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ORIGINAL ARTICLE

Standardizing The Analysis of 10-Hydroxy-Trans-2-Decenoic Acid (10-HDA) In Indian Royal Jelly Samples by LC-MS/MS Technique.

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ABSTRACT
Royal Jelly (RJ) is a natural bee-derived bioactive compounds, whose composition and contents vary based on geographical source, method of collection and species of bee. So in order to ensure the quality of the product, it is necessary to define and set quality control levels before its commercialization. 10-Hydroxy-trans-2-Decenoic acid (10-HDA) is a unique and major component of RJ. Although the uniqueness is well known, no attempt has been made to estimate the quality of Indian Royal Jelly. The present study is the first attempt in determining 10-HDA from Indian Royal Jelly samples. A rapid, precise, stable, and robust LC-MS/MS method was developed, validated, tested on Indian Royal Jelly (IRJ) samples procured from different regions of India. A Thermo C-18 column (100 mm × 4.6 mm, 5 µm) and a gradient mobile phase system (A: 0.1% formic acid in the water, B: 0.1% formic acid in methanol) at flow rate of 0.6 mL/min for 13 minutes at 35±2°C temperature was employed. The ESI source used for ionization was in negative mode with a scan range of 50–1000 m/z. The 10-HDA was eluted at 7.648. The linearity was established between 0.010-0.500 µg/mL with a correlation value of 0.9996. The molecular ion peak (m/z 185.00) and its fragment ions (m/z 139.00) confirms the identity of 10-HDA. The present technique was found capable, reliable and sensitive in detecting and quantifying 10-HDA in various samples of IRJ thus could be deployed as a quality control measure for determination of 10-HDA in IRJ samples.

Keywords: *Indian Royal Jelly, 10-HDA, HDAA, LC-MS/MS, Quality Control*

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INTRODUCTION

Bees, *Apis mellifera*, are valuable natural resources that produce a variety of precious natural products that contain health-promoting bioactive compounds. These products include honey, beebread, venom, bee pollen, propolis, and royal jelly (RJ) [1]. RJ is one of the most important naturally occurring substances produced by honeybees due to its high nutritional value, as well as its functional and biological properties. It is secreted by the hypopharyngeal glands of worker bees, and it is used to supply sustenance for both the queen bee and the colony's larvae [2]. It is the most important component of the diet of honeybee's larvae, workers bee, and nursing honeybees. RJ is provided to all bee larvae during the first three days, but only queen larvae are fed with RJ to the rest of their lives [3]. RJ is a thick jelly material that is typically not homogeneous owing to the existence of undissolved granules of varied sizes that have not been completely dissolved. It has a density of 1.1 g/mL in water and is only slightly soluble in it. Its color ranges from pale to yellow, with the yellow hue intensifying with storage. It has a sour and strong odor, and its flavor is a combination of tart and sweet. It has a low pH (3.1–3.9) and a high buffering capacity (between 4 and 7), making it a good choice for wound healing. [4].

The primary fatty acid found in RJ is hydroxy group-containing omega-hydroxy amino acid i.e. (E)-10-hydroxydec-2-enoic acid which has been shown to be beneficial in enhancing the immune system and anticancer activities, among other things. Despite the fact that it accounts for more than half of the free

fatty acid content, it has not been recorded in any other natural product, much alone in any other bee-related product [5].

As it is a bee-derived product, the composition and contents of RJ vary according to the species and geographical location in which it is harvested. The discrepancies found are most likely attributable to the variable number of samples collected in various locations and at different stages of the manufacturing process. RJ is also a naturally diverse but beneficial substance. It also becomes a possible source of adulteration since it is produced via natural means. To ensure the product's quality, it is necessary to define and set quality control levels prior to its commercialization. A variety of sophisticated hyphenated analytical techniques, like high-performance liquid chromatography [6-16], ultrahigh performance liquid chromatography [17], HPLC-mass spectrometry [18], capillary electrophoresis [19], gas chromatography [20], gas chromatography-mass spectrometry [21] and others, have been reported in numerous databases for determination of product quality , or solely for the determination of 10-hydroxy-2-decenoic acid (10-HDA), in RJ. However, it was observed that not much focus has been devoted to determine 10-HDA in royal jelly through LC-MS technique. Moreover, it was observed that only high resolution LC-MS method was reported for determination 10-HDA from royal jelly which resulted in the demand towards the development of an ideal method. In addition, it was observed that to date no specific method has been devised for determining the level of 10-HDA in RJ and RJ products marketed in India. This impelled us to develop a new LC-MS/MS method for the quantification of 10-HDA in RJ products marketed in the Indian market as well as in the global market.

MATERIAL AND METHODS

Samples and Chemicals:

Indian Royal Jelly (IRJ) samples from Southern Region (IRJ I- VIS /F /1), Northern region (IRJ II - 5 /HA /2020), and Central region (IRJ III - 4/ MA /2019) and Lyophilized powder of IRJ I from the southern region of India were collected, authenticated and supplied by Central Bee Research and Training Institute (CBRTI), Pune. 10-HDA (Purity 97.0%), HPG grade, was obtained from TCI, Bengaluru, India; Methanol, Formic acid (LC/MS grade) was obtained from Merck, India.

Preparation of Solutions

10-HDA standard working solution (100 µg/mL)*:* 10-HDA was accurately weighed and diluted to achieve (1000 µg /mL) stock solution with methanol, which was diluted further to get a standard working solution (100 µg/mL).

Preparation of Indian Royal Jelly Samples: Accurately weighed IRJ samples were transferred into10 mL volumetric flasks, 8 mL methanol was added, ultrasonicated with occasional shaking, and the volume was made up to the mark with methanol (10 mg/mL). All samples were filtered through a filtering cartridge and 0.45 µm nylon membrane using a disposable syringe and diluted with methanol to get 1000µg/mL working solution.

Instrumentation

Agilent Technologies® 6460 Triple Quad LC/MS system (Model: 1260 Infinity, QQQG6460C), consisting of 1260 Infinity autosampler, powered by Agilent Mass Hunter software was employed for the study. A Thermo C-18 column (100 mm × 4.6 mm; 5µm) was used for separation.

Preparation of mobile phase

A gradient mobile phase system (A: 0.1% formic acid in the water, B: 0.1% formic acid in methanol) was used for this study. The solution was degassed for 5 minutes with sonication before being filtered under vacuum through a 0.45 µm membrane filter.

Preparation of Diluent

Throughout the research, LC-MS grade formic acid: water (50: 50 v/v) was utilized as a diluent.

Chromatographic Condition

A Thermo C-18 column (100 mm × 4.6 mm, 5 µm) was used to develop the technique. The aforementioned gradient mobile phase system was employed at a flow rate of 0.6 mL/min for 13 minutes at 35±2°C temperature. The gradient system (A:B) includes: 50:50 v/v at 0 min; 50:50 v/v at 1 min; 30:70 v/v at 3 min; 20:80 v/v at 6 min; 50:50 v/v at 10 min; and 50:50 v/v at 13 min. The injection volume was 10 µL, and the assay was carried out at room temperature.

Method validation

The developed LC-MS/MS method was properly corroborated under the Q2A guideline and Q2B guideline of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and according to the United States Pharmacopoeia (USP).

Linearity and range:
The method's linearity was tested using six different concentrations (0.010-0.500 µg/mL) of the substance. The solutions were prepared using the diluent and an equal quantity was injected into the LC-MS/MS instrument to determine the peak area. On a linearity graph, the concentration and average area of each solute were plotted. The r^2 value of the regression coefficient was computed as well.

Accuracy:
The LC-MS/MS methods' accuracy was determined by estimating the recovery using the usual addition approach. To the pre-quantified sample solutions and the known quantities of standard solutions of 10-HDA were added at 50%, 100%, and 150% levels. Based on the specific concentrations, the process was executed in triplicate, and the obtained mean results were articulated in the form of a % recovery ± confidence interval with estimated % relative error.

Precision:
The method's precision (variability) was tested by injecting a 0.1µg/mL standard solution six times and three times in a single day (intra-day) and three days in a row (inter-day). The % RSD of the method was calculated.

Limit of detection:
The limit of detection (LOD) was estimated by injecting the smallest concentration sensed by this LC-MS/MS method. The LOD was estimated from the formula:

LOD = 3.3 (σ/S)

Where S signifies the calibration curve slope determined from the analyte calibration curve and σ refers to the standard deviation of response.

Limit of quantification:
The limit of quantification (LOQ) was determined by the lowest concentration which can perhaps be quantified consistently with a meticulous level of precision and accuracy. LOQ was estimated from the formula:

LOQ = 10 (σ/S)

Where S signifies the calibration curve slope determined from the analyte calibration curve and σ refers to the standard deviation of response.

Assay:
The assay was used to determine the 10-HDA present in Indian Royal Jelly samples collected from 3 different regions of India. Different concentrations of IRJ samples were analysed by the developed method.

Mass parameters
The following MS and ESI settings were used for analysis: The nozzle voltage was 500 V. For MS analysis a full scan MS mode with a resolution of 1 atomic mass unit, a scan range of 50–1000 m/z, was used. High-purity nitrogen gas was used for the ESI source, other specifications like Gas Temp: 300°C; Gas Flow: 8L/min; Nebulizer: 40 psi; Sheath Gas Temp: 400°C; Sheath Gas Flow: 9L/min was used The ESI source was configured in negative mode for ionization. All analyte were detected as $[M - H]^-$ ions.

RESULTS

Chromatographic conditions
In the LC-MS/MS method 10-HDA was determined. The spectra and graphics of the compound studied were recorded in negative mode **(Fig.1).** The developed method was able to identify the other fatty acid i.e. 10- Hydroxy decanoic acid (HDAA) simultaneously from IRJ. The HDA and HDAA are well separated and are eluted at 7.648 and 8.0 min, respectively. **(Fig.2)**

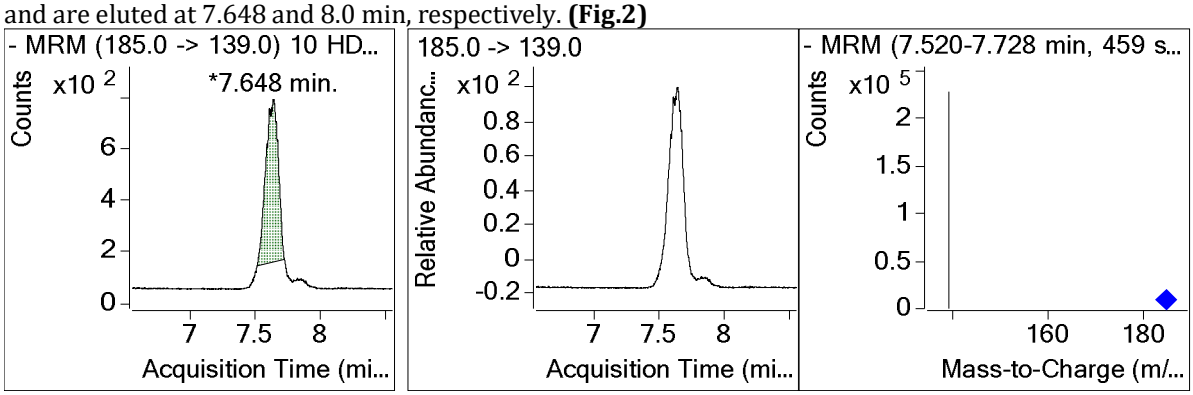

Fig.1 Represents graphics of the10-HDA compound.

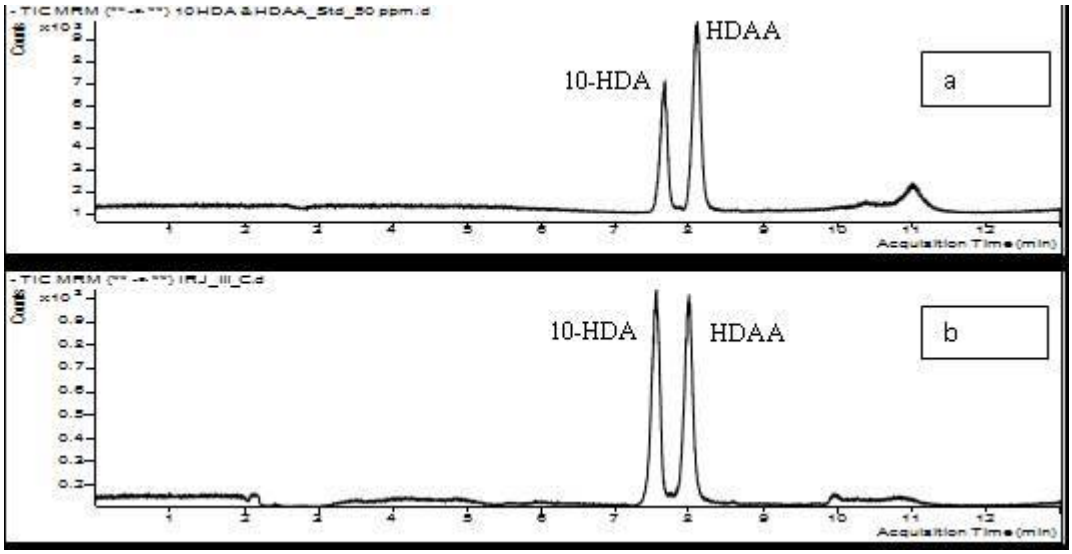

Fig.2 LC-MS Chromatogram of (a) 10-HDA with HDAA (b) Indian Royal Jelly

Method validation

Linearity and Range*:*

Linearity was established between concentration and peak area in the range of 0.01-0.5 µg/mL with regression equation obtained as of *y = 28.177005x – 1363.566830* (**Fig 3**), the regression coefficient value was 0.999, suggesting that there was a high degree of linearity. (**Table 1**).

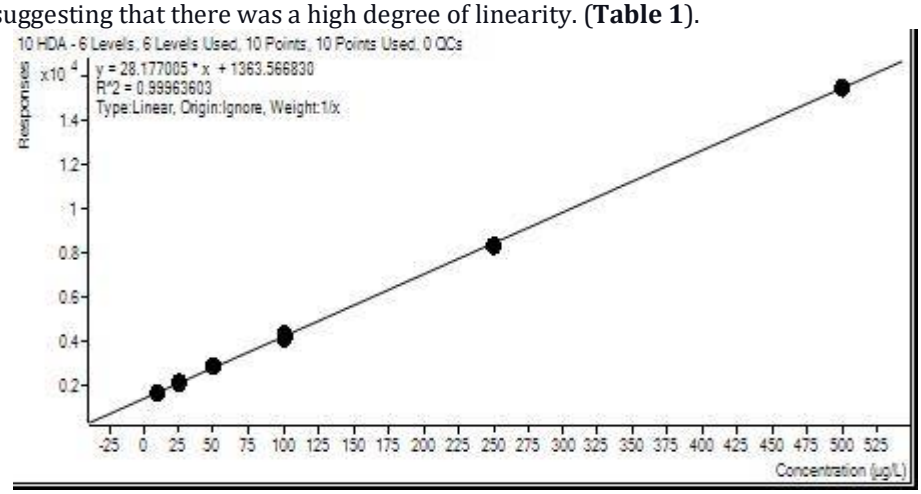

Fig.3 Linearity curve for 10-HDA

Precision*:* The repeatability of the target concentration (0.100 µg/mL) showed that the % RSD values were <2% which represents that the developed LC-MS/MS method possesses high precision. (**Table 1**).

Accuracy:

The trueness of the LC-MS/MS method was estimated for the recovery data using a calibration curve. The accuracy results of the study for IRJ were observed to be ~100% w/w. The % RSD at three specific concentrations; 50%, 100%, and 150% of the target concentration was <2% which shows the accurateness of the developed method. (**Table 1**).

LOQ and LOD*:*

The method was found to be quite sensitive in detecting 10-HDA in IRJ with LOQ and LOD values of 0.005 µg/mL and 0.003 µg/mL, respectively.

Table 1. Validation Results.

Parameters		**Results**
Linearity and Range	Range (µg/ml)	0.010-0.500
	Regression equations	y=28.177005x +1363.56
	Regression coefficient (r^2)	0.9996
Accuracy (%)	Standard Addition	98.95-101.54
Precision (% RSD)	Intraday	1.324
	Inter-day	1.519
Sensitivity	LOQ (µg/mL)	0.010
	LOD (µg/mL)	0.005

Assay:

IRJ samples were analyzed and presented in **Table 2**. In sample IRJ-II, the highest amount of 10-HDA was determined; i.e. 2.17% whereas the lowest amount was found in IRJ-III; i.e. 1.261% and 10-HDA was estimated at 6.071% for the lyophilized sample of IRJ.

Table 2. Estimation of 10-HDA from IRJ samples

IRJ Samples	Mean Concentration Injected (mg)	Area	Mean Area	SD	% CV	Mean % Content
		9411				1.993
IRJ I	11.34	9389	9407	16.37	0.174	
		9421				
		10031				2.175
IRJ II	11.072	10242	10205	159.20	1.560	
		10343				
		7874				1.261
IRJ III	14.988	7728	7838	97.14	1.239	
		7912				
		9189				6.071
IRJ IV	3.63	9166	9178	11.59	0.126	
		9180				

(n=3)

Mass parameters

10-HDA was successfully determined in the samples of IRJ. The appearance of a peak at m/z 139.1128 demonstrated that 10-HDA has been fragmented into a chemical component (fragment) of molecular formula $C_9H_{15}O_3$- (**Fig. 4**). The fragmentation pattern is provided in **Fig.5**. The exact masses of the deprotonated molecular ions (theoretical and measured) are summarized in **Table 3.**

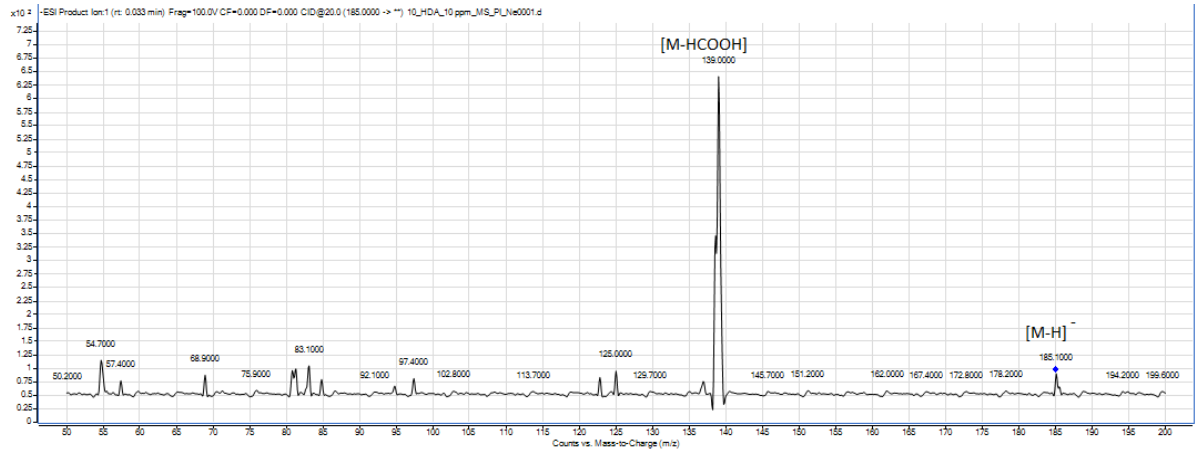

Fig.4 Fragmentation peak of 10-HDA

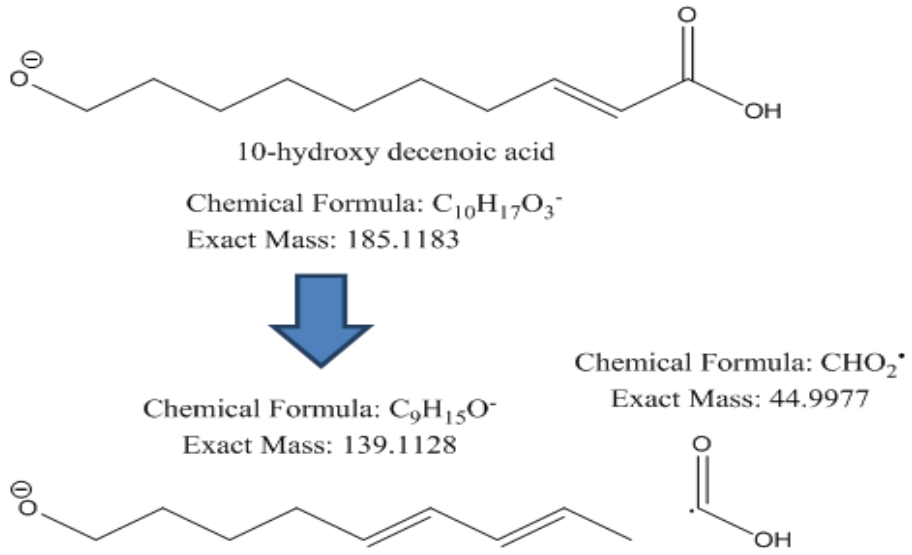

Fig.5 Fragmentation Pattern of 10-HDA

Table 3. Mass Spectral Results

Compound	Theoretical Mass	Practical Mass	Theoretical MS/MS	Practical MS/MS
HDA	185.1183	185	139.11	139
HDAA	187.134	187.1	141.12	141.2

DISCUSSION

Currently no International standards for bee products other than honey are in place, but some nations have set their quality standards or recommendations in their respective jurisdictions. Argentina was the first country to establish RJ standards in 1979, followed by other countries. An International Honey Commission (IHC) working committee produced a tentative proposal for the standardization of RJ based on information they had gathered a few years earlier. As per the International standards presented by the International Organization for Standardization (ISO), the queen bee acid, also known as 10-HDA, a characteristic bio-active molecule of RJ has been considered as a quality identifier for RJ products. So, attempts have been made to determine levels of 10-HDA in IRJ samples.

The first LC-HRMS analysis of free fatty acids in RJ was performed by Kokotou *et al.* which demonstrated the presence of 10-HDA, HDAA, 3-hydroxydecanoicacid, decanedioic acid, and 2-dodecenedioic acid. 10-HDA was found to appear at m/z 185.1181 [20]. In comparison, our LC-MS/MS study of the IRJ sample, abundant peak of 10-HDA produced at m/z 185.00 ($C_{10}H_{17}O_3^-$), and its fragment peak resulting to a loss of –COOH at 139.00 ($C_9H_{15}O^-$). It indicated presence 10-HDA in the sample and complying with HRMS data. Also proposed LC-MS/MS method, was able to identify and determine HDAA simultaneously from the Indian royal jelly sample.

CONCLUSION

New, simple, and sensitive LC-MS/MS method was developed to determine the 10-HDA in Indian Royal Jelly. The developed method showed the attributes of accuracy, precision, robustness, and reproducibility, and can be applied for the quality control of Royal Jelly. This study is a first attempt in determining 10-HDA from Indian Royal Jelly samples.

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Declaration of conflicting interests
Authors declare that there are no conflict interests to the work reported in this manuscript.

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