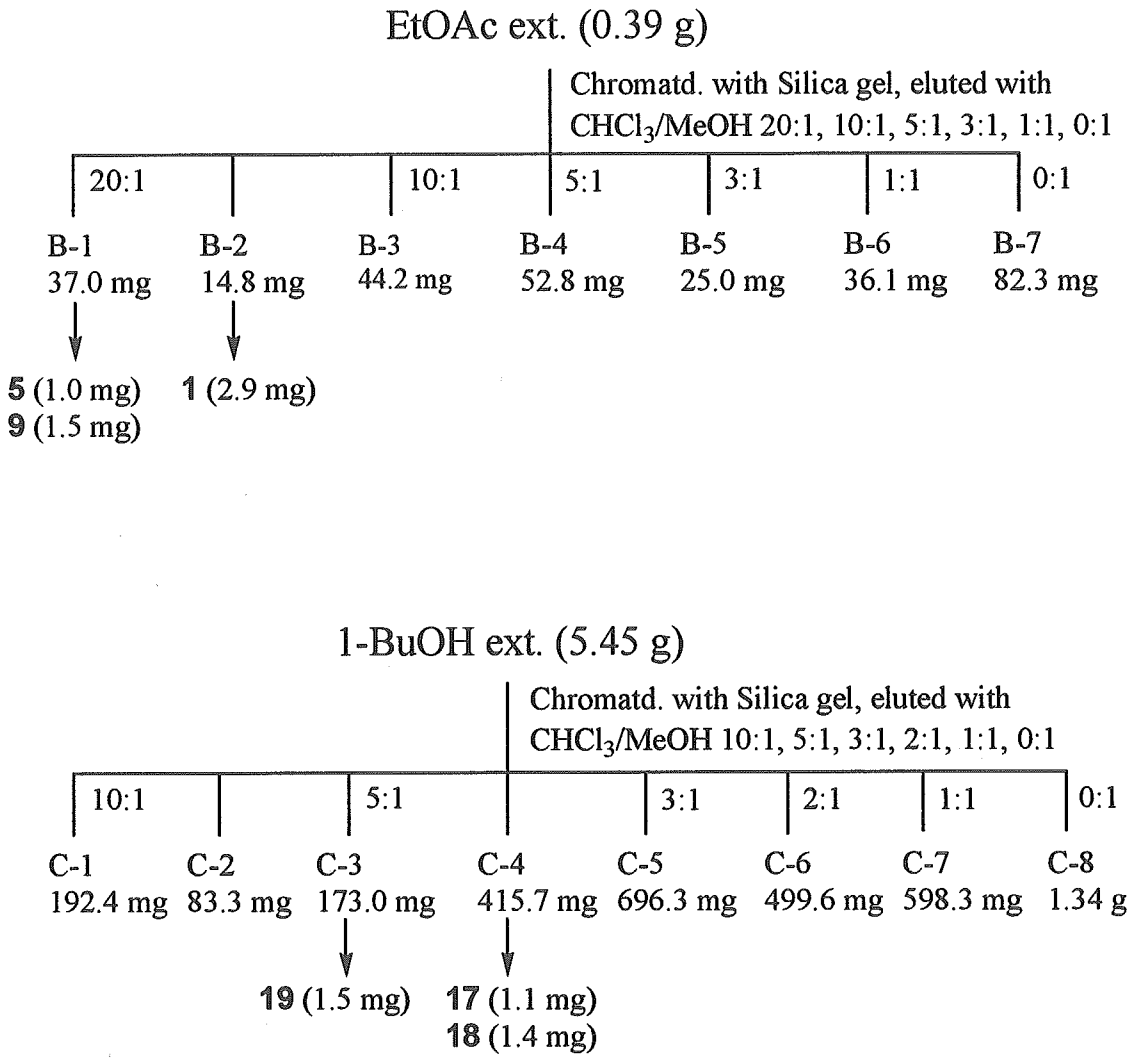


Chart 4. Isolation of compounds from *Impatiens balsamina* (2)

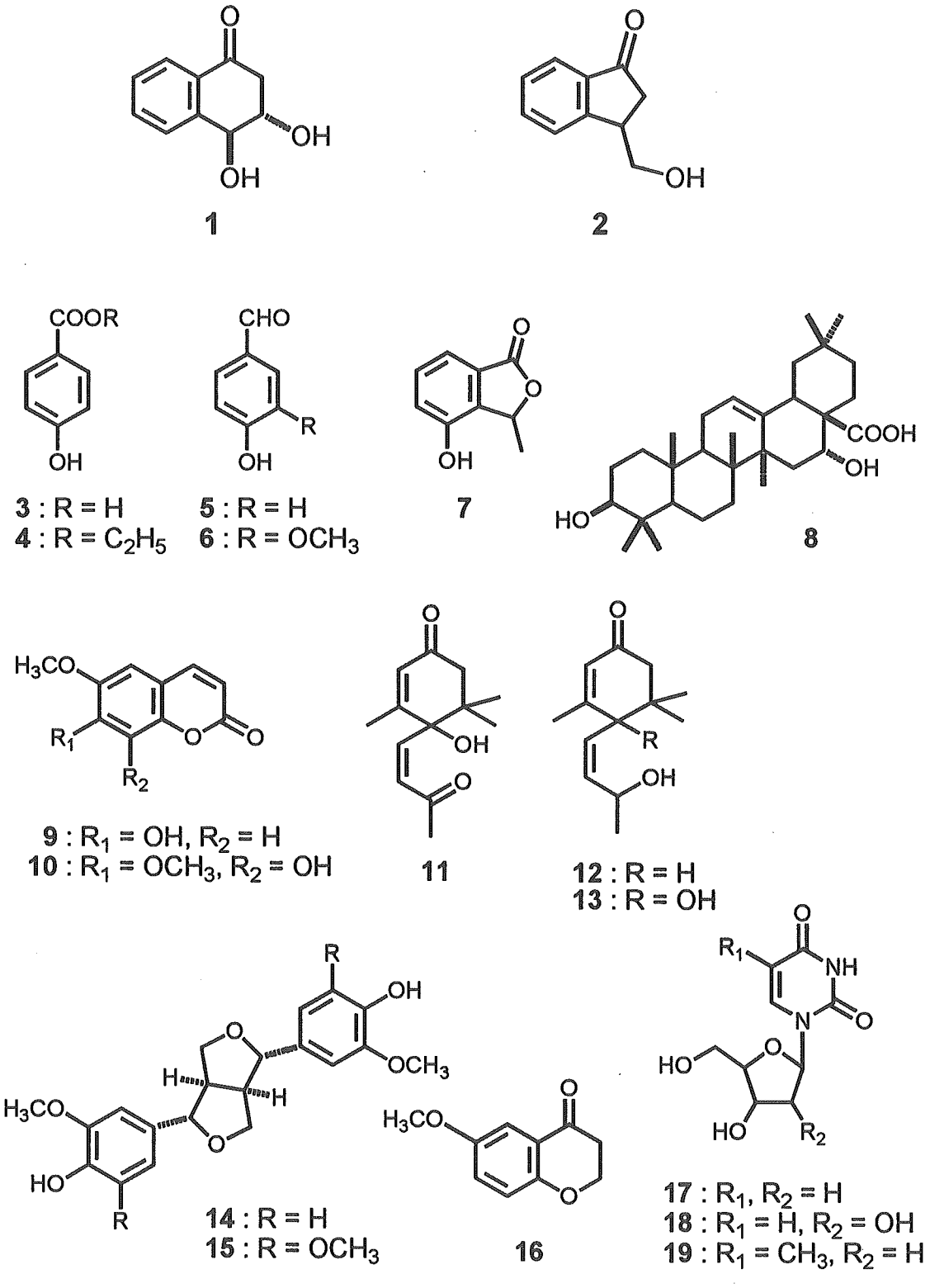
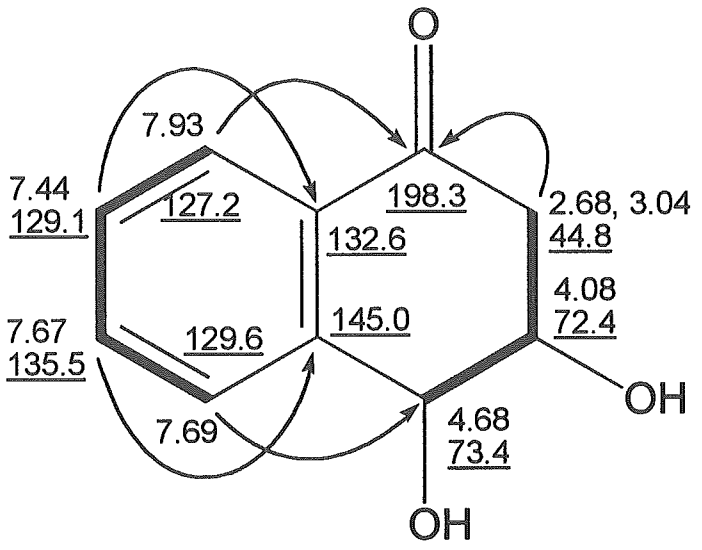


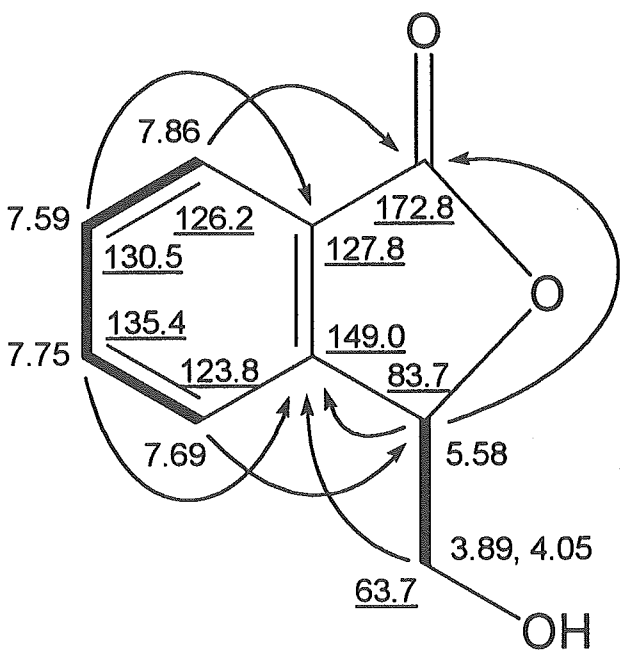
Chart 5. Chemical structure of compounds (**1-19**)



1

^{1}H-^{1}H COSY

HMBC



2

Fig. 1 ^{1}H-^{1}H and long-range ^{13}C-^{1}H correlations of **1** and **2**

研究成果の刊行に関する一覧表

雑誌

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Analysis of the Constituents in Jojoba Wax Used as a Food Additive by LC/MS/MS

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Jojoba wax is a natural gum base used as a food additive in Japan, and is obtained from jojoba oil with a characteristically high melting point. Although the constituents of jojoba oil have been reported, the quality of jojoba wax used as a food additive has not yet been clarified. In order to evaluate its quality as a food additive and to obtain basic information useful for setting official standards, we investigated the constituents and their concentrations in jojoba wax. LC/MS analysis of the jojoba wax showed six peaks with $[M+H]^+$ ions in the range from *m/z* 533.6 to 673.7 at intervals of *m/z* 28. After isolation of the components of the four main peaks by preparative LC/MS, the fatty acid and long chain alcohol moieties of the wax esters were analyzed by methanolysis and hydrolysis, followed by GC/MS. The results indicated that the main constituents in jojoba wax were various kinds of wax esters, namely eicosenyl octadecenoate (C20 : 1-C18 : 1) (I), eicosenyl eicosenoate (C20 : 1-C20 : 1) (II), docosenyl eicosenoate (C22 : 1-C20 : 1) (III), eicosenyl docosenoate (C20 : 1-C22 : 1) (IV) and tetracosenyl eiosenoate (C24 : 1-C20 : 1) (V). To confirm and quantify the wax esters in jojoba wax directly, LC/MS/MS analysis was performed. The product ions corresponding to the fatty acid moieties of the wax esters were observed, and by using the product ions derived from the protonated molecular ions of wax esters the fatty acid moieties were identified by MRM analysis. The concentrations of the wax esters I, II and III, in jojoba wax were 5.5, 21.4 and 37.8%, respectively. In summary, we clarified the main constituents of jojoba wax and quantified the molecular species of the wax esters without hydrolysis by monitoring their product ions, using a LC/MS/MS system.

Key words: natural gum base; jojoba wax; *Simmondsia californica* NUTT.; wax ester; food additive

Introduction

Simmondsia californica NUTT. is an evergreen desert shrub that is now cultivated in many arid and semi-arid countries. Its seeds contain about 50% of a light yellow oil, commonly called jojoba oil (Japanese name: hohoba oil), which is used in the cosmetic and pharmaceutical industries. Jojoba wax, a characteristically high melting point substance obtained from jojoba oil, is used as a natural gum base in the food industry. The List of Existing Food Additives in Japan[1] stipulates that jojoba wax is a substance composed mainly of eicosenyl eicosenoate obtained from jojoba fruits. Although the main constituents in jojoba oil have been reported[2)-4)], the constituents of jojoba wax used as a food additive have not been fully clarified.

In this study, in order to evaluate the quality of jojoba wax as a food additive and to obtain basic information useful for setting official standards, we investigated the constituents and their concentrations in jojoba wax, using the same sample as used for toxicity testing. We report on the fractionation of the main constituents in jojoba wax by preparative LC/MS and their structure elucidation by GC/MS. In addition, we also report a method for direct quantification of wax esters by LC/MS/MS.

Materials and Methods

Sample and reagents

A sample of jojoba wax (a colorless viscous liquid) taken from the same sample lot that was used for toxicity testing, and a sample of vegetable sterol (a white powder) used as a food additive, containing more than 92% phytosterols, were obtained through the Japan Food Additives Association. The oil reference standards (Supelco, Cat. No. O7756) containing various kinds of fatty acid methyl esters, methyl-*cis*-15-tetracosenoate and *cis*-11-eicosenoic acid were purchased from Sigma-Aldrich Co., Ltd., MO, USA. 11-Eicosenol manufactured by MP Biomedicals Co., Ltd., OH, USA was purchased from Wako Pure Chemical Industries Co., Ltd., Osaka, Japan. Oleic acid (*cis*-9-

octadecenoic acid) and *cis*-13-docosenol were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Trimethylsilylating reagent, TMSI-H®, was purchased from GL Sciences Co., Ltd., Tokyo, Japan. All other chemicals used were of HPLC or GC reagent grade and were used without further purification.

Instrumentation

The analytical LC/MS/MS system (MassLynx™, Waters Co., Ltd., Milford, MA, USA) was used in LC/MS mode or in LC/MS/MS mode, according to the experimental purposes. The system consisted of an Aliance 2,695 separation module, a 2,996 photodiode array detector (PDA) and a Quattro micro triple-quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface. The complete system was controlled by MassLynx software v.4.0. The APCI source was run at 10 μA corona ampere, 130℃ and 400℃ source and desolvation temperatures, respectively, and 300 and 50 L/hr desolvation and cone gas flow rates, respectively. The cone voltage was 25 V. Full-scan acquisition between *m/z* 200 and 700 was performed at a scan speed of 1.0 sec/scan with a 0.03 sec interscan delay. Positive-ion detection was applied. The on-line PDA detector was used for monitoring between 200 and 400 nm. For LC/MS/MS analysis, product ion scan and multiple reaction monitoring (MRM) analyses were performed by varying collision voltage at 15 V with argon as the collision gas.

The LC/MS-controlled preparative LC system (FractionLynx™ MS auto purification system, Waters) consisted of a 2,767 one-bed injection-collection Sample Manager, a 2,525 binary high-pressure LC pump, a column/fluidic organizer (CFO), a 2,996 photodiode array detector and a ZQ single-quadruple mass spectrometer (MS) equipped with a APCI interface. In addition, a reagent manager was used for the make-up liquid delivery. The CFO contained a 1 : 1000 accurate splitter, manufactured by LC Packings (Amsterdam, The Netherlands). The MS conditions were similar to those for the analytical LC/MS/MS, except for the scan conditions. Full-scan acquisition between *m/z* 200 and 700 was performed at a scan speed of 0.5 sec/scan with a 0.1 sec interscan delay. The solvent delivered to the APCI interface was split in a 1 : 4 ratio, delivering approximately 200 μL/min to the interface. The on-line PDA detector was used for monitoring between 200 and 400 nm. PDA spectra were used only for compound characterization and not as the collection trigger.

The GC/MS system (Shimadzu Co., Ltd., Kyoto, Japan) consisted of a GC-17A gas chromatograph equipped with a MS-QP5050 mass spectrometer in EI mode and an AOC-20i auto injector.

TLC analysis of jojoba wax

Jojoba wax was dissolved in hexane at 1 mg/mL and used for TLC analysis. Since it has been reported that the main components of jojoba oil are wax esters[2)-4)] and phytosterols are minor constituents[5)], we used as standard materials synthesized eicosenyl eicosenoate (a wax ester) and a vegetable sterol that is used as a food additive (phytosterols mixture). These standards were both dissolved in hexane at 1 mg/mL. A silica gel 60 F_{254} HPTLC plate (10 cm × 10 cm, Art. 5628, Merck Co., Ltd., Darmstadt, Germany) was developed with hexane–diethyl ether–acetic acid (80 : 20 : 1 (v/v/v)) as a solvent system and the spots were visualized with iodine.

LC/MS analysis of jojoba wax

A sample of jojoba wax (5 mg, liquid) was dissolved in 1 mL of acetone, and 10 μL of the solution was injected into the LC/MS mode system under the following conditions: column, Symmetry® C18 (2.1 i.d. mm × 150 mm, 3.5 μm, Waters); column temperature, 40℃; mobile phase, acetonitrile–acetone (7 : 3 (v/v)); flow rate, 0.4 mL/min; detection, UV (204 nm) and APCI positive scan.

Fractionation of the main constituents from jojoba wax using preparative LC/MS

A sample of jojoba wax (200 mg, liquid) was dissolved in 2 mL of acetone and the solution was injected into the preparative LC/MS system under the following conditions: column, XTerra RP18 (19 i.d. mm × 100 mm, 5 μm, Waters); mobile phase, acetonitrile–acetone (7 : 3 (v/v)); flow rate, 10 mL/min; injection volume, 400 μL; detection and collection trigger, APCI positive mode, *m/z* 561.6, 589.6, 617.5 and 645.6 for fractionation of peaks 2, 3, 4 and 5 in Fig. 2, respectively; make-up liquid, acetonitrile–acetone (7 : 3 (v/v)), 1.0 mL/min. This overall procedure was repeated 4 times to obtain large enough samples of the four fractions for structural analysis.

Derivatization of the fatty acid and long chain alcohol moieties of the wax esters

The compositions of the fatty acids and long chain alcohols of the wax esters were determined by GC/MS after methanolysis and hydrolysis, respectively.

For methanolysis, 3 mg of each of the jojoba wax products and peak fractions 2–5 were heated with 1 mL of 5% hydrogen chloride in methanol at 100℃ for 3 hr. The products were extracted twice with 3 mL of hexane. The extracts were evaporated to dryness and dissolved in 5 mL of hexane. The solution was used for GC/MS analysis of the fatty acid methyl esters. As authentic fatty acid methyl esters, a mixture of an oil reference standard containing various kinds of fatty acid methyl esters and methyl-*cis*-15-tetracosenoate was used.

For hydrolysis, 10 mg of each of the jojoba wax products and peak fractions 2–5 were heated with 1.5 mL of 0.5 mol/L KOH in 90% methanol at 100℃ for 3 hr. The products were then extracted with 3 mL of hexane. One-third of the extracts was evaporated to dryness, and the residue was reacted with TMSI-H in sealed tubes at 60℃ for 1 hr, followed by extraction of trimethylsilylated (TMS) alcohols using 1.5 mL of hexane.

Using the same method, authentic alcohols were also trimethylsilylated, and the products were used as authentic TMS alcohols. The ω9 *cis* fatty acids and fatty alcohols were used as authentic compounds, since double bonds in the alkyl chains in jojoba liquid waxes were previously found to be almost exclusively (98%) ω9[2] and unsaturated alcohols in jojoba wax esters were predominantly ω9 *cis* type[6]. Then, the TMS alcohols were subjected to GC/MS analysis.

GC/MS analysis of the fatty acid methyl esters and trimethylsilylated alcohols

GC/MS analysis of the fatty acid methyl esters and TMS alcohols was carried out on a DB-1 fused-silica capillary column (30 m×0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Folsom, CA). The injector and detector temperatures were set at 300℃ and 250℃, respectively, and the column temperature was programmed from 180℃ to 280℃ at 5℃/min, then from 280℃ to 300℃ at 15℃/min, and finally kept at 300℃ for 8 min. Samples (1 μL) were injected through a split-injector (1/4).

Synthesis of wax esters

The following three wax esters were synthesized according to a reported method[7,8]. To prepare eicosenyl octadecenoate (I) (C20 : 1–C18 : 1), eicosenyl eicosenoate (II) (C20 : 1–C20 : 1) and docosenyl eicosenoate (III) (C22 : 1–C20 : 1), equimolar amounts (0.13 mmol of each) of the appropriate ω9 *cis* alcohols and fatty acids, and eicosenol and octadecenoic acid, eicosenol and eicosenoic acid, and docosenol and eicosenoic acid, respectively, were mixed in 1.34 mL of benzene containing 0.26 mmol of *N,N*-dicyclohexyl carbodiimide with 4-dimethylaminopyridine as a catalyst. The reaction mixtures were allowed to stand for 24 hr with stirring under N_2, and then partitioned into hexane and 0.3 mol/L HCl. The synthesized wax esters were extracted in hexane, and then purified by preparative TLC on 2 mm layered silica gel plates (Silica gel 60 F_{254}, 20 cm×20 cm, Art. 1.05715, Merck) with hexane–diethyl ether (7 : 3 (v/v)) as a developing solvent. The purity of the synthesized compounds was confirmed by screening peaks at UV 200–400 nm, and by scanning at *m/z* 100–700, by LC/MS analysis, and no other peak was observed.

Quantification of wax esters in jojoba wax by LC/MS/MS

The concentrations of eicosenyl octadecenoate (I), eicosenyl eicosenoate (II) and docosenyl eicosenoate (III) in jojoba wax were determined with the LC/MS/MS system, using synthesized wax esters as authentic standards. Ten μL of the sample solution of jojoba wax (5 mg/mL of acetone) was injected into the LC/MS/MS system under the following conditions: column, Symmetry® C18 (2.1 i.d. mm×150 mm, 3.5 μm, Waters); column temperature, 40℃; mobile phase, acetonitrile–acetone (7 : 3 (v/v)); flow rate, 0.4 mL/min; detection, UV (204 nm) and APCI (positive mode, multiple reaction monitoring (MRM)), *m/z* 561.6→283.4 (for I), 589.6→311.4 (for II) and 617.6→311.4 (for III). The concentrations of the wax esters in jojoba wax were measured based on absolute calibration curves of the peak areas of the synthesized wax esters on the MRM chromatograms.

Results and Discussion

TLC analysis of jojoba wax

It has previously been reported that the main constituents of jojoba oil are wax esters[2-4]. Since jojoba wax is produced from jojoba oil, its main constituents are also thought to be wax esters. Therefore, to confirm the main constituents of jojoba wax, and in particular to evaluate what kind of simple lipid is predominant, TLC analysis of jojoba wax was performed following a reported method[9], in which various types of simple lipids and free fatty acids and alcohols migrate at different *Rf* values.

In Fig. 1, the Silica gel 60 F_{254} TLC profiles of jojoba wax and of authentic compounds are illustrated. Two spots were observed on the TLC profile for jojoba wax. The main spot was observed at $Rf=0.63$ and corresponded with that of a wax ester, synthetic eicosenyl eicosenoate. The weak spot ($Rf=0.08$) corresponded with that of a phytosterol mixture used as a food additive. No spots were observed at the *Rf* values corresponding to free fatty acids, free fatty alcohols, sterol esters and neutral fats. These results indicate that the main constituents in jojoba wax are wax esters.

LC/MS analysis of jojoba wax

To investigate the main constituents in jojoba wax, we employed analytical LC with PDA and APCI-MS detectors. The chromatograms at 204 nm and TIC of the jojoba wax are shown in Figs. 2A and 2B, in which six major peaks (1 to 6) can be seen. The APCI-MS

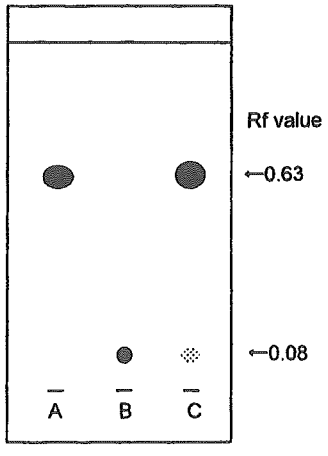

Fig. 1. TLC profile of jojoba wax

Solvent: hexane–diethyl ether–acetic acid = 80 : 20 : 1
Line: A, wax ester (eicosenyl eicosenoate); B, phytosterols (purified phytosterols mixture used as a food additive); C, jojoba wax
Spots were visualized with iodine.

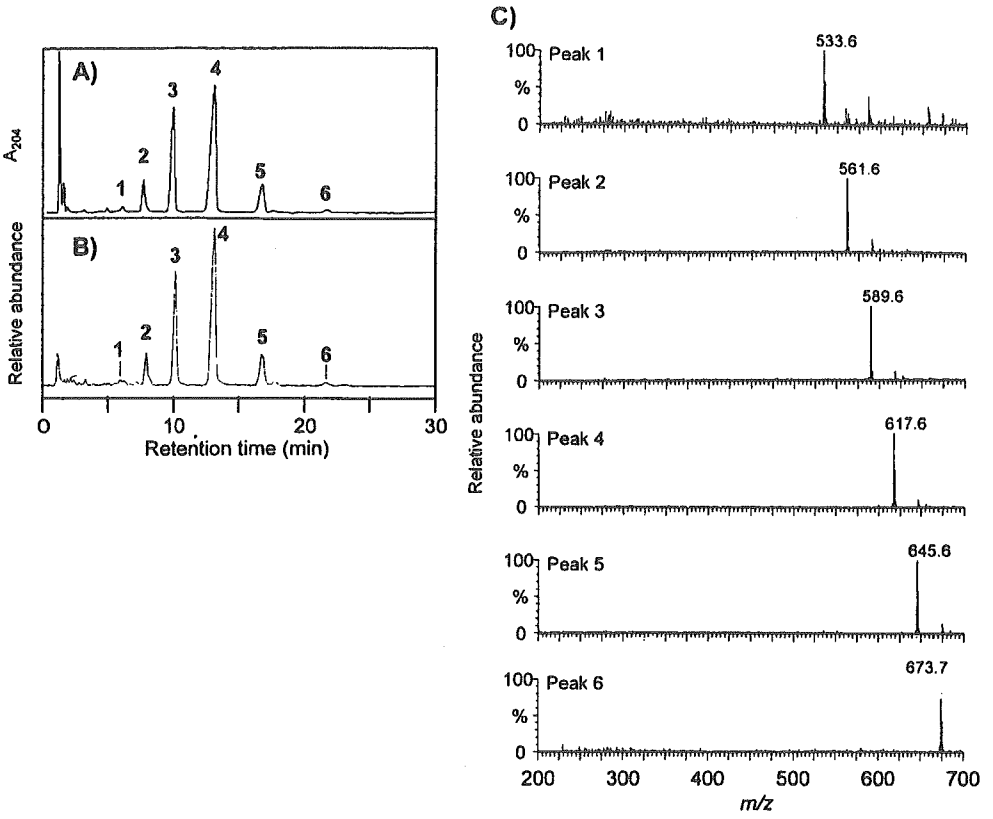

Fig. 2. Separation of the wax esters in jojoba wax by LC/MS

A) chromatogram at UV 204 nm, B) total ion chromatogram of the APCI positive mode, C) MS spectra of peaks 1 to 6 using APCI positive mode scanning.

(positive mode) spectra of peaks 1 to 6 are shown in Fig. 2C. Their $[M+H]^+$ ions appear at *m/z* 533.6, 561.6, 589.6, 617.6, 645.6 and 673.7. On the other hand, the major constituents in jojoba wax are expected to be wax esters based on the TLC results and a previous report on the main constituents in jojoba oil[2), 4), 10)]. Therefore, their molecular formulae could be represented as $C_nH_{2(n-m)}O_2$ (m=number of unsaturated bonds). It was reported that the wax esters in jojoba oil are mainly composed of monounsaturated fatty acids and monounsaturated fatty alcohols[2), 4), 10)]. Therefore, the major constituents in the jojoba wax with $[M+H]^+$ *m/z* 533.6, 561.6, 589.6, 617.6, 645.6 and 673.7 are suggested to be $C_{36}H_{68}O_2$, $C_{38}H_{72}O_2$, $C_{40}H_{76}O_2$, $C_{42}H_{80}O_2$ $C_{44}H_{84}O_2$ and $C_{46}H_{88}O_2$, respectively. These molecular formulae are consistent with wax esters. The percentage peak areas at UV 204 nm for peaks 1 to 6 are shown in Table 1, and their total was calculated to be 94.4%.

Identification of the wax esters

In order to elucidate the structures of the main constituents in jojoba wax, the compounds corresponding to the main peaks (peaks 2 to 5) were isolated by preparative LC/MS. Preparative LC/MS was carried out with four target masses: APCI positive mode, *m/z* 561.6, 589.6, 617.6 and 645.6 for the main peaks (2 to 5). The fatty acid and alcohol compositions of the wax esters in the jojoba wax and the four isolated peak fractions (peaks 2 to 5) were individually analyzed by GC/MS.

To determine the fatty acid moieties, jojoba wax and the four isolated peak fractions were analyzed after methanolysis. The peaks on the GC/MS chromatograms shown in Fig. 3, and their identification were based on the retention times and mass spectra of the authentic fatty acid methyl esters. Jojoba wax (Fig. 3A) contained hexadecanoic acid (C16 : 0), octadecenoic acid (C18 : 1), eicosenoic acid (C20 : 1), docosenoic acid (C22 : 1) and tetracosenoic acid (C24 : 1). The main fatty acids in the isolated peak fractions were octadecenoic acid (C18 : 1) in peak 2 (Fig. 3B), eicosenoic acid (C20 : 1) in peak 3 (Fig. 3C), and eicosenoic acid (C20 : 1) and docosenoic acid (C22 : 1) in peaks 4 and 5 (Figs. 3D and 3E). Table 1 shows the relative abundance of fatty acids based on the GC/MS total ion chromatograms.

To determine the alcohol moieties, jojoba wax and the isolated peak fractions were analyzed after hydrolysis and trimethylsilylation. Four peaks were observed on the GC/MS chromatogram (Fig. 4A). Based on the retention times and mass spectra of authentic TMS alcohol samples, the peaks at 11.3 min and 14.2 min were identified as being due to trimethylsilylated eicosenol (C20 : 1) and docosenol (C22 : 1), respectively. Peaks at 8.5 min and 17.2 min were considerd to be due

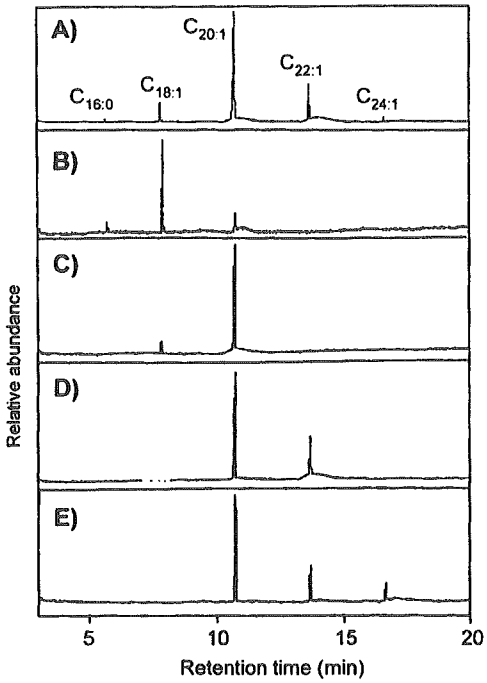

Fig. 3. GC/MS analysis of fatty acid methyl esters obtained by methanolysis of jojoba wax (A), peak fractions 2–5 (B to E) isolated by preparative LC/MS

The chromatograms show the result of total ion monitoring in the EI mode.

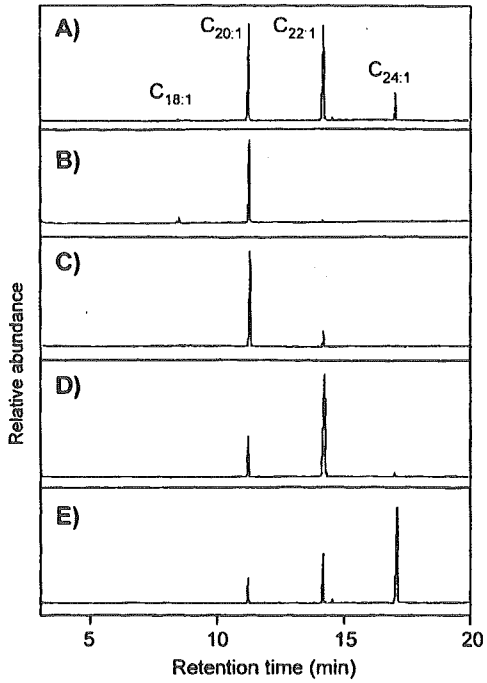

Fig. 4. GC/MS analysis of trimethylsilylated alcohols obtained by hydrolysis and trimethylsilylation of jojoba wax (A), peak fractions 2–5 (B to E) isolated by preparative LC/MS

The chromatograms show the result of total ion monitoring in the EI mode.

to trimethylsilylated octadecenol (C18 : 1) and tetracosenol (C24 : 1), respectively, from comparison of the mass spectra with the NIST 147 database and from the percentage abundance of fragment ions of octadecenol and tetracosenol determined in a previous report[5], in which the existence of ω9 unsaturated fatty alcohols in the jojoba wax esters was proved. The main trimethylsilylated alcohols in the isolated peak fractions corresponded to eicosenol (C20 : 1) in peaks 2 and 3 (Figs. 4B and 4C), eicosenol (C20 : 1) and docosenol (C22 : 1) in peak 4 (Fig. 4D), and docosenol (C22 : 1) and tetracosenol (C24 : 1) in peak 5 (Fig. 4E).

The GC/MS analysis of the composition of the fatty acids and alcohols suggested that the major esters were eicosenyl octadecenoate (I) (C20 : 1–C18 : 1) in peak 2, eicosenyl eicosenoate (II) (C20 : 1–C20 : 1) in peak 3, docosenyl eicosenoate (III) (C22 : 1–C20 : 1) and eicosenyl docosenoate (IV) (C20 : 1–C22 : 1) in peak 4 and tetracosenyl eicosenoate (V) (C24 : 1–C20 : 1) in peak 5. We confirmed that the retention times of the major peaks (2, 3 and 4) of jojoba wax in LC/MS analysis corresponded well with those of the synthesized wax esters I, II and III (Fig. 5).

The results shown in Table 1 indicate that the percentage peak areas of peaks 2 to 5 of jojoba wax at UV 204 nm were 7.2, 27.3, 48.0 and 9.5%, respectively. In addition, the percentage peak areas of the main fatty acids, namely octadecenoic acid (C18 : 1) in peak 2, eicosenoic acid (C20 : 1) in peak 3, eicosenoic acid (C20 : 1) and docosenoic acid (C22 : 1) in peak 4 and eicosenoic acid (C20 : 1) in peak 5 were 76.0, 92.2, 77.7 and 22.3, and 70.0%, respectively. Therefore, when the percentage peak areas of peaks 2 to 5 at UV 204 nm were multiplied by the percentage peak areas of the main fatty acids of peaks 2 to 5, the relative contents of I to V were calculated to be 5.5, 25.2, 37.3 and 10.7, and 6.7%, respectively. There are several reports on components of jojoba oil[2)-4),10)]. Two of them[2),10)] obtained the ratios of the molecular species of the wax esters with various

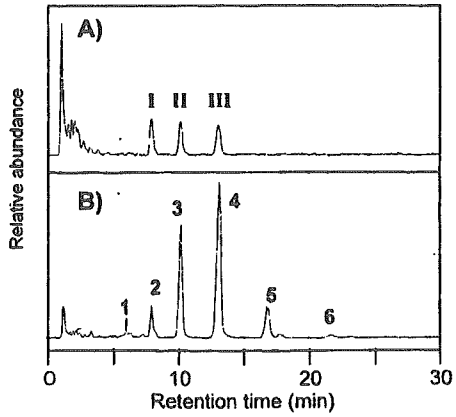

Fig. 5. Total ion chromatograms of synthesized wax esters I–III (A) and the wax esters of jojoba wax (B) by LC/MS analysis using APCI positive mode scanning

Table 1. Compositions of Wax Esters and Fatty Acid Moiety in Jojoba Wax

Peak*1 number	Chain length: unsaturation	Composition*2 at UV 204 nm (%)	Fatty acid moiety	
			Chain length	%*3
Jojoba wax (whole)			16 : 0	1.6
			18 : 1	16.1
			20 : 1	73.9
			22 : 1	7.4
			24 : 1	1.1
1	36 : 2	1.1	—*4	—
2	38 : 2	7.2	16 : 0	9.6
			18 : 1	76.0
			20 : 1	14.4
3	40 : 2	27.3	18 : 1	7.8
			20 : 1	92.2
4	42 : 2	48.0	20 : 1	77.7
			22 : 1	22.3
5	44 : 2	9.5	20 : 1	70.0
			22 : 1	20.4
			24 : 1	9.7
6	46 : 2	1.3	—	—

*1 Peak number shown in Fig. 2A.
*2 Composition of peak areas at UV 204 nm by HPLC analysis shown in Fig. 2A. The values mean the relative peak area percentages against the total peak area.
*3 Composition of peak areas of fatty acid methyl esters on total ion chromatograms by GC/MS analysis shown in Fig. 3.
*4 Not determined

combinations of fatty acids and alcohols by GC/MS/MS analysis[10] and by GC/MS analysis of hydrolyzed samples of HPLC-fractionated peaks[2]. The ratios in this paper are very similar to those reported in the previous papers[2)-4), 10].

Exact quantification of the wax esters in jojoba wax

The results of the GC/MS analysis showed that there were several kinds of wax esters with the same molecular weight, but with different fatty acid and alcohol compositions. Under the LC/MS conditions described in the experimental section, the fragment ion that related to the fatty acid and alcohol combination could not be clearly observed by varying the sampling-cone voltage for in-source collision induced decomposition (CID). Thus, it was difficult to discriminate between wax esters with the same retention time and the same molecular weight, but with different fatty acid and alcohol compositions. On the other hand, under the LC/MS/MS conditions, the product ions corresponding to the fatty acid moieties of wax esters were observed (Fig. 6).

Therefore, we could directly distinguish (without hydrolysis) the wax esters in jojoba wax that had the same retention time and molecular weight, but which had different fatty acid and alcohol compositions. We could precisely quantify eicosenyl octadecenoate (I) (C20 : 1–C18 : 1), eicosenyl eicosenoate (II) (C20 : 1–C20 : 1) and docosenyl eicosenoate (III) (C22 : 1–C20 : 1) in the jojoba wax by monitoring the product ions that corresponded

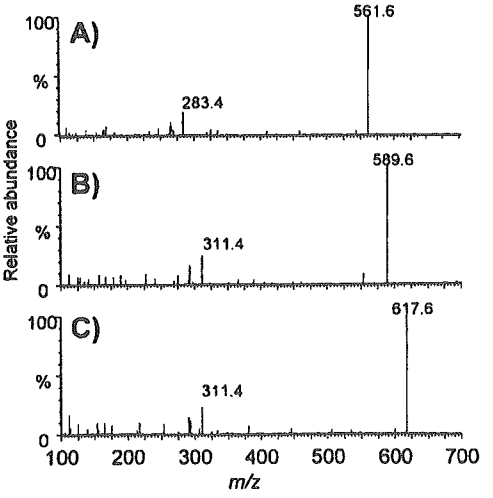

Fig. 6. Product ion scanning of the synthesized wax esters I (A), II (B) and III (C) by LC/MS/MS analysis using the APCI positive mode

to their fatty acid moieties (*m/z* 561.6→283.4, 589.6→311.4 and 617.6→311.4), as shown in Fig. 7. The concentration of the three wax ester compounds in jojoba wax was measured based on absolute calibration curves using the peak areas determined by multiple reaction monitoring (MRM) of the authentic wax esters. As a result, the concentrations of eicosenyl octadecenoate (I), eicosenyl eicosenoate (II) and docosenyl eicosenoate (III)

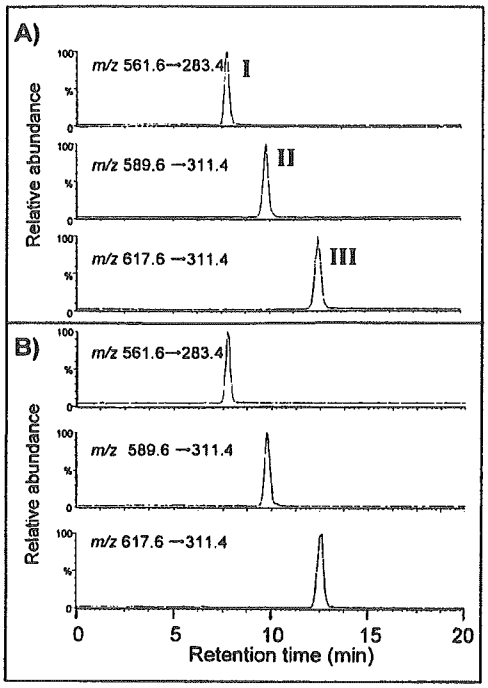

Fig. 7. Detection of product ions of the synthesized wax esters I–III (A) and the wax esters in jojoba wax (B) by LC/MS/MS analysis using the APCI positive mode scanning

were calculated to be 5.5, 21.4 and 37.8% per the weight of the jojoba wax, respectively. These results are comparable with the values calculated by multiplication of the percentage peak areas at UV 204 nm of peaks 2 to 4 and the percentage peak areas of the main fatty acids of peaks 2 to 4 in GC/MS analysis.

Some authors have investigated the molecular species of wax esters using various combinations of fatty acid and alcohol moieties[2), 4), 10)], though two of these studies[4), 10)] used GC/MS or GC/MS/MS. The other[2)] used LC/MS, but required post-column addition of Ag^+. In this paper, we demonstrated that direct quantification of certain molecular species of wax esters without hydrolysis could be performed by monitoring the product ions using LC/MS/MS. In order to evaluate the quality of food additives, it is important not only to have information about the constituents, but also their concentrations. Therefore, the direct quantification method used in this paper for each molecular species in wax esters using LC/MS/MS is expected to be very useful for helping to set official standards in the future.

Conclusion

Jojoba wax is a natural food additive used as a gum base in Japan. The findings of this study have confirmed, based on GC/MS and LC/MS/MS analyses, that the main constituents of jojoba wax are the wax esters, eicosenyl octadecenote (C20 : 1–C18 : 1), eicosenyl eicosenoate (C20 : 1–C20 : 1), docosenyl eicosenoate (C22 : 1–C20 : 1), eicosenyl docosenoate (C20 : 1–C22 : 1) and tetracosenyl eiosenoate (C24 : 1–C20 : 1). Docosenyl eicosenoate (C22 : 1–C20 : 1) and eicosenyl eicosenoate (C20 : 1–C20 : 1) were the major compounds. In this study, we clarified the main constituents of jojoba wax and directly quantified the fatty acid and alcohol composition of the wax without hydrolysis, by monitoring their product ions using a LC/MS/MS system. This is the first report that has identified the major constituents and determined their concentrations in jojoba wax used as a food additive.

Acknowledgments

We are grateful to the Japan Food Additives Association for providing jojoba wax samples. This work was supported by a grant for Risk Analysis Research on Food and Pharmaceuticals from the Ministry of Health, Labor and Welfare, Japan.

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厚生労働科学研究費補助金　食品の安心・安全確保推進研究事業
既存添加物の成分と品質評価に関する研究
平成 17 年度　総括・分担研究報告書　分冊　その 2

平成17年度 既存添加物の成分規格の設定に係る試験・研究及び調査

― 既存添加物の成分規格の設定に関する調査研究―

平成18年3月

日本食品添加物協会

主任研究者

山崎　壮

国立医薬品食品衛生研究所

研究報告書

既存添加物の成分規格の設定に関する調査研究

—規格策定のための含量・定量法及び確認試験法等に関する調査研究と自主規格(案)の策定—

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[はじめに]
当協会は，これまでも既存添加物の成分規格設定を目標に，行政並びに学識経験者のご指導のもと，当協会としての自主規格の策定を進めてきた。
平成14年11月には，これまで蓄積してきた189品目の自主規格を収載した「第三版既存添加物自主規格」を刊行した。しかしながら，既存添加物489品目（ただし，平成16年度中に通知により39品目が消除され，450品目）のうち，公定規格および自主規格策定済み品目は凡そ半数に止まっていたので，新規規格策定を継続し，平成15年度は19品目の自主規格の策定を行った。
また，第三版既存添加物自主規格既収載品目の成分規格については，平成16年度は，第8版公定書への収載候補品目を中心に，国立医薬品食品衛生研究所食品添加物部との間でその規格・試験法の妥当性を検討し，38品目について見直し改定を行った。
本年度は新たに9品目の既存添加物について自主規格の策定検討を推進し，1品目について見直し改定を行った。
これらの作業は，これまでと同様に当協会技術委員会の自主規格専門委員会が中心となって推進した。既存添加物を製造する企業が自社の品質管理に定めている規格・試験法等について調査を行い，総合的にその規格内容の妥当性を評価・検討した。必要に応じ，新しい試験法の開発検討も進め，自主規格(案)を策定するとともに，その妥当性評価を行なった。

研究結果の概要と考察

1. 研究方法

本研究は，当協会技術委員会の自主規格専門委員会が中心となって推進した。これまでと同様に既存添加物を製造する企業が自社の品質管理に定めている規格・試験法等について調査を行い，総合的にその規格内容の妥当性を評価・検討した。必要に応じ，新しい試験法の開発検討も進め，適切な安全性確保が図れるよう，自主規格(案)を策定し，その妥当性を評価した。

新規規格策定に当たっては，主成分の確認，定量法の開発検討等を中心に行い，規格・試験法の設定並びにその妥当性等に関して評価・検討を行った。

なお，これら評価・検討を行った自主規格専門委員会のメンバーは別紙に記したとおりである。

2. 研究結果の概要

2－1. 検討対象品目

本年度は以下の品目について，(1)新規規格設定のための調査研究と規格案の策定，及び，(2)当協会第三版既存添加物自主規格として定められている規格・試験法の内容についての見直しを行った。

必要に応じ新たな試験方法の導入を検討し，それらの妥当性に関しても評価・検討した。

(1)平成17年度 新規自主規格検討品目(8品目)

本年度新規規格検討品目は次のとおりである。

用途名(検討品目数)	自主規格検討品目
酵素 (8)	アクチニジン，α-アセトラクタートデカルボキシラーゼ，アントシアナーゼ，イヌリナーゼ，α-グルコシルトランフェラーゼ，ホスホジエステラーゼ，ラクトパーオキシダーゼ，リポキシゲナーゼ
製造用剤 (1)	高級脂肪酸(ステアリン酸)

(2)既設定規格の見直し品目(1品目)

平成14年11月，当協会の自主規格として「第三版 既存添加物 自主規格」を刊行した。

本年度は，「第三版 既存添加物 自主規格」収載品目のうち1品目の見直しを行った。見直しの結果，改訂の必要とされた規格項目に関しては，その妥当性を評価・検討した。

本年度見直しを行った品目及び見直しの概要は次の表のとおりである。

用途名(品目数)	検討項目	見直しの概要
着色料 (1品目)	アナトー色素	主成分とするものにより2つの成分規格に分割した。

3. 研究結果の概要

3－1. 新規作成検討品目

(1)酵素(8品目)

本年度は，次の8品目を選定し検討した。なお，これら品目はFCC-V 及びJECFA規格には収載されていない。

（アクチニジン，α-アセトラクタートデカルボキシラーゼ，アントシアナーゼ，イヌリナーゼ，α-グルコシルトランスフェラーゼ，ホスホジエステラーゼ，ラクトパーオキシダーゼ，リポキシゲナーゼ）

それぞれの品目について，性状，確認試験，純度試験，微生物限度，酵素活性測定法及び測定結果について調査研究を行い，この結果に基づいて規格案を策定し，その妥当性について検討した。 各品目とも規格，試験方法の妥当性が検証された。

規格の記載方法は公定書に準拠したが，第三版既存添加物自主規格の「酵素一般規格」に従って定義及び酵素特性を記載し，参考としてECナンバーを記載した。

確認試験は「それぞれの活性測定法に準じて試験を行うとき，酵素活性を示す」こととした。ただし，アントシアナーゼは，酵素活性測定法で酵素活性を示す方法では 一般的なβ-グルコシダーゼやβ-ガラクトシダーゼと識別できないので，赤色のアントシアン色素が退色することを確認する方法とした。又，α-グルコシルトランスフェラーゼについては，酵素活性測定法で酵素活性を示す方法と，酵素活性測定法が特異的でない場合として 基質，反応，及び生成

物を特定できる6つの方法を設定した。

純度試験については「酵素一般規格」に基づいて設定することとした。ただし，第8版食品添加物公定書の検討会にて，鉛規格の国際的整合対応として「JECFA 規格に対応でき，JECFA 規格に鉛規格以外の規格がない場合においては，重金属規格を削除できる」こととされ，酵素に関しては第8版公定書の成分規格から重金属を除くことで作業が進んでいることから，自主規格においても重金属規格を削除した。

微生物限度試験については，規格項目は「酵素一般規格」と同一としたが，細菌数の限度値は平成 15 年度食品・添加物等規格基準に関する試験検査等，食品添加物規格基準策定「食品用酵素剤の添加物指定に関する指針(案)」調査報告書において 10,000/g 以下を提案したことに基づき，規格値は 10,000/g 以下とした。

(2)製造用剤(1品目)

本年度は，高級脂肪酸(ステアリン酸)について検討した。含量，性状，確認試験，純度試験及び定量法について調査研究を行い，その結果に基づいて規格案を策定し，その妥当性について検討した。3サンプルについての繰り返し試験を行った結果，規格案の妥当性が確認された。なお，確認試験として赤外吸収スペクトルを追加検討したが，パルミチン酸の混在量で試験結果が異なるので見送った。

3－2. 既策定規格の見直し品目

(1)着色料(1品目)

今年度は，アナトー色素の「第8版食品添加物公定書」収載に向けた成分規格の策定のため，自主規格項目と試験法の見直しを行った。主成分とするものにより2つに分割した規格案について第三者機関およびメーカー各社による調査研究の結果，その妥当性があると考えられた。

4. 自主規格委員会メンバー

別紙のとおり。

5. 考察

今年度は9品目の新規自主規格策定検討及び1品目の既設定規格の見直しを行った。

規格検討内容の概要は既に述べてきた通りであるが，本年度も，規格案を作成した段階で当協会顧問の山田隆先生に規格案の全面的レビューと問題点の抽出をしていただき，必要に応じて修正するという一連の作業を繰り返し行った。これにより，本年度新規策定及び改定した規格内容は，より的確なものになったと考えている。

今後は，新規規格策定を継続するとともに，規格策定の可否についても見極めていく。また、第8版食品添加物公定書の公示が予定されていることから，自主規格内容の全面見直しを行い，第4版既存添加物自主規格の作成作業を進めて行く所存である。

本年度自主規格策定或いは見直し作業に関しては，国立医薬品食品衛生研究所食品添加物部の山崎壮先生を始めとする諸先生方並びに当協会顧問である山田隆先生には多大なるご指導を頂いた。この場をお借りし心より感謝申し上げる次第である。

以上

別　紙

自主規格専門委員会委員名簿

	委員氏名	企業名
技術委員長	高橋仁一	日本食品添加物協会
自主規格専門委員長	黄海三雄	オルガノ株式会社
自主規格専門委員	浅田敏	天野エンザイム株式会社
〃	植田実木生	扶桑化学工業株式会社
〃	長田裕次	東和化成工業株式会社
〃	唐澤昌彦	味の素株式会社
〃	香田隆俊	三栄源ｴﾌ･ｴﾌ･ｱｲ株式会社
〃	後藤康慶	三栄源ｴﾌ･ｴﾌ･ｱｲ株式会社
〃	滝口俊男	株式会社 ロッテ
〃	道津信夫	理研ビタミン株式会社
〃	深尾正	日本新薬株式会社
〃	古本重廣	武田キリン食品株式会社
〃	宮野信雄	株式会社タイショーテクノス
〃	村上和也	富田製薬株式会社
〃	大和谷　和彦	大日本住友製薬株式会社
〃	吉武繁廣	エーザイフード・ケミカル株式会社
技術顧問	山田隆	日本食品添加物協会

— 目　　次 —

平成18年3月

酵素8品目の新規自主規格（案）作成の調査研究

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ﾓﾊﾒﾄﾞ ｳｰﾋﾞﾋ　ノボザイムズ ジャパン(株)
和泉 昇　(株)林原
永井 幸枝　三井製糖(株)
金井 晴彦　ヤクルト薬品工業(株)
半谷 守弘　天野エンザイム(株)
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日本食品添加物協会 第七部会長

日本食品添加物協会・第七部会・酵素自主規格検討会は，既存添加物酵素8品目について新規に自主規格設定のための研究を行ったので，その概要を報告する。

既存添加物酵素は，「既存添加物名簿」に76品目が収載されている（ただし，内5品目は平成17年2月25日通知により消除された)。これまでに設定された規格としては，第7版食品添加物公定書に4品目，平成14年発行の第三版自主規格に17品目及び酵素一般規格が収載されている。尚，第三版自主規格に収載されているリゾチームは，第8版食品添加物公定書に収載される予定である。

平成13年度から16年度の調査研究として検討された34品目，及び平成17年度に新規に検討された8品目は，次の通りである。

第7版食品添加物公定書収載：パパイン，ブロメライン，ペプシン，トリプシン

第三版自主規格収載：　酵素一般規格，α-アミラーゼ，β-アミラーゼ，イソアミラーゼ，カタラーゼ，グルコアミラーゼ，グルタミナーゼ，シクロデキストリングルカノトランスフェラーゼ，セルラーゼ，トランスグルタミナーゼ，パンクレアチン，プルラナーゼ，プロテアーゼ，ペクチナーゼ，ヘミセルラーゼ，リゾチーム（第8版食品添加物公定書収載予定)，リパーゼ，レンネット

平成13年度新規検討品目：　インベルターゼ，α-ガラクトシダーゼ，β-ガラクトシダーゼ，グルカナーゼ，β-グルコシダーゼ，グルコースイソメラーゼ，グルコースオキシダーゼ，フィターゼ，ホスホリパーゼ

平成14年度新規検討品目：　アスコルビン酸オキシダーゼ，ウレアーゼ，キシラナーゼ，キトサナーゼ，α-グルコシダーゼ，酸性ホスファターゼ，5'-デアミナーゼ，トランスグルコシダーゼ，ペプチダーゼ，ポリフェノールオキシダーゼ

平成15年度新規検討品目：　アシラーゼ，アルギン酸リアーゼ，エキソマルトテトラオヒドロラーゼ，エステラーゼ，タンナーゼ，デキストラナーゼ，ナリンジナーゼ，フルクトシルトランスフェラーゼ，ヘスペリジナーゼ

平成16年度新規検討品目：　カルボキシペプチダーゼ，キチナーゼ，トレハロースホスホリラーゼ，パーオキシダーゼ，マルトースホスホリラーゼ，マルトトリオヒドロラーゼ

平成17年度（本年度）新規検討品目：　アクチニジン，α-アセトラクタートデカルボキシラーゼ，アントシアナーゼ，イヌリナーゼ，α-グルコシルトランスフェラーゼ，ホスホジエステラーゼ，ラクトパーオキシダーゼ，リポキシゲナーゼ