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 methanolyis and hydrolysis, followed by GC/MS. The results indicated that the main constituents in jojoba wax were various kinds of wax esters, namely eicosenyl eicosenoate (C20:1–C20:1) (I), eicosenyl eicosenoate (C20:1–C20:1) (II), docosenyl eicosenoate (C22:1–C20:1) (III), eicosenyl docosenoate (C20:1–C22:1) (IV) and tetracosenyl eicosanoate (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 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, 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. Oleic acid (cis-9-
the main components of jojoba oil are wax esters and phytosterols are minor constituents, used as standards 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<sub>254</sub> 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 acetonitrile and 10 μL of the solution was injected into the LC/MS mode system under the following conditions: column, Symmetry<sup>®</sup> C18 (2.1 i.d. mm × 150 mm, 3.5 μm, Waters); column temperature, 40°C; 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 acetonitrile and the solution was injected into the preparative LC/MS system under the following conditions: column, X Terra 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°C 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°C 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°C 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 \( \omega 9 \) \( \text{cis} \) 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\%) \( \omega 9 \) \( \text{cis} \) and unsaturated alcohols in jojoba wax esters were predominantly \( \omega 9 \) \( \text{cis} \) type\(^{[8]} \). 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 \( \times \) 0.25 mm i.d., film thickness 0.25 \( \mu \)m; J & W Scientific, Folsom, CA). The injector and detector temperatures were set at 300°C and 250°C, respectively, and the column temperature was programmed from 180°C to 280°C at 5°C/min, then from 280°C to 300°C at 15°C/min, and finally kept at 300°C for 8 min. Samples (1 \( \mu \)L) were injected through a split-injector (1/4).

**Synthesis of wax esters**

The following three wax esters were synthesized according to a reported method\(^{[9]} \). 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 \( \omega 9 \) \( \text{cis} \) 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 \( \times \) 20 cm, Art. 1.05715, Merck) with hexane and diethyl ether (80 : 20 : 1) 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 \( \mu \)L 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^\text{TM}^ {\oplus} C18 (2.1 i.d. \( \times \) 150 mm, 3.5 \( \mu \)m, Waters); column temperature, 40°C; 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
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 $\text{C}_n\text{H}_{2(n+m)}\text{O}_2$ ($m$ = 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 $\text{C}_{36}\text{H}_{68}\text{O}_2$, $\text{C}_{38}\text{H}_{72}\text{O}_2$, $\text{C}_{40}\text{H}_{76}\text{O}_2$, $\text{C}_{42}\text{H}_{80}\text{O}_2$, $\text{C}_{44}\text{H}_{84}\text{O}_2$ and $\text{C}_{46}\text{H}_{88}\text{O}_2$, 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 methanolation. 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 ($\text{C}_{16}:0$), octadecenoic acid ($\text{C}_{18}:1$), eicosenoic acid ($\text{C}_{20}:1$), docosenoic acid ($\text{C}_{22}:1$) and tetracosenoic acid ($\text{C}_{24}:1$). The main fatty acids in the isolated peak fractions were octadecenoic acid ($\text{C}_{18}:1$) in peak 2 (Fig. 3B), eicosenoic acid ($\text{C}_{20}:1$) in peak 3 (Fig. 3C), and eicosenoic acid ($\text{C}_{20}:1$) and docosenoic acid ($\text{C}_{22}:1$) 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 eicosanol ($\text{C}_{20}:1$) and docosanol ($\text{C}_{22}:1$), respectively. Peaks at 8.5 min and 17.2 min were considered to be due
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\textsuperscript{5}, in which the existence of \(\omega\) 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\textsuperscript{2–4,10}. Two of them\textsuperscript{2,10} obtained the ratios of the molecular species of the wax esters with various
combinations of fatty acids and alcohols by GC/MS/MS analysis and by GC/MS analysis of hydrolyzed samples of HPLC-fractionated peaks. The ratios in this paper are very similar to those reported in the previous papers.

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 docosyl eicosenoate (III) (C22:1–C20:1) in the jojoba wax by monitoring the product ions that corresponded 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 docosyl eicosenoate (III)
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 (C\(_{20}:1\)-C\(_{18}:1\)), eicosenyl eicosenoate (C\(_{20}:1\)-C\(_{20}:1\)), docosyl eicosenoate (C\(_{22}:1\)-C\(_{20}:1\)), eicosenyl docosenoate (C\(_{20}:1\)-C\(_{22}:1\)) and tetracosenyl eicosanoate (C\(_{24}:1\)-C\(_{20}:1\)). Docosyl eicosanoate (C\(_{22}:1\)-C\(_{20}:1\)) and eicosenyl eicosanoate (C\(_{20}:1\)-C\(_{20}:1\)) 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.

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