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Application of Solid/Liquid Extraction for the Gravimetric Determination of Lipids in Royal Jelly

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Gravimetric lipid determination is a major parameter for the characterization and the authentication of royal jelly quality. A solid/liquid extraction was compared to the reference method, which is based on liquid/liquid extraction. The amount of royal jelly and the time of the extraction were optimized in comparison to the reference method. Boiling/rinsing ratio and spread of royal jelly onto the extraction thimble were identified as critical parameters, resulting in good accuracy and precision for the alternative method. Comparison of reproducibility and repeatability of both methods associated with gas chromatographic analysis of the composition of the extracted lipids showed no differences between the two methods. As the intra-laboratory validation tests were comparable to the reference method, while offering rapidity and a decrease in amount of solvent used, it was concluded that the proposed method should be used with no modification of quality criteria and norms etablished for royal jelly characterization.

KEYWORDS: Royal jelly; lipids; extraction; quality

INTRODUCTION

Royal jelly is a secretion from mandibular and hypopharyngeal glands of *Apis mellifera* (Hymenoptera, Apidae) nurse bees involved in the sexual determination and longevity of the queen (*1*). Royal jelly contains sugars, vitamins, trace elements, fatty acids, and amino acids, and its biological activities are appreciated as a complement in human diet. As a result of the increasing interest of royal jelly in human health, the number of reports in the area of authentication and quality control have increased within the past few years. Methods have been developed to characterize the quality of royal jelly by determination of general parameters such as water content, sugars (*2*−*5*), lipids (*6*−*8*) proteins (*9*−*11*), or genuine compounds such as (*E*)-10-hydroxydec-2-enoic acid (*12*), by using high-pressure liquid chromatography, gas chromatography, or SDS−PAGE analysis.

Among these criteria, lipid determination provides useful information on quality based on the presence of natural lipids in royal jelly. Exogenous lipids, due to harvest process or fraudulently introduced in the product, can also be easily identified by gas chromatographic analysis using appropriate standards.

Gravimetric determination of lipids was also a major parameter to certify the composition of royal jelly.

Until now, extraction of lipids was based on a discontinuous liquid/liquid extraction with diethyl ether of an aqueous solution of royal jelly as previously developed in our laboratory (*13*). By using this reference method, the gravimetric determination of the dry extract was referred to as the lipid content. No other protocol has been proposed to date. Because this extraction is complex, and solvent- and time-consuming, we report the development of an alternative quantitative method and its evaluation in comparison to the reference method.

MATERIALS AND METHODS

Safety. Boron trifluoride in methanol is a very dangerous reactive. Precautionary handling procedures must be associated with its use.

Reagents. All solvents were of analytical grade. Ultrapure deionized (18 MΩ), degassified water (Elgastat UHQ II, Elgale, England) was used for the aqueous solutions. 10-Hydroxydecanoic acid, sebacic acid, and palmitic acid for standard solutions, and boron trifluoride dimethanol complex (20% w/v) for lipid derivatization, were purchased from Sigma Aldrich (St. Quentin Fallavier, France). (*E*)-10-Hydroxydec-2-enoic acid was provided by Nippon Shogi Kaisha (Osaka, Japan). (*E*)-9-Hydroxydec-2-enoic acid and (*E*)-9-oxodec-2-enoic acid were purchased from Phero Tech Inc. (Delta ,BC).

Equipment. For the alternative method, a Soxtech system HT2 1045 unit (Tecator, Hoganas, Sweden) with a digital temperature controller was used as an extractor apparatus. The timer of the device was used to control the duration of the different steps involved in the extraction. Aluminum extraction cups manufactured by Foss Electric (Nanterre, France) and cellulosic thimbles 33 × 80 mm (Schleicher & Schüll GmbH, Dassel, Germany) were used. For the reference method, glassware involved in extraction was manufactured according to the procedures recommended by quality assurance (Mod'Verre, Biot,

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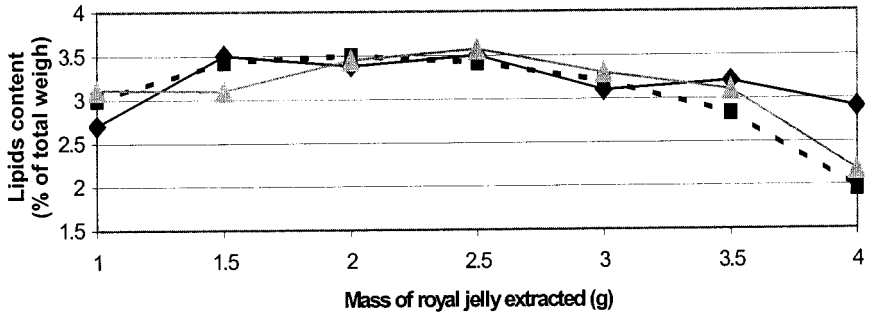

Figure 1. Recovery of lipid content (%) by the alternative method according to the amount of royal jelly. Value (3.83%) obtained by the reference method was considered as the reference value. Boiling and rinsing times were fixed at 15 and 20 min, respectively. Symbols: (♦), (■), (▲) triplicates.

France) and used with an appropriate liquid-phase rotative extractor (Crouzet, France).

Samples. A unique sample of fresh royal jelly from Thailand was used in a sufficient quantity required for all the experiments. Commercial samples for comparison of the two methods were provided by beekeeper associations. All samples were stored at 4 °C until analysis.

Extraction. For the alternative method, lipids were extracted by continuous solid/liquid extraction with refluxing diethyl ether using the following procedure. A known amount of royal jelly was spread on the thimble. Diethyl ether (50 mL) was added in a weighed extraction cup and the temperature control was set at 80 °C according to the manufacturer's recommendation. The extraction process included three steps: boiling (thimble immersed in diethyl ether), rinsing (thimble hung above the solvent), and solvent evaporation from the cup. This procedure was applied three times per sample to ensure a total recovery of lipids, whatever the physical properties of the royal jelly. Each extract was equilibrated at room temperature for 30 min. Gravimetric determination gave the lipid content. Blank analysis without royal jelly showed no influence of the solvent and thimbles.

In the reference method, 3 g of royal jelly was suspended in 150 mL of ultrapure water and extracted with an equivalent volume of diethyl ether. Thirty rotations (6 min per rotation) were performed. After filtration (Whatman phase separator, silicone treated, 1PS), the organic layer was collected in a weighed extraction cup and then evaporated on a hot-plate set at 45 °C. The aqueous layer was completed with 150 mL of diethyl ether. The extraction process was then repeated twice using the same conditions. Each extract was equilibrated at room temperature for 30 min before gravimetric determination. In these conditions, at least 570 min was necessary for a total cycle of extraction.

Calculations. Evaluation of the alternative method during the optimization step was based on lipid content. This parameter was expressed either as the resulting amount of lipid in grams or as the resulting amount of lipids relative to the amount of the sample of royal jelly (%).

Derivatization. Gas chromatography analyses were used to evaluate the influence of the extraction process on the final composition of the lipidic extract. Prior to injection, lipids were derivatized into the corresponding methyl esters as follows. The dry extracts of lipids were dissolved in a 50-mL flask using 5 mL of diethyl ether; the extraction cup was rinsed twice with 5 mL of diethyl ether to avoid loss of matter. Boron trifluoride dimethanol complex (5 mL) was added, and the resulting solution was heated at reflux for 3 min. Heptane (4 mL) was then introduced into the flask and heated at reflux for 3 min. A saturated solution of NaCl (5 mL) was added to the reaction mixture. After decantation of the mixture, the organic layer was transferred into a 10-mL glass vial containing anhydrous $MgSO_4$. Despite its high toxicity and known reaction of isomerization of double bonds *(14)*, the use of boron trifluoride in methanol is still the reference method for gas chromatographic analysis of fatty acid in royal jelly.

Gas Chromatography Analytical Conditions. Gas chromatography−flame ionization detector (GC−FID) analyses were performed with a 6890 Hewlett-Packard chromatograph using a split/splitless injector. A column (PE-WAX, 30 m × 0.32 mm i.d.) was operated with 0.9 mL/min carrier gas (N_2). The oven temperature was programmed from 150 °C at 1 °C/min to 220 °C, then isothermally. The injector and flame ionization detector were operated at 220 and 300 °C, respectively. The injection volume was 1 μL (splitless 0.43 min). Gas chromatography−mass spectrometry conditions were the same as those used for FID detection, using a 6890 chromatograph connected to an HP 5973N quadrupole mass spectrometer. Electron impact masses were recorded at 70 eV and helium was used as carrier gas.

Identification and Quantification. Chemical assignment of methylated compounds was made by comparing gas chromatography retention time to those of authentic standards. Standard additions in the heptane extract were used to ensure the identification. The identification was completed by comparison of the mass spectra obtained from the samples and authentic compounds. Unidentified compounds were referred to as unknown because authentic standards were not commercially available to confirm the assignments reported in the literature. Characterization of the extract was based on peak area normalization, using HP GC Chemstation (version A.07) sofware.

RESULTS AND DISCUSSION

Optimization of the Amount of Royal Jelly and the Extraction Time. For the evaluation of the solid/liquid method, the extraction step was optimized by determining the appropriate amount of royal jelly and the time of extraction. The lipid contents of the extracts obtained by the two methods were compared by gravimetric analysis. For the amount of royal jelly, optimization was performed on samples from 1 to 4 g. According to the manufacturer's recommendation, boiling and rinsing times were fixed at 15 and 20 min, respectively. With these parameters of time, **Figure 1** shows the importance of the amount of sample on the extraction efficiency. Amounts of royal jelly <2 g or >3 g led to lower and variable yields of lipids. These results agreed with the observed variations for amounts lower than 2 g during the previous optimization of the reference method. Two hypotheses can explain these variations. First, the variability for the lowest sizes of samples may be correlated with heterogeneous distribution of lipids in the royal jelly. Lipids in royal jelly are present in a proportion lower than 5% and similar variations are regularly evoked during the development of analytical methods for natural and complex food products (*15*). Second, the spread of royal jelly onto the thimble also appeared to be a critical parameter. A high amount of royal jelly led to an increase in the thickness on the timble, resulting in a decreased extraction efficiency. According to these experimental results, the optimal amount of sample was 2.5 g. A lipid content of 3.83% was obtained from the extraction of the sample by the reference method.

We further determined the optimal durations for the boiling and rinsing phases. As shown in **Figure 2**, both boiling and rinsing times had an influence on lipid yield. The optimization was performed according to the boiling/rinsing ratio. According to the data, boiling and rinsing times were fixed at 20 and 40 min, respectively. This combination gave a good yield in comparison to the reference value (3.83%) and a convenient

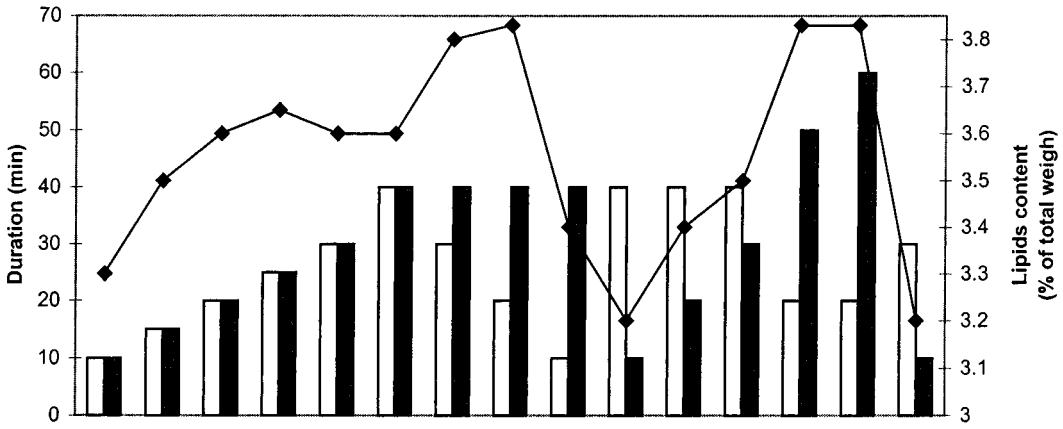

Figure 2. Recovery of lipid content (%) by the alternative method according to the duration of boiling and rinsing steps. The reference lipid content was 3.83%. Symbols: (♦) mean of triplicates; (□) boiling duration; (■) rinsing duration.

Table 1. Comparative Performance Results for the Reference and Alternative Methods Obtained with 20 Replicates of the Same Fresh Sample of Royal Jelly

method	size of sample (g)	lipid content g	lipid content %	S_r	RSD (%)
reference	3.0	0.117	3.9	0.0019	1.6
solid/liquid	2.5	0.098	3.9	0.0017	1.7

time of extraction (60 min). Longer boiling and rinsing times did not improve the recovery yields.

Repeatability. Twenty runs were performed with 2.5 g from the same sample of royal jelly. Calculations applied were those dedicated to validation of repeatability tests as previously published (*16, 17*). The results of the gravimetric determination (**Table 1**) showed a good repeatability for the two methods with repeatability standard deviations of $S_r = 0.0017$ for the alternative method and 0.0019 for the reference method. Maximum tolerable differences for repeatability were defined by $r = 3.20 \times S_r$ for a 95% confidence level. The data revealed no measurement outside these limits, and the extraction methods were considered repeatable.

Intra-Laboratory Reproducibility of the Solid/Liquid Method. Statistical variances between two analysts were examinated in order to determine whether there were any differences due to the operator. The means for the amounts of lipids extracted with 7 replicates were 0.098 g ($S_r = 0.0011$) and 0.097 g ($S_r = 0.0012$). Following the evaluation of variances of each analyst using a F test ($p > 0.05$), the paired *t*-test did not reveal any significant differences between the two analysts ($p > 0.05$): therefore, the alternative method proved to be reproducible.

Comparison of the Alternative and Reference Methods. Performance statistics of both methods were first compared using the same test material. The results, included in **Table 1**, showed comparable S_r values for the two methods. Therefore, if we consider the quantification resulting from the reference method as the correct value, the soxtech and reference method results were in good agreement.

To ascertain whether there was no difference between the methods, 10 different samples containing various amounts of lipids were analyzed. Lipid contents from 3.5 to 4.2% were calculated. These values are representatives of the natural variation usually found in royal jelly analysis. Maximal difference of 0.1% was obtained in the lipid content for each sample when the extraction methods were compaired. Using a paired *t*-test, no significant differences for the gravimetric determination of lipid content (%) were observed between the two methods ($p > 0.05$).

Table 2. Mean ($n = 3$) and Relative Standard Deviation (RSD) of Normalized Area of Fatty Acid Methyl Esters in Lipid Extracts Obtained Using Reference and Alternative Methods

fatty acid	reference method mean (%)	reference method RSD (%)	solid/liquidmethod mean (%)	solid/liquidmethod RSD (%)
unknown[a]	3.74	2.6	3.82	2.4
unknown[a]	2.34	2.1	2.47	2.1
palmitic acid	0.37	20.3	0.32	25.6
sebacic acid	7.87	1.1	7.80	1.2
10-hydroxydecanoic acid	20.46	0.4	20.66	0.3
(*E*)-10-hydroxydec-2-enoic acid	49.02	0.3	49.14	0.3
(*E*)-9-hydroxydec-2-enoic acid	3.07	3.7	3.13	0.3
unknown[a]	27.56	0.4	27.53	0.4

[a] Not structurally identified.

To test the selectivity of the extraction on the fatty acids of royal jelly, analyses of triplicates obtained by the two methods were performed by gas chromatography after derivatization. As summarized in **Table 2**, relative percentages of methyl ester fatty acids showed a good agreement between the two methods. Saturated and unsaturated fatty acids appeared to be unaffected by the experimental conditions of the solid/liquid extraction, in comparison to the reference method. The relative standard deviations were identical to those generally obtained during the development of extraction protocol. The reference literature suggest that the unknown compound representing 27% of the total peak area is (*E*)-9-oxodecanoic acid. However, the injection of standard associated with the comparison of mass spectra have not confirmed this identification. Currently, work is in progress concerning the characterization of this compound.

These results suggest the interest of the solid/liquid extraction for quantitative determination of natural lipids in royal jelly. However, for this purpose, a new protocol should be developed in order to eliminate the known limitation due to the use of boron trifluoride in methanol. It could be suggested that this method was also appropriate for the detection and the characterization of exogenous lipids in royal jelly.

Of particular interest, this study has shown similar results regarding the gravimetric determination and the individual quantification of fatty acids with both methods. This is particularly important because the alternative method could be used without modification of the current quality criteria of royal jelly or any correction factor. However this method should be

used routinely with a strict control of the identified critical factors: the spread of royal jelly onto the thimble and the duration of boiling/rinsing steps. Although this method still uses the hazardous solvent diethyl ether, the amount of solvent has been drastically reduced in comparison to that of the reference method.

Moreover, it is known that extraction methods based on polar solvents are not only selective for fat compounds, but also lead to extract traces of non fat compounds. As a consequence, this approach addresses the problem of the accuracy of the gravimetric determination. However, in this case, the influence of such compounds seems to be identical with both methods. Even if the derivatization process minimizes the importance of nonlipidic compounds in the chromatographic analysis, the development of other protocols could be useful to improve the gravimetric determination of lipids content in royal jelly. A promising method could be superfluid critical extraction which is involved in recent developments for fat extraction (*18, 19*). Before the implementation of this new approach, the method described in this report can be considered as an interesting alternative to the current reference method.

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