INTRODUCTION

Royal jelly is a milky-white colored secretion produced by the hypopharyngeal and mandibular glands located in the head of young worker honeybees to feed the larvae in the colony (Münstedt and von Georgi, 2003). If a queen bee is desired, a chosen larva will be provided a large quantity of royal jelly as its only food source, which induces rapid differentiated growth and morphology. Because of this specialized nutrition, queen bees are different from worker bees in several ways including their size, production of eggs, and longevity.

Both pure royal jelly creams and dietary supplements of royal jelly are commercially available. These products claim various health benefits such as immune-regulation, anti-aging, cholesterol-lowering, anti-inflammation, tumor vascularization inhibition, wound-healing, and antibiotic effects (Iannuzzi, 1990; Hashimoto et al., 2005; Salazar-Olivo and Paz-González, 2005; Erem et al., 2006; Hattori et al., 2007). Researchers believe that such health benefits are attributed to various ingredients present in royal jelly such as B-complex vitamins, fatty acids, adenosine, and trace minerals. The most unique and chemically interesting component in royal jelly is its fatty acid, trans-10-hydroxy-2-decenoic acid (10-HDA) (Barker et al., 1959; Blum et al., 1959; Lercker et al., 1981; Howe et al., 1985).

The amount of 10-HDA in pure royal jelly varies depending on the origin of the jelly (Ferioli et al., 2007). Since other bee products do not contain 10-HDA, the presence of 10-HDA can be used as a marker to validate the quality of royal jelly.
from other bee products in many countries (Weaver and Law, 1960; Antinelli et al., 2003). According to the Korean Beekeeping Association, for example, the amount of 10-HDA should not be less than 4.0, 1.6, and 0.56 % for lyophilized powder, pure royal jelly, and royal jelly products, respectively (Kim et al., 1989). Although details of requirements are slightly different, other countries like Australia, China, Japan, Brazil, and Thailand have comparable policies that dictate the minimum concentration of 10-HDA in various royal jelly products (Genç and Aslan, 1999; Garcia-Amoedo and de Almeida-Muradian, 2003; Ferioli et al., 2007; Zhou et al., 2007).

The quantification of 10-HDA in royal jelly products was generally conducted by high performance liquid chromatography (HPLC) and gas chromatography (GC) (Lercker et al., 1981; Bloodworth et al., 1995; Genç et al., 1999; Garcia-Amoedo and de Almeida-Muradian, 2003; Ferioli et al., 2007; Zhou et al., 2007). In the case of GC analysis, 10-HDA should be extracted from the sample and chemically converted to a volatile compound prior to GC analysis. This process generally results in the loss of significant amounts of 10-HDA and made HPLC the preferred technique (Bloodworth et al., 1995). Although specific experimental conditions are different, a hydrophobic C18 column and a polar mobile phase were frequently adopted in previous HPLC analysis.

For the last two decades, there have been increasing numbers of pure royal jelly creams and other types of royal jelly products (e.g., dietary supplements) available in the United States. However, there are no federal or local regulations regarding the concentration of 10-HDA in royal jelly products despite their increasing consumption. In this paper, the concentration of 10-HDA in pure royal jelly creams and dietary supplements containing lyophilized royal jelly commercially available in the United States via HPLC is reported.

**MATERIALS AND METHODS**

**Materials**

Deionized water used in the preparation of samples, internal standard solutions, and mobile phase and in the cleaning of the experimental apparatus was produced by a Millipore Water Purification System with a minimal resistivity of 18.0 MΩ·cm. trans-10-hydroxy-2-decenoic acid (10-HDA, 98%, Larodan Fine Chemical AB), methyl 4-hydroxybenzoate (MHB, >99.0%, Fluka), and methanol (HPLC grade, 99.9%, Acros), and phosphoric acid (H₃PO₄, ACS reagent, 85%, Acros) were purchased and used as received without further purification. Three pure royal jelly creams (Sample A, B, and C) were directly purchased from bee farms. They were delivered in an insulated box with a blue ice pack and stored at -4°C before use. Eleven dietary supplements of royal jelly (Sample D to N) were purchased from local supermarkets and national distributors. According to the supplement facts, these products contain varying amounts of lyophilized royal jelly.

**Instrumentation**

HPLC analysis was performed using a Hewlett-Packard (HP) 1050 Series HPLC system, equipped with a variable wavelength UV absorbance detector (VWD, part no. 79853C) and an Agilent Zorbax Eclipse XDB-C18 column (150 × 4.6 mm) adjusted to 25°C as a column temperature. The maximum absorbance of 10-HDA was confirmed to be 215 nm by UV-visible absorption measurements. The mobile phase was a mixture of methanol, water, and phosphoric acid (55:45:2.7, v/v/v) and the flow rate was 1.0 mL/min. The total run time of each injected sample was 10 min. For each sample, the injection volume was 3 µL. Five injections were made to gain the averages and standard deviations reported in this paper. Control of HPLC, data collection, and determination of retention time were conducted by ChemStation Software (version 9.01, Agilent Technologies). UV-visible absorption spectra ranging from 200 to 400 nm were obtained using on a
HP8452A diode array spectrophotometer. Absorption measurements were conducted three times to gain the averaged absorption data presented in this paper.

**Preparation of standard solutions and samples**

0.1 g of methyl 4-hydroxybenzoate (MHB) as an internal standard was dissolved in a solvent (methanol and water, 50:50, v/v) to produce a final volume of 1000 mL with a concentration of 100 µg/mL. Precisely weighed 0.016 g of 10-HDA was completely dissolved in a solvent (methanol and water, 50:50, v/v) to prepare 100 mL of stock solution with a concentration of 160 µg/mL. By diluting this stock solution, a series of 10-HDA solutions were prepared with concentrations of 0.1, 0.5, 1, 5, 10, 20, 40, 80, and 160 µg/mL. Finally, solutions for the construction of a standard curve were prepared by mixing an equal volume of MHB and 10-HDA solutions.

About 50 mg of samples were accurately weighed, dissolved in 25 mL of solvent (methanol and water, 50:50, v/v), and sonicated for at least 30 min. Following sonication, sample solutions were filtered twice using disposable syringe filtering cartridges with 0.45 and 0.2 µm nylon membranes, respectively. Finally, solutions for HPLC injection were prepared by mixing an equal volume of sample solutions and 100 µg/mL of the MHB solution.

**Recovery evaluation**

The accuracy of HPLC analysis was estimated by a recovery test using honey samples with the known amount of 10-HDA as reported previously (Genç and Aslan, 1999; Ferioli et al., 2007; Zhou et al., 2007). A precisely weighed 50 mg of honey, purchased from a local mart was completely mixed with 25 mL of 10-HDA solutions containing 160, 80, 40, and 20 µg/mL which had been previously prepared. The mixture was sonicated until complete dissolution for 30 min, filtered twice using disposable syringe filtering cartridges with 0.45 and 0.2 µm nylon membranes, respectively, and diluted by mixing an equal volume of 100 µg/mL MHB solution.

**RESULTS AND DISCUSSION**

Previous quantifications of 10-HDA in various types of royal jelly samples via HPLC equipped with UV detector were conducted at the absorption wavelength ranging between 210 and 225 nm. Prior to our sample analysis, UV-visible spectra of 10-HDA, MHB, and one sample (Sample J) in the range between 200 to 400 nm were obtained to optimize the absorption wavelength of the UV detector. Results are presented in Fig. 1. The concentrations of these samples were intentionally adjusted to be about 10.0, 6.25, and 588 µg/mL for 10-HDA, MHB, and Sample J, respectively, in order to keep the maximum absorbance over the entire spectral range below 1. While 10-HDA shows a strong absorption peak around 215 nm, spectra of MHB and Sample J contain no absorption around 215 nm. Therefore, our HPLC analysis was determined to be conducted at 215 nm. In addition, an absorption peak at 260 nm is observed in the spectra of MHB and Sample J, although their intensities significantly differed from each other. Although they are not included in Fig. 1, all UV-visible spectra from pure royal jelly creams and dietary supplements are similar to each other.

Fig. 2 shows chromatograms of 10-HDA, MHB, and Sample J, obtained by monitoring absorbance at 215 nm and using a flow rate of 1.0 mL/min and an eluent composed of methanol, water, and phosphoric acid (55:45:2.7, v/v/v). The retention times of 10-HDA and MHB were found to be about 6.473 and 3.602 min, respectively, by our HPLC system (Fig. 2a and 2b). In addition, no detectable interferences were found in the chromatogram of Sample J (Fig. 2c). Further data collections extended for 30 min do not show any observable peaks in the chromatograms of 10-HDA, MHB, and Sample J.

Fig. 3 shows a standard curve constructed from concentrations of 10-HDA versus the ratio of two peak areas from 10-HDA and MHB. A linear relation was found in the concentration of 10-HDA ranging from 0.05 to 80 µg/mL ($r^2 = 0.9999$). The
ratio of relative response factors (RF) of 10-HDA and MHB (RF10-HDA/RFMHB) was nearly constant over the 10-HDA concentration ranging from 5 to 80 µg/mL, and it was found to be 1.09 ± 0.03. Although royal jelly samples with known amounts of 10-HDA are preferred for the recovery evaluation, we opted to use 10-HDA spiked honey samples, which were prepared as previously reported (Genç and Aslan, 1999; Ferioli et al., 2007; Zhou et al., 2007). Table 1 shows the results of recovery of 10-HDA in honey. The overall recovery rate lies in between 97.4 and 105.9% with the relative standard deviation (RSD) of 2.4 - 3.4% over the spiked concentrations ranging from 10 to 80 µg/mL of 10-HDA. The result indicates that our proposed experimental condition was efficient enough to detect about 100% of 10-HDA present in samples.

Limit of detection (LOD) and limit of quantification (LOQ) were estimated by evaluating the baseline noise of chromatograms where LOQ and LCQ are defined as a signal to a noise ratio (S/N) of 3 and 7, respectively. By lowering the concentration of 10-HDA in honey, LOD and LOQ were estimated to be about 0.05 and 0.1 µg/mL via the experimental conditions outlined in this study. When a large amount of sample (5 and 10 µL) was injected, LOD and LCQ were not noticeably changed. LOD and LOQ from our results are comparable to those reported previously (Ferioli et al., 2007; Zhou et al., 2007).

Finally, the average concentrations of 10-HDA in 14 samples (3 pure royal jelly creams and 11 supplements) was determined by our optimized HPLC method and the results are listed in Tab. 2. It was found that the concentration of 10-HDA ranges from 1.85 to 2.15% for pure royal jelly creams and from 0.43 to 6.28% for supplemental products. Our results shows that the concentrations of 10-HDA in our pure royal jelly creams were comparable to those from China (1.26 - 2.21%), Turkey (1.02 - 2.38%), Italy and other European countries (0.8 - 3.2%), and Brazil (1.58 - 3.10 %) (Genç and Aslan, 1999; Garcia-Amoedo and de Almeida-Muradian, 2003; Ferioli et al., 2007; Zhou et al., 2007).

Since royal jelly supplements contain different amounts of royal jelly, concentrations of 10-HDA in samples were estimated by the concentration of pure royal jelly in samples claimed by manufacturers.
**Table 1**

Recovery of 10-HDA from samples with different concentrations of 10-HDA in honey (n = 5)

<table>
<thead>
<tr>
<th>Concentration of 10-HDA (µg/mL)</th>
<th>Mean recovery ± standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.0</td>
<td>97.4 ± 2.4</td>
</tr>
<tr>
<td>40.0</td>
<td>102.6 ± 2.6</td>
</tr>
<tr>
<td>20.0</td>
<td>100.4 ± 3.4</td>
</tr>
<tr>
<td>10.0</td>
<td>105.9 ± 2.7</td>
</tr>
</tbody>
</table>

**Fig. 2.** HPLC chromatogram of internal standard (a), 10-HDA (b), and Sample J (c)
Fig. 3. Standard curve constructed from various concentrations of 10-HDA versus the ratio of two peak areas from 10-HDA and MHB

Table 2

The average and relative standard deviation (RSD, %) of 10-HDA concentrations in pure royal jelly creams and royal jelly supplements (n = 5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>10-HDA concentration (%) by HPLC</th>
<th>Claimed 10-HDA concentration (%) by manufacturer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure creams</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.85 ± 0.37</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>2.15 ± 0.55</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>2.07 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>Supplements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.31 ± 0.22</td>
<td>2.69</td>
</tr>
<tr>
<td>E</td>
<td>0.91 ± 0.37</td>
<td>1.59</td>
</tr>
<tr>
<td>F</td>
<td>2.48 ± 0.14</td>
<td>5.41</td>
</tr>
<tr>
<td>G</td>
<td>0.43 ± 0.57</td>
<td>0.28</td>
</tr>
<tr>
<td>H</td>
<td>0.54 ± 0.67</td>
<td>2.36</td>
</tr>
<tr>
<td>I</td>
<td>1.73 ± 0.19</td>
<td>3.64</td>
</tr>
<tr>
<td>J</td>
<td>6.28 ± 0.33</td>
<td>7.66</td>
</tr>
<tr>
<td>K</td>
<td>0.97 ± 0.25</td>
<td>1.66</td>
</tr>
<tr>
<td>L</td>
<td>5.79 ± 0.21</td>
<td>5.98</td>
</tr>
<tr>
<td>M</td>
<td>1.66 ± 0.40</td>
<td>2.15</td>
</tr>
<tr>
<td>N</td>
<td>0.64 ± 0.70</td>
<td>2.26</td>
</tr>
</tbody>
</table>

* Claimed concentration of 10-HDA was estimated by the use of the average concentration of 10-HDA in pure royal jelly creams of Samples A, B, and C and the amount of pure royal jelly in supplements
and concentration of 10-HDA in pure royal jelly (Tab. 2). We assumed that the concentration of 10-HDA in pure royal jelly is the average of 10-HDA from our samples (2.02%) although the origin of royal jelly in supplements was not confirmed. It was noticeable that all royal jelly supplements contain less 10-HDA than the claimed amount except Sample G. Such a difference is presumably caused by the freeze-drying process (lyophilization). It was reported that lyophilization for commercialization significantly affects the contents of major ingredients in pure royal jelly creams (Messia et al., 2005).

**SUMMARY AND CONCLUSIONS**

Reversed phase HPLC was employed to analyze the concentrations of 10-HDA in pure royal jelly creams and supplemental products purchased in the United States. Optimized experimental conditions including the mobile phase and chromatographic column, allowed for the determination of 10-HDA concentrations with enhanced sensitivity and reliability. Our results show that the concentrations of 10-HDA in pure royal jelly creams are nearly in the range of 1.85 - 2.18%, which is comparable to those of royal jelly creams in other countries. However, the concentration of 10-HDA in dietary supplements ranges from 0.43 to 6.28%, which is less than the claimed concentrations with the exception of one sample.

**ACKNOWLEDGEMENT**

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**REFERENCES**


Przeprowadzono analizę ilościową kwasu trans-10-hydroksy-2-decenowego (10-HDA) w próbkach świeżego mleczka pszczelego oraz suplementach diety z mleczka pszczelego dostępnych w Stanach Zjednoczonych, z użyciem techniki wysokosprawnej chromatografii cieczowej w układzie odwróconych faz. Rozdział badanego związku, 10-HDA, w próbkach oraz wewnętrznego standardu, 4-hydroksybenozesangu metylu (MHB), przeprowadzono z wykorzystaniem kolumny Zorbax Eclipse XDB-C18 (150 × 4,6 mm) z fazą mobilną składającą się z metanolu i wodnego roztworu kwasu fosforowego w temperaturze 25°C. Szybkość przepływu wynosiła 1,0 mL/min, detekcję UV prowadzono przy długości fali 215 nm. Średni odzysk 10-HDA wyniósł 97,4 - 100,4% przy względem odchyleniu standardowym (RSD) 2,4 - 3,4% dla stężeń z zakresu od 10 do 80 µg/mL. Granicę wykrywalności (LOD) i granicę oznaczalności (LOQ) określono odpowiednio na poziomie około 0,05 i 0,25 µg/mL. Otrzymane wyniki wykazują, iż stężenie 10-HDA wynosi od 1,85 do 2,18% dla świeżego mleczka pszczelego oraz od 0,43 do 6,28% dla suplementów z mleczka pszczelego.

Słowa kluczowe: mleczko pszczele, kwas trans-10-hydroksy-2-decenowy (10-HDA), wysokosprawna chromatografia cieczowa (HPLC).