holding coil	1	2	50
19	Dispensing solution to waste	5	Empty	500

*This discarding step was the aspiration of the solution which was held in the line outside the reactor.
**The discarding step was the aspiration of the solution of the mixture at higher volume than the remaining solution to ensure complete removal.

different parameters, such as the aspiration sequence, the number of flow reversal, the concentrations of Fe(II) and SCN^- solution, the aspirated volume of sample, the reaction temperature, and the reaction time, were investigated.

As shown in Figure 2, three aspiration sequences were tested for determining the lipid hydroperoxides. As a result, sequence B was found to provide good sensitivity and required the shortest time to aspirate all of the reagents onto the holding coil. Therefore, the aspiration sequence B was selected for further experiments.

The solution mixing in the system initially occurred in the holding coil, but the solutions could not mix very well without additional processes. Between zero to four flow reversal steps were examined to determine the effectiveness of the number of flow reversal steps for improving the mixing of the solutions and increasing the yield of the product. Consequently, the peak height increased as the number of flow reversal steps increased, and the best sensitivity was found using three flow reversal steps. The signal was somewhat lower using four reversal steps than using three steps, because the mixed solution was diffused further in the carrier stream with each additional flow steps. Therefore, three flow reversal steps were chosen for the proposed SIA system.

Sample aspiration volumes of 20 – 150 μL were tested using a 0.5 mmol/L standard benzoyl peroxide, and the peak height was found to increase as aspiration volume increased. Using a high sample aspiration volume, the amount of analyte used was also increased to enhance the Fe(III)–thiocyanate complex. From economic view, a 100 μL of sample aspiration volume was selected for further experiments.

The effect of reaction temperature on the coloration of the Fe(III)–thiocyanate complex was investigated between 50 and 70°C. The coloration of the Fe(III)–thiocyanate complex was found to be influenced by the increasing temperature, and for our SIA system, 60°C was selected as the reaction temperature.

The effect of reaction time, in the range of 0 – 60 sec, was investigated using a reaction temperature of 60°C. The peak height was found to increase as the incubation time increased up to 40 sec, and it increased slightly after that time. Therefore, a reaction time of 40 sec was selected for the proposed SIA system.

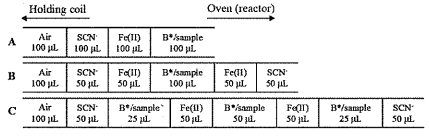

Figure 2. Aspiration sequences for the proposed SIA system.
*B: benzoyl peroxide

In the range of 0.6 – 4.4 mmol/L, the effect of the Fe(II) concentration on the coloration was investigated using the experimental conditions that had already been selected, as described above, except for the SCN^- concentration. The peak height was found to reach a maximum and remain constant for the Fe(II) concentrations of over 3.1 mmol/L. Therefore, 3.1 mmol/L of Fe(II) was chosen.

Fe(II) aspiration volumes of 20 – 80 μL were used to determine the assay condition using the experimental conditions that had already been selected, as described above, except for the SCN^- concentration. The results of the study were expected to determine highest sensitivity that could be achieved using a low volume of Fe(II) solution. An Fe(II) aspiration volume of 60 μL was concluded to provide the best sensitivity in terms of the peak height. Over this volume, the peak height of signal decreased due to the effect of diffusion in the mixed solution.

Different SCN^- concentrations (2 – 8% (w/v)) were also tested using the experimental conditions that had already been selected. The peak height was found to increase as the SCN^- concentration increased up to 6%, and the sensitivity slightly ameliorated at higher concentrations. Therefore, the recommended SCN^- concentration for determining the lipid hydroperoxide using our system was 6%.

The effects of aspiration volume of SCN^- solution was also tested between 20 and 100 μL. The peak height increased as the volume of SCN^- solution was increased up to 60 μL, and the sensitivity remained constant at higher volume. Therefore, 60 μL of SCN^- solution was used for the determination of lipid hydroperoxides.

3.2. Analytical characteristics

As shown in Figure 3, the response signals, using benzoyl peroxide as the standard reagent to quantify the lipid hydroperoxides, were linearly increased over the 0 – 0.5 mmol/L in the proposed system using the selected conditions. The significant linearity was recognized between the concentration of benzoyl peroxide and the peak height ($r = 0.998$, $n = 6$, $p < 0.01$). The detection limit (defined as the concentration giving a signal to noise ratio of 3) was estimated to be 0.015 mmol/L. The reproducibility of the method was determined by analyzing a 0.3 mmol/L standard benzoyl peroxide 11 times. The relative standard deviation was 1.3%. It was possible to analyze 10 samples per hour (6 min per analysis cycle), including all of the steps (aspiration, incubation, detection, and cleaning).

3.3. Recoveries

Two kinds of oil samples, rapeseed and olive oils, were each spiked with 0.1 and 0.2 mmol/L of standard benzoyl peroxide and analyzed to determine the recoveries using the developed SIA system (Table 2). The recoveries were 97.2 – 99.5%. This shows that the SIA system provided appropriate recoveries for determining total lipid hydroperoxides in edible oils.

3.4. Application to edible oil samples

The proposed SIA system was applied to determine the lipid hydroperoxides in eight edible oils. Before the analysis using SIA system, a 0.2 g aliquot of each oil sample was dissolved in 5 mL of isopropanol, and then it was further diluted fourfold with isopropanol. The concentrations of lipid hydroperoxides are presented in Table 3, expressed in mmol benzoyl peroxide equivalent (eq.) per litter. The POV (meq/kg) was also determined using the AOCS method in order to compare the results (Table 3). Consequently, the results obtained using the SIA system and the AOCS method were linearly-related with a correlation coefficient of 0.987 ($n = 8$, $p < 0.01$).

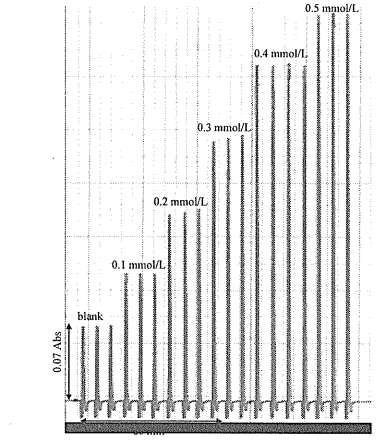

Figure 3. Typical response signals for benzoyl peroxide obtained using the proposed SIA system.

Table 2. Recovery tests of the lipid hydroperoxides in edible oils using the proposed SIA system (n = 4).

Edible oils	Lipid hydroperoxide (mmol benzoyl peroxide eq./L) Found	Spiked	Found in total	Recovery (%)
Rapeseed	0.052 ± 0.001	0.1	0.152 ± 0.004	99.4 ± 3.6
		0.2	0.251 ± 0.007	99.5 ± 3.6
Olive	0.136 ± 0.002	0.1	0.234 ± 0.005	97.2 ± 4.9
		0.2	0.332 ± 0.004	98.0 ± 2.1

Table 3. Determination of the lipid hydroperoxides in edible oils using the proposed SIA system and the AOCS method.

Edible oils	SIA system (n = 4) Lipid hydroperoxide (mmol benzoyl peroxide eq./L)	AOCS method (n = 2) POV (meq/kg)
Rapeseed	0.052 ± 0.001	1.1 ± 0.3
Soybean	0.065 ± 0.001	1.6 ± 0.1
Sesame	0.068 ± 0.001	1.8 ± 0.6
Olive	0.436 ± 0.002	7.2 ± 0.1
Grape seed	0.119 ± 0.001	2.2 ± 0.0
Corn	0.113 ± 0.002	2.4 ± 0.1
Perilla	0.426 ± 0.004	8.4 ± 0.1
Flaxseed	0.107 ± 0.001	1.6 ± 0.3

4. Conclusion

Lipid hydroperoxides, which are the primary products of the lipid peroxidation process, could be determined using a simple, rapid, and automatic SIA system with spectrophotometric detection. The concentrations of lipid hydroperoxides determined using the proposed SIA method correlated well with the POV determined using the AOCS official method ($r = 0.987$, $n = 8$, $p < 0.01$). The proposed system produces less organic waste than the conventional methods; the proposed SIA system used 240 μL of reagents for detection of lipid hydroperoxides and 4000 μL of career solution for moving of analyte and cleaning of line, whereas the ACOS method used approximately 55 mL of reagent per titration. In addition, the high reproducibility (RSD = 1.3%, n = 11) and high throughput (10 samples per hour) were obtained in the proposed SIA system. Thus, we concluded that the SIA system proposed here can be used as an alternative method for the routine determination of lipid hydroperoxides in edible oils.

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References

[1] F. Shahidi, Quality Assurance of Fats and Oils: "Bailey's Industrial Oil and Fat Products", John Wiley & Sons, Inc., Hoboken, NJ, 2005.

[2] M. S. Bloomfield, *Analyst*, **124**, 1865 (1999).

[3] K. Akasaka, T. Takamura, H. Ohrui, H. Meguro, and K. Hashimoto, *Biosci. Biotechnol. Biochem.*, **60**, 1772 (1996).

[4] K. Tian and P.K. Dasgupta, *Anal. Chem.*, **71**, 2053 (1999).

[5] G.P. Avila, A. Salvador, and M. De La Guardia, *Analyst*, **122**, 1543 (1997).

[6] American Oil Chemists' Society (4th Ed.), Method Cd 8b-90, American Oil Chemists' Society, Champaign, IL, 1996.

[7] Association of Analytical Communities (17th Ed.), Method 965.33, Peroxide Value of Oils and Fats, AOAC International, Gaithersburg, MD, 2002.

[8] M.H. Moh, T.S. Tang, Y.B.C. Man, and O.M. Lai, *J. Food Lipid*, **6**, 261 (1999).

[9] G. Setiowaty, Y.B. Che Man, S. Jinap, and M.H. Moh, *Phytochem. Anal.*, 11, 74 (2000).

[10] M.D. Guillén and N. Cabo, *Food Chem.*, **77**, 503 (2002).

[11] S. Armenta, S. Garrigues, and M. de la Guardia, *Anal. Chim. Acta*, **596**, 330 (2007).

[12] K. Ma, F. van de Voort, J. Sedman, and A. Ismail, *J. Am. Oil Chem. Soc.*, **74**, 897 (1997).

[13] P.G. Nouros, C. A. Georgiou, and M.G. Polissiou, *Anal. Chim. Acta*, **389**, 239 (1999).

[14] B. Saad, W.T. Wai, B.P. Lim, and M.I. Saleh, *Anal. Chim. Acta*, **565**, 261 (2006).

[15] J.P. Bunting, and D.A. Gray, *J. Am. Oil Chem. Soc.*, **80**, 951 (2003).

[16] J.H. Sohn, Y. Taki, H. Ushio, and T. Ohshima, *Lipids*, **40**, 203 (2005).

[17] A. Lips, R. Chapman, and W. McFarlane, *J. Am. Oil Chem. Soc.*, **20**, 240 (1943).

[18] R. Chapman and K. Mackay, *J. Am. Oil Chem. Soc.*, **26**, 360 (1949).

[19] I.M. Kolthoff and A.I. Medalia, *Anal. Chem.* **23**, 595 (1951).

[20] C.M. Stine, H.A. Harland, S.T. Coulter, and R. Jenness, *J. Dairy Sci.* **37**, 202 (1954).

[21] B. Mihaljević, B. Katušin-Ražem, and D. Ražem, *Free Radic. Biol. Med.*, **21**, 53 (1996).

[22] D. Hornero-Méndez, A. Pérez-Gálvez, and M. Mínguez-Mosquera, *J. Am. Oil Chem. Soc.*, **78**, 1151 (2001).

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Quantification of acesulfame potassium in processed foods by quantitative ^{1}H NMR

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ABSTRACT

Acesulfame potassium (AceK), a high-intensity and non-caloric artificial sweetener, is used in various processed foods as a food additive. In this study, we established and validated a method for determining the AceK content in various processed foods by solvent extraction and quantitative ^{1}H NMR, using a certified reference material as the internal standard. In the recovery test, the proposed method gave satisfactory recoveries (88.4–99.6%) and repeatabilities (0.6–5.6%) for various processed foods. The limit of quantification was confirmed as 0.13 g kg^{-1}, which was sufficiently low for the purposes of monitoring AceK levels. In the analysis of commercially processed foods containing AceK, all AceK contents determined by the proposed method were in good agreement with those obtained by a conventional method based on dialysis and HPLC. Moreover, this method can achieve rapid quantification and yields analytical data with traceability to the International System of Units (SI) without the need for an authentic analyte standard. Therefore, the proposed method is a useful and practical tool for the determination of AceK in processed foods.

1. Introduction

Acesulfame potassium (AceK) is a high-intensity, non-caloric artificial sweetener with a sweetening strength approximately 200 times that of sucrose. As such, AceK is consumed as a sugar substitute in order to restrict sugar intake and control blood glucose levels [1]. In addition, this compound is used as a food additive in more than 100 countries [2] due to its high stability under acidic conditions and heating. Many countries regulate the use of AceK according to specific legislation regarding food additives based on acceptable daily intake (ADI) values (0–15 mg/kg of body weight per day) established by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) [3]. The maximum usage level of AceK in various foods is determined by the Codex Committee. In Japan, AceK can currently be used in specific processed foods such as chewing gum, jam, ice cream, soft drink, and beverages containing *Lactobacillus*. The maximum level is set between 0.35 g kg^{-1} and 15 g kg^{-1}. Accordingly, reliable analytical methods are necessary to monitor AceK content in processed foods and to assure regulatory compliance.

Several analytical methods have been applied in the determination of AceK in foodstuffs such as processed foods and beverages. Among them, chromatographic methods including HPLC [4–6], ion chromatography [7], and HPLC–MS/MS [8] are used most frequently in the quantification of AceK. In these chromatographic methods, an authentic standard, such as a certified reference material (CRM) with a proven identity and purity determined by metrological valid procedures, is required in order to obtain reliable analytical results.

On the other hand, quantitative NMR (qNMR) is a considerably powerful tool to quantify analytes without the need for identical standards, and is considered to be a primary ratio method [9,10]. In particular, quantification using ^{1}H NMR (qHNMR) is widely applied in the quantification of medicines [11,12], beverage components [13,14], and natural products in medicinal plant extracts [15–17] due to its high sensitivity and the widespread presence of ^{1}H nuclei in organic molecules [18]. In qHNMR, the content or concentration of the analyte is determined using the ratio between the integral values of a specific signal of the analyte to that of an internal standard (IS). The integral values of the analyte and IS are directly proportional to the number of protons per resonance line times the molar concentration of the analyte and the IS, respectively. Therefore, the absolute quantitative value of the analyte can be determined with metrological traceability to the International System of Units (SI), if a specific CRM is used as the IS in qHNMR. Recently, qHNMR with IS, also called AQARI (accurate quantitative NMR with internal reference substance), was reported [19]. We previously demonstrated that qHNMR using an SI-traceable reference material combined with solvent extraction could be utilized to determine the absolute content of preservatives in processed foods [20,21].

In this study, we developed a novel method for the determination of AceK in processed food using solvent extraction and qHNMR. To determine the accuracy and precision, we also applied and validated the proposed method using various processed foods. Furthermore, we compared the proposed method to a conventional method based on dialysis and HPLC.

Abbreviations: AceK, Acesulfame potassium; ADI, acceptable daily intake values; CRM, certified reference material; qNMR, quantitative NMR; IS, internal standard; LOQ, limit of quantification

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2. Materials and methods

2.1. Processed food samples

Twelve processed foods without AceK (chewing gum, red bean paste, candy, jelly, biscuits, ice cream, jam, salad dressing, vegetable pickled in soybean sauce, sherbet, a soft drink, and a beverage containing *Lactobacillus* species) and five processed foods that specified the use of AceK on the label (candy, two types of jellies, biscuits, and a soft drink) were purchased from markets in Tokyo, Japan.

2.2. Chemicals and reagents

Analytical grade AceK (purity: > 98%) and 2,2-dimethyl-2-silapentane- 5-sulfonate-d_6 sodium salt (DSS-d_6) (Code no. 048-31071, Lot. EPL1095, purity: 92.2 ± 0.7%), the certified reference material, were obtained from Wako Pure Chemical Industries, Ltd. DMSO-d_6 and HPLC grade acetonitrile were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Ultrapure water (purified to 18 MΩ cm) using a Millipore (Danvers, MA, USA) Milli-Q water purification system was used. All other chemicals were of analytical grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hexane saturated with acetonitrile and acetonitrile saturated with hexane were prepared as follows: 500 mL of acetonitrile and 500 mL of hexane were transferred to a separating funnel and were shaken vigorously. After the upper and lower layers were completely separated, they were used as hexane saturated with acetonitrile and acetonitrile saturated with hexane, respectively. Acesulfame was prepared by the acid hydrolysis of AceK as follows: AceK (8 g) in 5% sulfuric acid (100 mL) was allowed to sit at room temperature for 15 min. Subsequently, the solution was partitioned with ethyl acetate (100 mL^2) and the ethyl acetate layer was evaporated to yield acesulfame (6 g). Prepared acesulfame was completely characterized using DART-TOFMS and NMR.

2.3. Instruments

^{1}H NMR was measured on a JNM-ECA 600 spectrometer (JEOL Ltd., Tokyo, Japan). HPLC was performed on a Shimadzu HPLC system (LC-10A) equipped with an SPD-M10Avp diode array detector (Shimadzu Corporation, Kyoto, Japan). Homogenization was carried out using an ULTRA-TURRAX T 25 digital homogenizer (IKA Works, Wilmington, NC, USA). An XP2U (Mettler-Toledo AG, Greifensee, Switzerland) ultra-micro balance was used to weigh the analytical grade AK for qHNMR analysis, and DSS-d_6. A ME235S (Sartorius, Bloomington, MN, USA) semi-micro balance was used to weigh the processed foods, DSS-d_6 stock solution, and DMSO-d_6.

2.4. Pretreatment of the processed foods

2.4.1. Solvent extraction

The four processed foods (chewing gum, candy, jelly, and vegetable pickled in soy bean sauce) were cut into small pieces before weighing. Portions (5 g) of all processed foods were accurately weighed in glass centrifuge tubes. A saturated solution of sodium chloride (20 mL), 10 vol% sulfuric acid (4 mL), and diethyl ether (20 mL) were added, and the mixture was subjected to high-speed homogenization for approximately 1 min. The homogenate was centrifuged at 1500*g* for 5 min, and then the upper layer was transferred to a clean flask. The residue was homogenized again with 20 mL of diethyl ether and centrifuged at 1500*g* for 5 min. The upper layers were combined and then evaporated at 30 °C to yield the qHNMR sample.

For the biscuit, ice cream, jam, and salad dressing, 10 mL of methanol was added to the extract to remove oil components after the evaporation of the upper layer, as described above, and then the samples were centrifuged. Finally, the methanol layer was transferred to a clean flask and then evaporated to yield the qHNMR sample.

For chewing gum, 20 mL of hexane saturated acetonitrile and acetonitrile saturated hexane was added to the extract following the evaporation of the upper layer, as described above, and then the sample was centrifuged. Subsequently, the acetonitrile layer was evaporated to obtain the qHNMR sample.

2.5. Dialysis

The dialysis procedure utilized in this study was the same as described previously [4]. Briefly, the solid processed foods were cut into small pieces prior to weighing. Accurately weighed portions (20 g) of all processed foods were packed into cellulose tubes with 20 mL of 0.01 mol L^{-1} hydrochloric acid containing 10% sodium chloride and set to glass vessel. After the total volume was adjusted to 200 mL by the addition of 0.01 mol L^{-1} hydrochloric acid in a glass vessel, the sample was dialyzed against 0.01 mol L^{-1} hydrochloric acid for 48 h. Then, 20 mL of dialysate and 2 mL of 0.1 mol L^{-1} tetra-*n*-propyl ammonium bromide were transferred to a volumetric flask and the volume was adjusted to 25 mL by the addition of water. Five milliliters of the sample solution was passed through a Sep-Pak Vac C_{18} column. After washing the column with 10 mL of water, the sample was eluted with 10 mL of 40% methanol. The obtained eluate was passed through a Bond Elut SAX column for further purification. After washing the column with 5 mL of 0.5% phosphoric acid and water, AceK was eluted with 5 mL of 0.3 mol L^{-1} hydrochloric acid. This eluate was transferred to a volumetric flask, and the volume was adjusted to 5 mL by the addition of water. The final solution was filtered with a 0.45 μm syringe filter and used for HPLC analysis.

2.6. qHNMR measurements

2.6.1. qHNMR measurement parameters

qHNMR was measured using previously described parameters [20,21]. The data were processed using the qNMR analysis software, Alice 2 for qNMR "PURITY" (JEOL RESONANCE Ltd.). The signal integral value calculated with this software was used for quantitative analysis. The chemical shift of all data was referenced to DSS-d_6 at δ_H 0.00.

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

The reference material, DSS-d_6 (21.91 mg), was dissolved in 100 g of DMSO-d_6 to obtain the stock solution. The concentration

of DSS-d_6 in the stock solution was calculated to be 0.202 mg g^{-1}, based on the purity of DSS-d_6 (92.2%).

2.8. qHNMR measurement of prepared acesulfame

Three samples of acesulfame (10 mg; prepared as described in Section 2.2) were accurately weighed using an ultra-micro balance. Each sample was then placed in a separate vial, and the stock solution (1 g) was added to each vial to fully dissolve the samples. Each solution was then transferred to a 5 mm (outer diameter) NMR tube (Kanto Chemical Co., Inc., Tokyo, Japan) to a height of 4 cm from the bottom of the tube and was subjected to qHNMR analysis. The purities of acesulfame were calculated using the following equation:

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

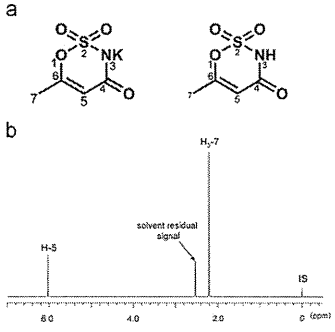

Fig. 1. Chemical structure of AceK (left) and acesulfame (right) (a) and ^{1}H NMR spectrum of acesulfame in DMSO-d_6 containing DSS-d_6 (b). IS, internal standard.

Table 1
Purity of acesulfame determined by qHNMR.

Signal (δ, ppm)	Number of protons	Purity (%)[a]	RSD (%)
2.20	3	97.8	0.1
6.04	1	97.7	0.1

[a] Values represent the mean of three independent experiments.

where I_{AF} and I_{DSS} are the signal integral values for the acesulfame and DSS-d_6, respectively. H_{AF} and H_{DSS} are the number of protons for the acesulfame and DSS-d_6 signals, respectively. M_{AF} and M_{DSS} are the molecular weights of acesulfame and DSS-d_6, respectively. C_{AF} and C_{DSS} are the acesulfame concentration (10 mg g^{-1}) and DSS-d_6 concentration (0.202 mg g^{-1}) in the stock solution.

2.9. Recovery test

The recovery was determined by spiking acesulfame in 12 processed foods and obtaining triplicate measurements. The four levels (0.50, 1.0, 2.5, and 5.0 g kg^{-1}) of acesulfame in each processed food were close to each maximum usage levels of AceK permitted in Japan. Accordingly, these four levels were used as the high concentration levels of each food in this test. After solvent extraction of each spiked food sample, the samples were subjected to qHNMR measurements. The recoveries of each food sample were calculated using the following equation:

$$\text{Recovery}\ (\%) = \frac{\text{Measured concentration}}{\text{Spiked concentration}} \times 100 \quad (2)$$

$$\text{Measured concentration}\ (g\ kg^{-1}) = \frac{I_{AF}/H_{AF}}{I_{DSS}/H_{DSS}} \frac{M_{AF}/W_{FD}}{M_{DSS}/C_{DSS}} \quad (3)$$

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

Table 2
Comparison of gravimetric and experimental values.

Gravimetric value (mg g^{-1})[a]	δ_H 2.20			δ_H 6.04		
	Experimental value (mg g^{-1})[a]	RSD (%)[b]	Relative error (%)[c]	Experimental value (mg g^{-1})[a]	RSD (%)[b]	Relative error (%)[c]
0.15	0.15	0.1	−0.7	0.15	0.9	−2.0
0.30	0.30	0.1	−0.8	0.30	0.0	−0.9
0.61	0.61	0.2	−0.8	0.61	0.5	−0.8
1.2	1.2	0.2	−0.4	1.2	0.3	−0.3
2.4	2.4	0.1	−0.9	2.4	0.1	−0.7
4.9	4.9	0.3	−0.6	4.9	0.3	−0.6
9.8	9.8	0.1	0.5	9.8	0.1	0.3
20	20	0.2	0.8	20	0.2	0.1
25	25	0.2	0.3	25	0.2	0.5

[a] Each gravimetric and experimental values represent the mean of three independent experiments.
[b] RSD, relative standard deviation.
[c] Relative error calculated with the following equation: Relative error= [(experimental value − gravimetrical value)/gravimetrical value] × 100.

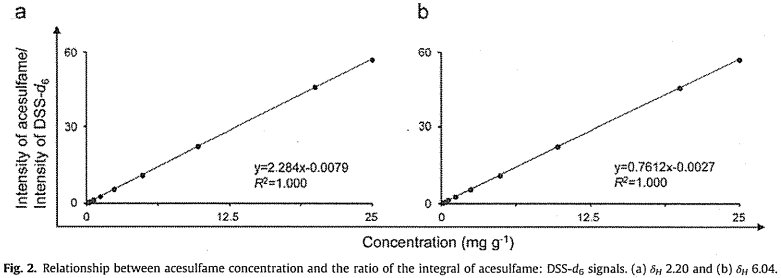

Fig. 2. Relationship between acesulfame concentration and the ratio of the integral of acesulfame: DSS-d_6 signals. (a) δ_H 2.20 and (b) δ_H 6.04.

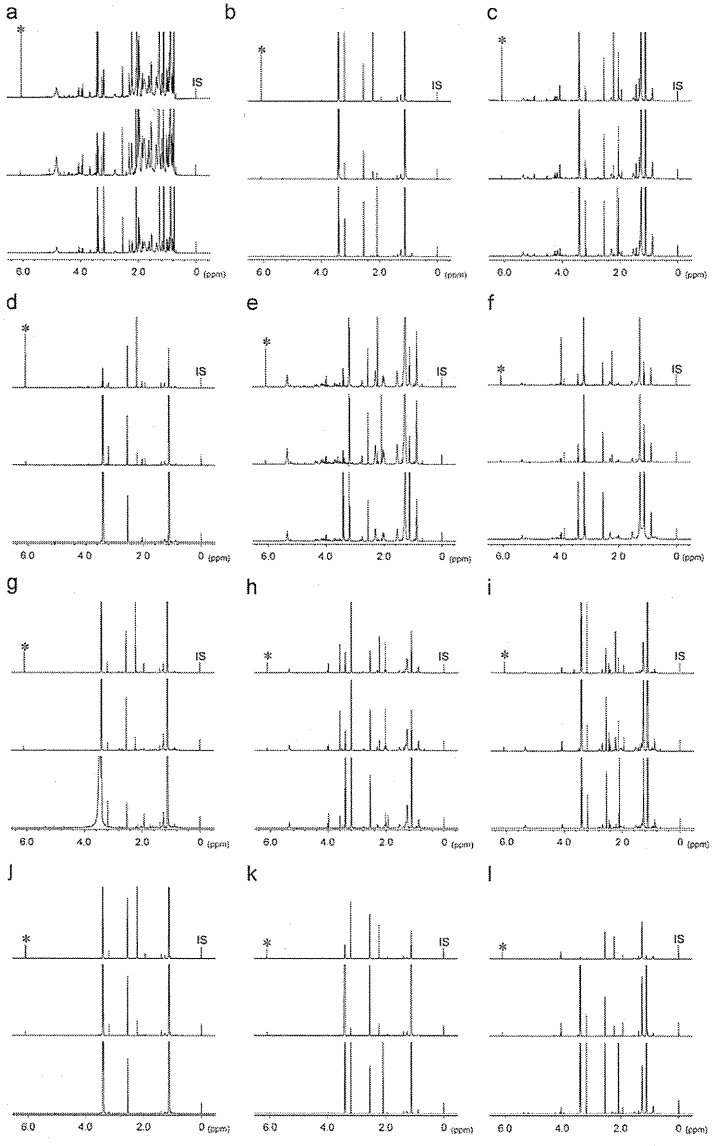

Fig. 3. ^{1}H NMR spectra of samples extracted from processed foods spiked with acesulfame at the high concentration level (top), 0.13 g kg^{-1} (middle), and blank (bottom). The signals marked with asterisks were used for quantification. (a) Chewing gum, (b) red bean paste, (c) candy, (d) jelly, (e) biscuit, (f) ice cream, (g) jam, (h) salad dressing, (g) vegetable pickled in soybean sauce, (h) sherbet, (i) soft drink, and (j) beverage containing *Lactobacillus* species. IS, internal standard (DSS-d_6).

2.10. qHNMR measurement of AceK in the processed foods

The extract obtained from the solvent extraction of each of the processed foods was dissolved in 1 g of stock solution and subjected to qHNMR. In qHNMR, AceK the from respective food samples was detected as acesulfame owing to the acid hydrolysis of AceK during extraction. The AceK contents of the food samples were calculated using the following equation:

$$\text{Content (g kg}^{-1}) = \frac{I_{AF}/H_{AF}}{I_{DSS}/H_{DSS}} \frac{M_{AF}/W_{FD}}{M_{DSS}/C_{DSS}} \times 1.233, \quad (4)$$

where 1.233 is the ratio of the molecular weights between AceK and acesulfame.

2.11. HPLC measurement

The sample solution obtained by dialysis was subjected to HPLC analysis at 230 nm using a Capcell pak NH_2 (4.6 mm i.d. × 250 mm, particle size 5 μm; Shiseido, Tokyo, Japan) at 40 °C and a flow rate of 1.5 mL/min with 1% phosphoric acid/acetonitrile (2:3) as the mobile phase. The AceK contents of the food samples were calculated using the following equation:

$$\text{Content (g kg}^{-1}) = \frac{C \times 200 \times 25}{1000 \times W \times 20}, \quad (5)$$

where C is the concentration of the acesulfame in the sample solution (μg mL^{-1}) and W is the weight of the sample (g).

3. Results and discussion

3.1. qHNMR measurement of acesulfame

To ensure whether qHNMR could be used for quantification of the AceK content in the processed foods, the acesulfame was first analyzed using qHNMR, because AceK was converted to acesulfame through acid hydrolysis during pre-treatment of processed foods. As shown in Fig. 1, the ^{1}H NMR spectrum displayed the two characteristic singlet signals of acesulfame due to a methyl group at δ_H 2.20 and an olefinic proton at δ_H 6.04, in addition to signals of DSS-d_6, the internal standard, at δ_H 0.00 and residual proton of DMSO-d_6 in the stock solution. We considered that these two signals could be applied in the qHNMR quantification of acesulfame, because these signals were well separated from other signals. Therefore, the absolute purity of acesulfame was determined from the ratio of the integrated signal intensity of each the two characteristic signals to the signal of DSS-d_6. The results shown in Table 1 indicate that the purities calculated using the signal of asesulfame at δ_H 2.20 were not significantly different those calculated using the signal at δ_H 6.04. These results suggested that the two signals were suitable for the accurate quantification of asesulfame.

3.2. Linearity

To determine the linearity and the measuring range of the three signals, acesulfame solutions at nine different concentrations including 0.15, 0.30, 0.61, 1.2, 2.4, 4.9, 9.8, 20, and 25 mg g^{-1} were prepared and the calibration curves of each signal were subsequently plotted based on the linear regression analysis of the ratio of the integrated signal intensity of each signal to that of DSS-d_6 (Y) versus the concentration of acesulfame (X, mg mL^{-1}). As shown in Fig. 2, clear linear regressions with the coefficient of determination of 1.0 were obtained in the range of 0.15–25 mg g^{-1} for the signal at δ_H 2.20 and 0.30–25 mg g^{-1} for the signal at δ_H 6.04, indicating a good linear relation in the method's response. In these concentration ranges, relative errors between the experimental values obtained using qHNMR and the gravimetric value of each signal were also less than 1%, as shown in Table 2.

3.3. Accuracy and precision of the proposed method

To develop a quantitative method for AceK in processed foods using qHNMR, the method combining extraction method for pre-treatment and subsequent qHNMR analysis was examined to determine the AceK in processed foods. Although dialysis is generally used as a conventional pre-treatment [4,22] for the quantification of AceK in processed foods, it requires 48 h to purify AceK from processed foods. To avoid this time-consuming process, we utilized solvent extraction using diethyl ether as the extraction solvent based on a previous report [20,21].

To assess the intra-day accuracy and precision of the proposed quantitative method, we performed the recovery test using the samples of 12 kinds of the processed foods spiked with known amounts of acesulfame with triplicate measurements at each level. Fig. 3 shows a representative ^{1}H NMR spectra of acesulfame-spiked sample extracts and blank sample extracts. In these spectra, whole or partial overlaps between the signal at δ_H 2.02 in acesulfame and those in other ingredients of the foods were observed in some samples. These overlaps considerably affected the accurate quantification of spiked asesulfame, especially at low concentrations. On the other hand, with the quantification using the signal at δ_H 6.04, the signals of other ingredients were minimal in the range of δ_H 5.8–δ_H 6.2. Therefore, we utilized the signal at δ_H 6.04 to determine the acesulfame content in all food samples. As shown in Table 3, the recoveries and RSDs of all samples spiked with the high concentration level ranged from 88.4% to 99.6% and from 0.8% to 4.1%, respectively. At a concentration of 0.13 g kg^{-1}, the recoveries were in the range of 90.4–99.2% with RSDs less than 5.6%. Consequently, the results suggested that the proposed method was reliable in the determination of AceK in processed foods.

Table 3
Recovery of acesulfame from various processed foods.

Sample	0.13 g kg^{-1} Spiked		High concentration level spiked		
	Recovery (%)[a]	RSD (%)[b]	Level (g kg^{-1})	Recovery (%)[a]	RSD (%)[b]
Chewing gum	97.8	5.6	5.0	88.4	2.4
Red bean paste	97.7	3.0	2.5	96.5	1.3
Candy	96.7	0.6	2.5	98.2	2.4
Jelly	92.6	1.6	2.5	98.0	1.7
Biscuit	90.9	2.7	2.5	95.1	3.4
Ice cream	99.2	0.6	1.0	92.4	4.1
Jam	99.3	1.4	1.0	99.6	0.8
Salad dressing	90.4	1.1	1.0	96.8	1.7
Vegetable pickled in soybean sauce	96.5	4.8	1.0	98.6	2.7
Sherbet	97.1	1.7	1.0	98.5	1.5
Soft drink	98.2	0.9	0.50	94.6	1.5
Beverage containing *Lactobacillus* species	99.0	2.6	0.50	95.6	4.0

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

Table 4
Inter-day recoveries, repeatability, and intermediate precisions of acesulfame in chewing gum, ice cream, and vegetable pickled in soybean sauce.

Sample	0.13 g kg^{-1}			High concentration level spiked		
	Recovery (%)[a]	RSD_r (%)[b,c]	RSD_{ip} (%)[c,d]	Recovery (%)[a]	RSD_r (%)[b,c]	RSD_{ip} (%)[c,d]
Chewing gum	94.3	1.9	4.8	86.2	2.9	2.9
Ice cream	97.0	2.9	3.7	91.4	4.4	4.5
Vegetable pickled in soybean sauce	94.3	3.7	4.0	93.9	3.6	4.7

[a] Each recovery value represents the mean of results on five different days (two trials per day).
[b] RSD_r, the relative standard deviation for repeatability.
[c] RSD_{ip}, the relative standard deviation for intermediate precision.
[d] RSD_r and RSD_{ip} are calculated by one-way analysis of variance of the recovery values obtained on five different days.

3.4. Intermediate precision

To evaluate the intermediate precision and accuracy of the proposed method, we performed the recovery test on five different days, as described in the section entitled accuracy and precision of the proposed method. In this test, we selected three food samples (chewing gum, ice cream, and vegetable pickled in soybean sauce). As shown in Table 4, the proposed method gave precise intermediate data from all spiked samples, for which the RSD ranged from 2.9% to 4.8%. In addition, the recoveries of all samples ranged from 86.2% to 91.7%. As such, the proposed method was highly reliable and reproducible.

3.5. Limit of quantification of the proposed method

According to previous reports [20,21], the limit of quantification (LOQ) was determined based on the accuracy (recovery), inter- and intra-day precisions (RSD) in the recovery test, and signal-to-noise (S/N) ratio of the signal for quantification. The recoveries for all samples spiked at 0.13 g kg^{-1}, the lowest spiked concentration, were larger than 90% while their intra- and inter-day precision were lower than both 6%. In terms of the S/N ratio, Malz and Jancke reported that an S/N ratio at least 150 was necessary for qHNMR measurements with uncertainty of less than 1% [23]. Additionally, we previously demonstrated that the S/N ratio for quantification should be at least 100 to precisely determine the level of some preservatives in processed foods [20,21]. In this study, the means of the S/N ratios of all samples spiked at 0.13 g kg^{-1} of acesulfame were larger than 100. These results indicate that the proposed method could efficiently determine acesulfame or salts such as AceK in processed foods at concentrations of at least 0.13 g kg^{-1} using the signal at δ_H 6.04. As such, we estimated that the LOQ of the proposed method is 0.13 g kg^{-1} in processed foods. As the maximum usage levels of AceK in processed foods are in the range of 0.35–15 g kg^{-1} in Japan, the

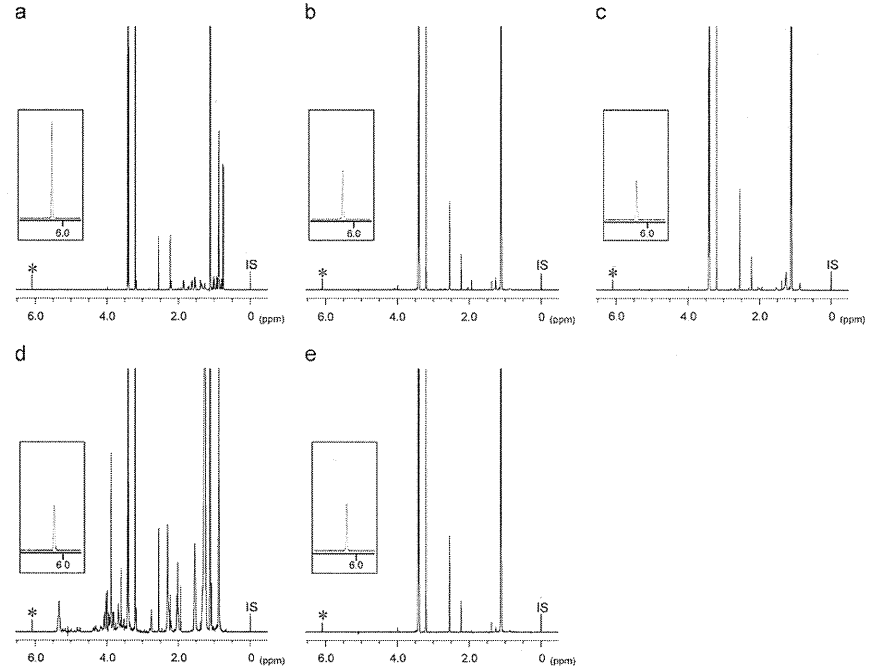

Fig. 4. ^{1}H NMR spectra of sample solutions from commercially produced foods containing AceK. The signals (δ_H 6.04) marked with asterisks were used for quantification. The signals at δ_H 6.04 are highlighted. (a) Candy, (b) Jelly I, (c) Jelly II, (d) biscuit, and (e) soft drink. IS, internal standard (DSS-d_6).

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

Sample	Proposed method (solvent extraction/qHNMR)		Conventional method (dialysis/HPLC)	
	Content (g kg^{-1})	RSD (%)	Content (g kg^{-1})	RSD (%)
Candy	0.52	0.4	0.50	3.6
Jelly I	0.25	1.9	0.25	1.4
Jelly II	0.21	1.3	0.21	9.1
Biscuit	0.22	4.1	0.24	3.2
Soft drink	0.20	0.4	0.20	3.0

proposed method can be applied for the monitoring of AceK in processed foods for regulatory purposes.

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

To clarify the applicability of the proposed method for the quantification of commercial processed foods containing AceK, the method was applied using candies, jellies, biscuits, and soft drinks containing AceK and was compared with conventional methods that use a combination of dialysis and HPLC. Fig. 4 shows representative ^{1}H NMR spectra of the four food samples. We selected the signal at δ_H 6.04 for to determine the amount of acesulfame potassium in the processed foods, because this signal was well separated from the interference signals in all processed foods. As shown in Table 5, no significant differences between the AceK levels determined using the proposed method and those determined using the conventional method were found in any food samples by statistically evaluation ($P < 0.05$) with the *t*-test. Although, the RSDs of the proposed method with Jelly I and biscuits were slightly larger than those of the conventional method, the RSDs of all samples were lower than 9.1%. These results suggested that the proposed method was as reliable as the conventional method. Therefore, we conclude that the proposed method is suitable for the determination of AceK in various processed foods.

4. Conclusion

In this study, we developed and validated a method for the determination of AceK content in various processed foods using a combination of solvent extraction and qHNMR. Although quantification of AceK in cola beverages using ^{1}H NMR with an external calibration curve was previously reported [24], this report exemplifies the first use of qHNMR to determine AceK levels in various processed foods. Our study demonstrates the advantages of the proposed method including high accuracy, intra-day and inter-day precision, as well as good linearity. Compared with the conventional method, the proposed method exhibited similar capabilities in the determination of AceK in various samples, but was significantly faster at 65 min in comparison to 49 h in the conventional method. The breakdown of the timeline of the conventional method includes 48 h of dialysis, 50 min of HPLC analysis including the analysis of the standard to create the calibration curve, followed by 10 min for the determination of AceK. On the other hand, the proposed method requires an initial solvent extraction which requires 45 min, while the following qHNMR analysis accounts for 15 min, and the final determination of AceK requires 5 min. Moreover, an additional advantage of the proposed method is that absolute quantification with SI-traceability can be performed in the determination of AceK in processed foods, without the need for an authentic and identical analyte standard. As such, the proposed method is a very useful and efficient tool for determining AceK content in processed foods. In addition, this method could potentially be used as an alternative at inspection centers or quarantine laboratories to assess the suitability of usage levels and compliance with labeling provisions for quality control, and to monitor of AceK consumption for consumer safety.

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References

[1] M. Fukuda, T. Terata, K. Tsuda, M. Sugawara, N. Kitatani, Y. Semo, Food Sci. Technol. Res. 16 (2010) 457–466.
[2] P. Shankar, S. Ahuja, K. Sriram, Nutrition 29 (2013) 1293–1299.
[3] WHO Technical Report Series, no. 806, Evaluation of Certain Food Additives and Contaminants ,Thirty-Seventh Report of the Joint FAO/WHO Expert Committee on Food Additives, 1991, pp. 20–21.
[4] T. Moriyasu, M. Nakazato, C. Kobayashi, Y. Kikuchi, K. Hayano, Y. Tamura, Shokuhin Eiseigaku Zasshi 37 (1996) 91–96.
[5] N. Dossi, S. Toniolo, S. Susmel, A. Pizzariello, G. Bontempelli, Chromatographia 63 (2006) 557–562.
[6] A. Wasik, J. McCourt, M. Buchgraber, J. Chromatogr. A 1157 (2007) 187–196.
[7] Y. Zhu, Y. Guo, M. Ye, F.S. James, J. Chromatogr. A 1085 (2005) 143–148.
[8] H.-S. Lim, S.-K. Park, J.-S. Kwak, H.-I. Kim, J.-H. Sung, S.-J. Jang, M.-Y. Byun, S.-H. Kim, Food Sci. Biotechnol. 22 (2013) 233–240.
[9] T.J. Quinn, Metrologia 34 (1997) 61–65.
[10] R.J. Wells, J. Cheung, J.M. Hook, Accredit. Qual. Assur. 9 (2004) 450–456.
[11] A. Zoppi, M. Linares, M. Longhi, J. Pharm. Biomed. Anal. 37 (2005) 627–638.
[12] A.A. Salem, H.A. Mossa, B.N. Barsoum, J. Pharm. Biomed. Anal. 41 (2006) 654–661.
[13] C. Almeida, I.F. Duarte, A. Barros, J. Rodrigues, M. Spraul, A.M. Gil, J. Agric. Food Chem. 54 (2006) 700–706.
[14] F.H. Larsen, F. van den Berg, S.B. Engelsen, J. Chemom. 20 (2007) 198–208.
[15] G.F. Pauli, B.U. Jaki, D.C. Lankin, J. Nat. Prod. 70 (2007) 589–595.
[16] M. Paoli, A. Bighelli, V. Castola, F. Tomi, J. Casanova, Magn. Reson. Chem. 51 (2013) 756–761.
[17] J. Staneva, P. Denkova, M. Todorova, L. Evstatieva, J. Pharm. Biomed. Anal. 54 (2011) 94–99.
[18] I. Rundlof, M. Mathiasson, S. Bekiroglu, B. Hakkarainen, T. Bowden, T. Arvidsson, J. Pharm. Biomed. Anal. 52 (2010) 645–651.
[19] M. Tahara, N. Sugimoto, T. Ohtsuki, A. Tada, H. Akiyama, Y. Goda, Y. Harashi, Environ. Sci. 27 (2014) 142–150.
[20] T. Ohtsuki, K. Sato, N. Sugimoto, H. Akiyama, Y. Kawamura, Talanta 99 (2012) 342–348.
[21] T. Ohtsuki, K. Sato, N. Frusho, H. Kubota, N. Sugimoto, H. Akiyama, Food Chem. 141 (2013) 1222–1227.
[22] C. Kobayashi, M. Nakazato, H. Ushiyama, Y. Kawai, Y. Tateishi, K. Yasuda, Shokuhin Eiseigaku Zasshi 40 (1999) 166–171.
[23] F. Malz, H. Jancke, J. Pharm. Biomed. Anal. 38 (2005) 813–823.
[24] P. Maes, Y.B. Monakhova, T. Kuballa, H. Reusch, D.W. Lachenmeier, J. Agric. Food Chem. 60 (2012) 2778–2784.

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Abstract

Glycyrrhizic acid (GA), a triterpenoid saponin containing two glucuronic acid (GlcA1 and GlcA2) units, is found in the roots of *Glycyrrhiza* plants, and has been widely used as a natural sweetener for foods as well as a natural medicine. Purified GA is commercially available from various manufacturers as an analytical standard or a biochemical reagent. While producers describe the configurations of GlcA1 and GlcA2 as α and β-forms, respectively, reports of the structural elucidation of GA have proposed that both GlcA units are β-form. To clarify this point, commercial GA from various sources was analyzed by 1D and 2D NMR studies. Results confirmed that the actual configuration of both GlcA units in GA is β-form.

Keywords : glycyrrhizic acid, glucuronic acid, natural sweetener

I Introduction

Glycyrrhizic acid (GA) is a triterpenoid saponin found in *Glycyrrhiza* plants such as *Glycyrrhiza glabra* (licorice), *G. inflata*, and *G. uralensis*. Since GA is 30–50 times sweeter than sucrose, root extracts of *Glycyrrhiza* (known as licorice root extract) have been used as a natural sweetener for foods[1]. In addition, it has been extensively reported that GA has several pharmacological activities, including anti-inflammatory[2], immunomodulatory[3], anti-ulcer[4] and anti-tumorigenic effects[5, 6]. Moreover, licorice root is a well-known oriental and occidental herbal medicine that is frequently prescribed for the treatment of various diseases. Purified GA is commercially available, and is utilized as an authentic standard in natural medicines and as a research agent for the investigation of biochemical and pharmaceutical activities.

GA is composed of a triterpenoid aglycone, glycyrrhezinic acid (GLA), and two glucuronic acid units (GlcA1 and GlcA2). The two GlcA units are connected *via* a 1 → 2 glycoside linkage (Fig. 1) and the GlcA1 connects to position 3 of the aglycone GLA *via* a glycoside linkage. With respect to the configuration of the two GlcA units, recent papers dealing with GA prepared from *Glycyrrhiza* plants proposed that both were β-form[7, 8]. However, we noted that the labels of commercial GA and GA salts indicate the configurations of GlcA1 and GlcA2 as α-form and β-form, respectively. In our investigation of commercial reagent labeling, all commercial GA and GA salts were labeled as containing the α-form configuration of GlcA1 in their product catalogues. The origin of the labeling might be an authentic database such as the Chemical Abstract Service (CAS) that states that the structure of GlcA1 is α-form, e.g., GA (CAS Registry Number: 1405-86-3), GA ammonium salt (CAS Registry Number: 53956-04-0), and GA dipotassium salt hydrate (CAS Registry Number: 68797-35-3). These inconsistencies create confusion in analytical and biochemical investigations using commercial GA reagents as authentic standards.

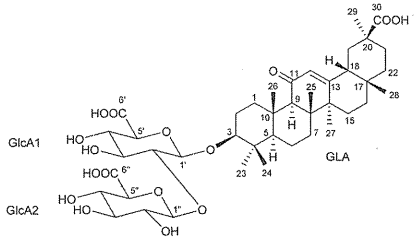

Fig. 1. Structure of glycyrrhizic acid (GA)

In order to clarify the situation, we present the correct structural determination of commercial GA reagents purchased from various distributors using high-resolution NMR analysis.

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II Material and Methods

Reagents: Two GA reagents and five GA ammonium salt reagents were purchased from seven sources as follows: glycyrrhizic acid standard (Wako Pure Chemical Industries, Ltd., Osaka, Japan), glycyrrhizic acid (Tokyo Chemical Industry co., Ltd., Tokyo Japan), glycyrrhizic acid monoammonium salt (Sigma-Aldrich, MO, USA), glycyrrhizic acid monoammonium salt standard (Kanto Chemical Co., Inc., Tokyo, Japan), glycyrrhizic acid ammonium salt, trihydrate (LKT Labs, Inc., MN, USA), glycyrrhizic acid ammonium salt (ChromaDex, Inc., CA, USA), and glycyrrhizic acid monoammonium salt (Acros Organics, Geel, Belgium). Commercial catalogues of these sources noted the configurations of GlcA1 and GlcA2 in all reagents as α-form and β-form, respectively.

NMR study: 1D (^{1}H and ^{13}C) and 2D NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC, and NOESY experiments) spectra of GA were recorded on JEOL ECA instruments (600 MHz) in CD_3OD as the solvent at 25°C. The methyl proton signal at 3.30 ppm in CD_3OD was referenced on the NMR measurement.

III Results

Seven commercial GA or GA ammonium salts were analyzed using NMR in this study. The commercial catalogues supplied by seven sources indicated the configuration of GlcA1 in GA as α-form, and that of GlcA2 as β-form. To confirm the configuration of GlcA units in GA, the standard GA reagent purchased from Wako Pure Chemical Industries was first analyzed using NMR. The chemical shifts (δ_H and δ_C) of GA were recorded in CD_3OD at 25°C and all signals were assigned by the analysis of 1D (^{1}H and ^{13}C) and 2D NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC, and NOESY experiments). The ^{13}C NMR spectrum of GA showed 42 signals, including a typical ketone signal (δ_C 202.7) corresponding to C11 on GLA and three carboxyl signals (δ_C 180.4, 172.6 and 172.1) corresponding to C30, C6′ and C6″, respectively (Table 1). The aglycone GLA is known to be an 18 β-H-oleanane-type compound (18β-GLA)[9]. The 18α-epimer

Table 1. Chemical shifts of glycyrrhizic acid (GA) in CD_3OD

position	δ^1H (multiplet, *J* Hz)	δ^{13}C
GLA		
1	0.99 (td, 13.0, 3.5) 2.66 (dt, 10.3, 3.5)	40.2
2	1.74 (m) 1.81 (m)	27.0
3	3.16 (dd, 11.7, 4.4)	90.8
4		40.6
5	0.76 (brd, 12.0)	56.5
6	1.43 (m) 1.60 (br, 12.0)	18.4
7	1.43 (m) 1.72 (m)	33.8
8		46.7
9	2.43 (s)	63.1
10		38.0
11		202.7
12	5.56 (s)	128.9
13		172.4
14		44.6
15	1.04 (m) 1.24 (brd, 14.0)	27.6
16	1.87 (m) 2.14 (td, 13.8, 4.5)	27.4
17		33.0
18	2.18 (dd, 13.5, 3.5)	50.0
19	1.70 (m) 1.83 (m)	42.4
20		44.9
21	1.39 (m) 1.94 (m)	32.0
22	1.39 (m) 2H	39.0
23	0.81 (s) 3H	16.8
24	1.04 (s) 3H	28.2
25	1.12 (s) 3H	17.0
26	1.12 (s) 3H	19.3
27	1.42 (s) 3H	23.8
28	0.82 (s) 3H	29.2
29	1.16 (s) 3H	28.7
30		180.4
GlcA1		
1′	4.51 (d, 7.5)	105.3
2′	3.50 (t, 9.5)	84.0
3′	3.58 (t, 9.8)	77.4
4′	3.54 (t, 9.8)	72.9
5′	3.76 (d, 9.0)	76.3
6′		172.6
GlcA2		
1″	4.62 (d, 7.7)	106.3
2″	3.28 (m)	76.3
3″	3.38 (t, 9.8)	77.2
4″	3.52 (t, 9.5)	73.1
5″	3.74 (d, 9.7)	77.6
6″		172.1