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Research Article

Development and validation of a rapid gas chromatography for simultaneous quantification of major fatty acids in royal jelly

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Abstract: Fatty acids are distinctive components of royal jelly (RJ), serving as a crucial indicator for assessing the quality of RJ. This study aimed to establish a rapid and simultaneous quantification method for various fatty acids in RJ utilizing gas chromatography. An optimized two-step extraction process, incorporating ethanol and diethyl ether followed by derivatization with *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide, was developed to enhance sensitivity and precision in detecting fatty acids. Validation of the methodology revealed excellent linearity ($R^2 > 0.999$), precision (relative standard deviation (RSD) < 1%), repeatability (RSD < 1%), and recoveries (94.4%–104%). Furthermore, the limits of detection and quantification were found to be low. The results indicated that the method offered reliable and consistent quantification of major fatty acids, thereby improving quality control for RJ and facilitating its applications in food, pharmaceutical, and biochemical research.

Keywords: royal jelly; fatty acids; rapid determination; gas chromatography

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1 Introduction

Royal jelly (RJ) is a yellowish, creamy, and acidic secretion produced by the hypopharyngeal and mandibular glands of worker bees aged between 5 and 15 d[1]. It plays an important role in developing the bee colony, especially in nurturing the queen bee and young larvae[2]. The composition of RJ is complex, consisting of proteins, carbohydrates, lipids, vitamins, minerals, etc.[3]. Notably, lipids constitute 8%–19% of lyophilized RJ, with free fatty acids accounting for 80%–90% of the total lipids[4]. The fatty acids in RJ are primarily medium-chain fatty acids containing 8–10 carbon atoms that are hydroxylated at the terminal and internal positions[5]. Additionally, they possess a second terminal carboxylic acid functionality that may be saturated or unsaturated at the 2-position[6]. Fatty acids, including 10-hydroxy-2-decenoic acid (10-HDA), 10-hydroxydecanoic acid (10-HDAA), 3-hydroxydecanoic acid (3-HDAA), sebacic acid (SEA), 8-hydroxyoctanoic acid, 2-decene-1,10-dicarboxylic acid and so on, are one of the significant components of RJ with diverse biological activities[7], such as antioxidant[8], anti-inflammation[9], anti-bacteria[10], and anti-tumor[7]. In particular, 10-HDA is the primary fatty acid in RJ and is considered a critical characteristic substance. Therefore, it serves as an essential indicator for assessing the quality of RJ[11].

In recent years, diverse techniques have been extensively employed for the detection of fatty acids in RJ, including high performance liquid chromatography (HPLC)[12], gas chromatography (GC)[13], and enzyme-linked immunosorbent assay (ELISA)[14]. Numerous studies have concentrated on enhancing pre-treatment methods and conditions to reduce detection times, improve accuracy, and lower limits of detection[15–16]. These advancements have significantly refined existing methodologies, providing greater convenience and reliability in detecting RJ. Moreover, innovative approaches such as near-infrared spectroscopy[17], capillary zone electrophoresis (CZE)[18], and high resolution mass spectrometry[19] have emerged for the detection of fatty acids in RJ. These advanced techniques provide additional options for rapid, non-destructive, and precise quantification of fatty acids in RJ[20]. Among these methods, HPLC is widely utilized in quality control of RJ since it serves as the reference method for detecting 10-HDA according to the GB 9697–2008 *Royal jelly*.

Analytical methods for detecting fatty acids in RJ predominantly concentrate on the principal component 10-HDA[20]. However, studies on other essential functional fatty acids in RJ, such as 8-hydroxyoctanoic acid and 2-decene-1,10-dicarboxylic acid, have not been sufficiently quantified[19]. Furthermore, the methods commonly used for the simultaneous detection of multiple fatty acids in RJ often involve complex procedures and costly equipment, making it challenging to detect various fatty acids in RJ effectively[21]. Consequently, establishing quantitative measurement techniques for multiple fatty acids in RJ is crucial for enhancing quality control systems associated with this valuable substance. Improvements to RJ quantitative detection methods will deepen our understanding of its biochemical properties and ensure its consistency and efficacy across various applications. Therefore, a method for rapidly and simultaneously quantifying various commonly found fatty acids in RJ was developed in this study. Subsequently, comprehensive validation was carried out to demonstrate its effectiveness. The aim of this study was to establish conditions for detecting a variety of fatty acids, and provide technical support for adulteration

identification, authenticity verification, and other related applications concerning RJ.

2 Materials and methods

2.1 Chemicals

Methanol was acquired from Merck (Darmstadt, Germany). *N*,*O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA), methyl 4-hydroxybenzoate, and pyridine was purchased from Sigma-Aldrich (MO, USA). Ethanol, HCl, and ether were obtained from Shanghai Hushi Laboratory Equipment Co., Ltd. (Shanghai, China). 10-HDAA, SEA, 8-hydroxyoctanoic acid, and methyl *p*-hydroxybenzoate were purchased from Macklin (Shanghai, China). 3-HDAA was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). 2-Decene-1,10-dicarboxylic acid was synthesized by Accela (Shanghai, China). The reagents used above are chromatographic purity. Deionized water was used to prepare all the working solutions.

2.2 Preparation of standard solutions and samples

A standard stock solution was prepared by dissolving 0.013 4 g of 10-HDAA, 0.004 9 g of 3-HDAA, 0.002 0 g of SEA, 0.008 2 g of 8-hydroxyoctanoic acid, and 0.008 0 g of 2-decene-1,10-dicarboxylic acid in ethanol in a 50 mL volumetric flask. The solution was then stored at 4 °C in a refrigerator. Calibration standards were freshly prepared by adding 0.5, 1, 2, 3, 4, or 5 mL of the standard stock solution and 2 mL of the internal standard solution, adjusting the final volume to 10 mL. The internal standard solution was prepared by weighing 0.162 5 g methyl *p*-hydroxybenzoate and diluting it to 25 mL with ethanol.

RJ (0.500 0 g) was transferred to a 25 mL volumetric flask, 1.5 mL of 0.01 mol/L HCl solution was added and mixed using a vortex mixer, 5 mL of internal standard solution was added and diluted to the flask with ethanol. The mixture was dispersed in an ultrasonic bath for 15 min, and 1 mL of it was filtered with a 0.22 μm nylon membrane and placed in a 75 °C oven to evaporate the solvent. Then, 1 mL ether was added and put in a 75 °C oven to evaporate the solvent. And then, 400 mL pyridine and 80 mL BSTFA were added, sealed, and heated in a 70 °C water bath for 1 h to derivatize samples. Mixed standard stock solution (1 mL) was subjected to the same derivatization pretreatment.

2.3 GC analysis

GC analysis was performed using an 7890A gas chromatograph (Agilent, CA, USA) equipped with a HP-5ms capillary column (30 m × 0.25 mm, 0.25 μm) (Agilent, CA, USA) and a flame ionization detector (FID). The GC conditions were set with an injection volume of 1.0 μL and a split ratio 1:5. The injector temperature was 250 °C and the detector temperature was 330 °C. The oven temperature program was set as follows: an initial temperature of 50 °C was held for 5 min, followed by an increase to 300 °C at a rate of 5 °C/min, and held constant at 300 °C for 5 min. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min.

2.4 Method validation

According to the protocol described by the International Conference on Harmonization (ICH) and Association of Official Analytical Chemists (AOAC) International, several parameters were assessed, including linearity, the limit of detection (LOD), limit of quantification (LOQ), precision, repeatability, and accuracy, were determined to validate the method.

2.4.1 Linearity

The linearity of calibration curves was assessed for those generated for the determination of 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid, respectively, and evaluated by examining the linear regression coefficient (R^2) of the curves constructed with 6 levels of concentration. Six standard solutions with different concentrations were prepared by diluting them with ethanol. The calibration curves were constructed by plotting the peak areas of 5 fatty acids versus their concentrations. The linearity of these calibration curves was then assessed using the least squares method.

2.4.2 LOD and LOQ

The LOD and LOQ were estimated by performing 10 replicate measurements of low-concentration analytes, with the limits determined as 3 times and 10 times the signal-to-noise ratio (R_{SN}), respectively.

2.4.3 Precision

The precision of the method was assessed by calculating the relative standard deviation (RSD) from intra-day and inter-day measurements. Intra-day precision was determined by analyzing the same sample 6 times within the same day. In contrast, inter-day precision was evaluated by analyzing the same sample in 6 replicates across two consecutive days.

2.4.4 Repeatability

The repeatability of the developed method represents the degree of agreement between a series of measurement results obtained by taking the same sample independently 6 times.

2.4.5 Accuracy

Recovery assessment was conducted by adding standard solutions at spiking levels of 50%, 100%, and 150% of their initial concentrations in RJ samples. The amount of analyte recovered from the spiked samples was determined by comparing the measured concentration to the known amount added.

2.5 Statistical analysis

Statistical analysis, linear regression, calculations, and graphs were performed using Prism software (GraphPad Software, CA, USA) and Excel (Microsoft, Redmond, USA). Results were expressed as the mean ± standard deviation of 3 or 6 replicates unless otherwise specified.

3 Results and discussions

3.1 Confirmation of extraction and derivatization conditions

Extracting fatty acids from the viscous matrix of RJ presents technical challenges. This study employed HCl for acid hydrolysis before extraction. The results revealed a marked difference in peak areas when comparing the sample with and without HCl treatment,

underscoring the necessity of hydrolyzing RJ with HCl before extraction. This study focused on extracting major fatty acids from RJ, including 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid. These 5 fatty acids are notably polar due to their two functional groups: either a carboxyl and a hydroxyl group or two carboxyl groups[5]. Ethanol and ether, solvents with polarity similar to the fatty acids in RJ, are commonly used for their extraction[22]. While ethanol could effectively extract a broad range of fatty acids from RJ, it often led to peak chromatogram overlap. On the other hand, ether provided excellent peak resolution in chromatographic analysis but tended to cause RJ to solidify during extraction, resulting in lower extraction efficiency compared to ethanol. Therefore, a two-step extraction process was employed in this study. First, the fatty acids were extracted using ethanol. After filtration and evaporation, a subsequent extraction with ether was performed to enhance the efficiency and resolution of the fatty acids extracted from RJ. In conclusion, this study finally determined that the optimal approach involved hydrolyzing RJ with HCl, followed by a sequential extraction process utilizing ethanol and ether, thus greatly reducing the interference from extraneous substances, thereby enhancing separation efficiency.

Due to the high boiling points of fatty acids, derivatization is generally necessary for fatty acid analysis via GC, particularly for fatty acids containing more than 10 carbon atoms[23]. Derivatization methods include methylation and silylation[24]. Silylation entails converting the carboxyl and hydroxyl groups of fatty acid into silyl derivatives, which is especially advantageous for analyzing polar compounds such as hydroxyl and amino acids[25]. This process enhances the separation efficiency of these compounds in GC. Since the fatty acids in RJ possess hydroxyl groups, BSTFA was employed for sample preparation in GC analysis in this study.

3.2 Optimization of instrumental analysis condition

The obtained derivative solutions were separated and analyzed using a GC system equipped with an HP-5ms capillary column. For the analysis, 1 μL of the sample was injected into the system. The injector temperature was maintained at 250 °C, while the detector was set to a higher temperature of 330 °C to ensure optimal detection sensitivity. The oven temperature program was set as follows: an initial temperature of 50 °C was held for 5 min, followed by a ramping increase to 300 °C at a rate of 5 °C/min, and then held constant at 300 °C for a additional 5 min to ensure complete elution of higher boiling point compounds. Hydrogen, recognized for its efficiency as a carrier gas in GC due to its high diffusivity and low viscosity, was used at a constant flow rate of 1 mL/min, under these conditions, the chromatograms of the 5 fatty acids were shown in Figure 1. The chromatograms also displayed peaks corresponding to relevant standards, demonstrating the capability of this method to separate and identify these compounds effectively.

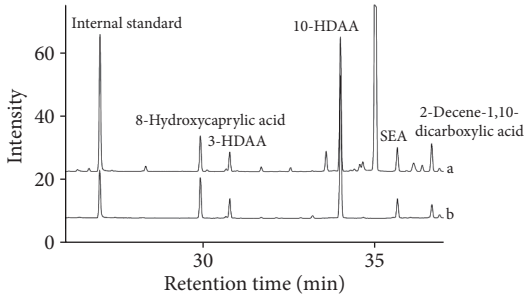

Figure 1 GC chromatogram of fatty acids in RJ and corresponding standards. a, RJ; b, standards.

3.3 Validation of method performances

3.3.1 *Linearity result*

Linearity refers to the capacity of the method to produce results directly proportional to the analyte concentration within a given range. Essentially, it implies that as the concentration of the measured substance increases, the response should increase proportionally[26]. The linearities for 5 fatty acids in RJ, including 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid were demonstrated by evaluating various concentrations of each compound against their corresponding instrumental response. The key parameters, such as regression equation and correlation coefficient (R^2), for the calibration curves used in quantifying these 5 fatty acids were summarized in Table 1, respectively.

The absolute values of *y*-intercept for the calibration curves were well within acceptable limits, with measurements of 0.045 9 for 10-HDAA, 0.008 2 for 3-HDAA, 0.006 4 for SEA, −0.041 8 for 8-hydroxycaprylic acid, and −0.009 3 for 2-decene-1,10-dicarboxylic acid. These low *y*-intercept values indicated a high accuracy in detecting the 5 fatty acids at very low concentrations. Furthermore, the R^2 for the calibration curves of all 5 fatty acids exceeded 0.999, highlighting the excellent fit of the calibration models to the data. The elevated R^2 underscored the stability, repeatability, and linearity of the measurement system and affirmed the reliability and precision of the method in quantifying these fatty acids.

While these findings demonstrate the method's efficacy regarding linearity, further validation is essential to ensure a comprehensive evaluation of its performances. This includes assessing additional parameters, such as LOD and LOQ, and evaluating the method's precision, repeatability, and accuracy[27]. These factors are critical for a thorough understanding of the method's capabilities and limitations, thereby providing a more holistic assessment of its suitability for routine analysis of fatty acids in RJ.

Table 1 Determination of the linear range for major fatty acids in RJ.

Fatty acid	Linear range (μg/mL)	Regression equation	Correlation coefficient (R^2)
10-HDAA	13.4–134	$y = 0.883\,1x + 0.045\,9$	0.999 5
3-HDAA	4.9–49	$y = 0.667\,8x + 0.008\,2$	0.999 5
SEA	2.0–20	$y = 0.770\,0x + 0.006\,4$	0.999 7
8-Hydroxycaprylic acid	8.2–82	$y = 0.585\,4x - 0.041\,8$	0.999 3
2-Decene-1,10-dicarboxylic acid	8.0–80	$y = 0.746\,4x - 0.009\,3$	0.999 8

3.3.2 LOD and LOQ results

LOD is defined as the lowest concentration of an analyte that can be detected in a sample under specified conditions, while LOQ refers to the lowest concentration of an analyte in a sample that can be quantitatively determined with appropriate precision and accuracy. Both parameters are crucial for validating the sensitivity and reliability of analytical methods[28]. As shown in Table 2, the LODs and LOQs demonstrated the high sensitivity and suitability for accurate detection and quantification of 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid in RJ.

Table 2 LODs and LOQs for determination of fatty acids in RJ (μg/mL).

Fatty acid	LOD	LOQ
10-HDAA	0.719	2.398
3-HDAA	0.944	3.147
SEA	0.807	2.690
8-Hydroxycaprylic acid	1.085	3.615
2-Decene-1,10-dicarboxylic acid	0.695	2.316

3.3.3 Precision result

Precision is defined as the degree of closeness among repeated individual measurements of an analyte under specified conditions, and it is considered one of the most critical steps in method verification[29]. Six independent extractions were performed on the same sample following the established extraction procedure to assess the precision of the analytical method. As presented in Table 3, each extract was analyzed individually, and the RSDs for the concentrations of 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid were calculated. The RSDs obtained were 0.902%, 0.078%, 0.119%, 0.226%, and 0.135%, respectively, all below 1%, which indicating minimal measurement variations, confirming that the method consistently produced results across multiple trials. This high level of precision ensures the accurate detection and quantification of specific fatty acids in RJ, making the method ideal for applications where accuracy and reproducibility are critical.

3.3.4 Repeatability result

Repeatability refers to the precision achieved under consistent operating conditions, including analysts and equipment, over a short interval. Generally, the repeatability of the analytical method is evaluated by assessing intra-day and inter-day precision. Intra-day precision is determined by analyzing multiple replicates of the same sample within a single day, while inter-day precision is assessed through repeated analysis over several consecutive days[30]. The intra- and inter-day precision of 5 fatty acids in RJ were evaluated through 6 replicates in this study. As shown in Table 4, the intra-day and inter-day precision of 10-HDAA were 0.908% and 1.308%, respectively, the intra-day and inter-day precision of 3-HDAA were 0.132% and 0.947%, respectively, the intra-day and inter-day precision of SEA were 0.191% and 0.453%, respectively, the intra-day and inter-day precision of 8-hydroxycaprylic acid were 0.276% and 0.338%, respectively, and the intra-day and inter-day precision of 2-decene-1,10-dicarboxylic acid were 0.338% and 0.480%, respectively. The low variation in repeatability across intra-day and inter-day assessments suggested that the method could consistently produce reliable results.

Table 4 Repeatability for determination of fatty acids in RJ ($n = 6$).

Fatty acid	Intra-day precision (%)	Inter-day precision (%)
10-HDAA	0.908	1.308
3-HDAA	0.132	0.947
SEA	0.191	0.453
8-Hydroxycaprylic acid	0.276	0.338
2-Decene-1,10-dicarboxylic acid	0.338	0.480

3.3.5 Accuracy result

The accuracy of the analyte was evaluated by introducing spiked concentrations into the matrix at low, middle and high-quality

Table 3 Precision for determination of fatty acids in RJ ($n = 6$).

Item	Sample					
	1	2	3	4	5	6
Sample quality (g)	0.512 34	0.505 01	0.496 82	0.509 80	0.491 02	0.508 42
Internal standard quality (g)	0.003 25	0.003 25	0.003 25	0.003 25	0.003 25	0.003 25
Peak area ratio of 3-HDAA and internal standard	0.147 385	0.144 619	0.141 119	0.144 361	0.138 734	0.146 530
Peak area ratio of 10-HDAA and internal standard	1.041 047	1.037 682	1.019 053	1.049 264	0.973 961	1.033 674
Peak area ratio of SEA and internal standard	0.195 401	0.191 578	0.188 956	0.193 090	0.182 239	0.191 797
Peak area ratio of 8-hydroxycaprylic acid and internal standard	0.279 169	0.277 734	0.269 320	0.278 305	0.260 092	0.274 578
Peak area ratio of 2-decene-1,10-dicarboxylic acid and internal standard	0.223 805	0.216 750	0.217 813	0.221 774	0.211 246	0.223 101
3-HDAA content (%)	0.093	0.093	0.092	0.092	0.092	0.094
Mean ± RSD (%)	0.093 ± 0.078					
10-HDAA content (%)	0.660	0.668	0.667	0.669	0.645	0.661
Mean ± RSD (%)	0.662 ± 0.902					
SEA content (%)	0.124	0.123	0.124	0.123	0.121	0.123
Mean ± RSD (%)	0.123 ± 0.119					
8-Hydroxycaprylic acid content (%)	0.177	0.179	0.176	0.177	0.172	0.176
Mean ± RSD (%)	0.176 ± 0.226					
2-Decene-1,10-dicarboxylic acid content (%)	0.142	0.139	0.142	0.141	0.140	0.143
Mean ± RSD (%)	0.141 ± 0.135					

Table 5 Recovery for determination of fatty acids in RJ (%).

Fatty acid	Low concentration recovery	Middle concentration recovery	High concentration recovery	Average spiked recovery
10-HDAA	103.0	105.0	104.0	104.0
3-HDAA	94.8	94.5	93.9	94.4
SEA	95.9	98.2	95.6	96.6
8-Hydroxycaprylic acid	102.1	102.4	102.0	101.7
2-Decene-1,10-dicarboxylic acid	100.7	98.3	99.8	99.6

control levels[31]. The evaluation was conducted by spiking the samples with a known number of standards at concentrations corresponding to 50%, 100%, and 150% of their expected levels. Table 5 presents the recoveries of 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid at spiking levels of 50%, 100%, and 150% of their initial concentrations in RJ samples. The result indicated that the average spiked recoveries for different concentrations of these fatty acids were as follows: 104.0% for 10-HDAA, 94.4% for 3-HDAA, 96.6% for SEA, 101.7% for 8-hydroxycaprylic acid, and 99.6% for 2-decene-1,10-dicarboxylic acid. These findings indicated consistent and reliable recovery performance for each fatty acid under varying concentration conditions. The high recoveries for fatty acids in RJ suggested excellent analyte retention and minimal loss during the analytical process, which was crucial for accurate quantification. These findings supported the method's potential application across various fields, including food quality control, pharmaceutical analysis, and biochemical research.

4 Conclusion

This study established and validated a straightforward standard GC for quantifying multiple fatty acids in RJ. By optimizing the extraction and derivatization processes, along with precise chromatographic conditions, the technique facilitated accurate detection and measurement of 10-HDAA, 3-HDAA, SEA, 8-hydroxycaprylic acid, and 2-decene-1,10-dicarboxylic acid. The method exhibited excellent linearity, precision, repeatability, and accuracy, making it a valuable tool for the quality control of RJ. These findings significantly contribute to ensuring the consistency and efficacy of RJ in various applications, ranging from food products to pharmaceuticals. Furthermore, this method can be further expanded to detect more fatty acids in RJ, as well as other bee products such as honey and bee pollen. Additionally, applying this method to large-scale quality control and authentication processes could substantially improve the standardization and commercialization of RJ products, ensuring their safety and effectiveness for consumers worldwide.

Conflict of interest

The authors declare no conflict of interest.

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