

Simultaneous estimation of three triterpenoids from *Carissa carandas* using validated high performance liquid chromatography

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Background: *Carissa carandas* L. (Apocynaceae), an important fruit of tropical and subtropical arid region, has a great potential for the development of a new phytotherapeutic medicine. Due to an increase in commercial products of this fruit in the global market, an extensive phytochemical comparison seems imperative to understand its internal quality. **Aims and Objective:** To develop and validate an HPLC-DAD method for simultaneous estimation of three triterpenoids from *C. carandas*. **Materials and Methods:** A simple, sensitive and rapid HPLC-DAD method was developed for simultaneous estimation of lupeol, stigmaterol, and β -sitosterol from *C. carandas* fruits. Isocratic elution at a flow rate of 1.0 mL/min was employed on C₁₈ Cosmosil column with PDA detection at 210 nm using acetonitrile and ethanol. Different parts of *C. carandas* and its fruits collected from different geographical region were screened for lupeol, stigmaterol, and β -sitosterol contents. **Results:** Proposed method for simultaneous estimation of lupeol, stigmaterol and β -sitosterol was found highly specific, reliable, and sensitive which allowed good recovery of all analytes. It was validated as per ICH guidelines and proved to have good linearity, precision, repeatability, and stability. It was efficiently applied to screen different samples of *C. carandas* providing a mean to assess the quality and authenticity. **Conclusion:** The HPLC method could be an effective technique for routine analysis of lupeol, stigmaterol, and β -sitosterol simultaneously and monitoring the quality of fruit and its available products in cosmetic and pharmaceutical companies.

Key words: *Carissa carandas*, simultaneous estimation, triterpenoids, validation

INTRODUCTION

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed.^[1]

Fruits, stem bark, leaves and roots of *Carissa carandas* L. (Apocynaceae) have been extensively used in traditional and folklore medicine either in the treatment or prevention of amenorrhea,^[2] rheumatoid

arthritis,^[3] piles,^[4] nervine disorders, cardiac disorders, splenomegaly, diabetes, pyrexia,^[5] hepatic disorders, tuberculosis,^[6] renal dysfunction and leprosy.^[7]

In recent years, there has been a growing interest in the chemical composition and biological activities of *C. carandas*. Among the various phytochemical found in fruits, stem bark, leaves and roots of *C. carandas*, sterols particularly triterpenoids are widely regarded as major biologically active components which may contribute to their reputed and diversified health benefits.

Previous phytochemical studies on *C. carandas* reported the presence of lupeol, β -sitosterol, 16 β -hydroxybetulinic acid, α -amyrin, carisone, carindone, stigmaterol, carinol, carissin, linalool, β -caryophyllene, carissol and carissic acid using various analytical techniques.^[5]

Since *C. carandas* is distributed in different parts of the world,^[3] content of its bioactive markers vary greatly depending on their geographical locations, climatic conditions and other factors.^[8] Availability of many species of *Carissa* in the same location makes identification and collection even more difficult. Considering the above facts, there is an urgent

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need to develop a satisfactory method for controlling the quality of *C. carandas*. In the present study, an RP-HPLC-DAD method has been developed by optimizing the extraction, separation and analytical conditions for simultaneous estimation of three triterpenoids, namely lupeol, stigmasterol and β -sitosterol from *C. carandas*.

MATERIALS AND METHODS

Materials

Different parts of *C. carandas* were collected from Kalyan (plains of Maharashtra, India). Ripe fruits were collected from different provinces in Maharashtra. Plant specimens were authenticated by Agharkar Research Institute (Voucher Specimen No. Auth 11-123), Pune, India and deposited at Department of Botany for future reference.

Reference Standards and Reagents

Lupeol, stigmasterol and β -sitosterol (LSB, 98% purity) were purchased from Sigma Aldrich, Germany. HPLC grade solvents were procured from Merck Specialties Pvt. Ltd, Mumbai, India.

Preparation of Standard Solutions

Standard stock solutions (1000.0 $\mu\text{g}/\text{mL}$) of LSB were prepared in methanol. Working standard solutions containing LSB were prepared and diluted with methanol to obtain appropriate concentrations for establishment of calibration curves (aliquots of 2.0–350.0, 5.0–250.0 and 5.0–200.0 $\mu\text{g}/\text{mL}$ for LSB respectively). Three quality control samples (LQC: MQC: HQC; lower, middle and higher respectively) of lupeol (3.5, 30.0, 280.0 $\mu\text{g}/\text{mL}$), stigmasterol (7.5, 40.0, 200.0 $\mu\text{g}/\text{mL}$) and β -sitosterol (7.5, 35.0, 160.0 $\mu\text{g}/\text{mL}$) were prepared for method validation studies and stored at 4°C.

Instrumentation and Chromatographic Conditions

High performance thin layer chromatography

Chromatographic analysis was performed on HPTLC plates pre-coated with silica gel 60 F₂₅₄ (E. Merck) of 0.2 mm thickness with aluminium sheet support. Samples (10.0 μL) were spotted using Camag Linomat IV (Switzerland) equipped with syringe (Hamilton, 100.0 μL). Plates were developed in a glass twin trough chamber (Camag) presaturated with toluene: methanol (8:1, v/v) and scanned with Camag TLC Scanner II conjugated with Cats III software. The temperature was maintained at 20 \pm 2°C. For detection of LSB the plate was derivatised with 10% methanolic sulphuric acid and scanned at 366 nm. Camag Reprostar 3 system was used for photo documentation.

High performance liquid chromatography

Chromatographic analysis was performed at room

temperature using Jasco's HPLC system comprising of two PU-1580 pumps (HG-1580-31), rheodyne injector (20.0 μL loop) and Photo Diode Array (PDA) detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on Cosmosil C₁₈-column (150 \times 4.6 mm, 5.0 mm) using acetonitrile: ethanol (40:60, v/v) delivered at a flow rate of 1.0 mL/min. After a 10 min of equilibration period, the samples were injected into the HPLC system. Peaks were recorded at 210 nm.

Selection of Suitable Chromatographic Technique for Method Validation

Triterpenoids are compounds with a carbon skeleton based on six isoprene unit which are derived biosynthetically from the acyclic C₃₀ hydrocarbon, squalene.^[9] Most of the triterpenoids are phytosterols and they differ structurally from cholesterol because of an additional alkyl substitute at C-24 and/or a double bond at C-22.^[10]

Although HPTLC has recently emerged as an analytical tool for fingerprinting and quantitation of a wide range of marker compounds from herbal drugs,^[11] separation of few triterpenoids like stigmasterol and β -sitosterol was proved difficult in the present work. Lupeol (C₃₀H₅₀O) being a pentacyclic triterpenoid easily resolved from β -sitosterol (C₂₉H₅₀O) and stigmasterol (C₂₉H₄₈O) on HPTLC plate, whereas structural similarities between β -sitosterol and stigmasterol (both with tetracyclic ring)^[12] made their separation difficult. Hence, in the current work separation and resolution of these two tetra cyclic compounds along with lupeol was achieved using HPLC.

To validate the HPLC method, a series of assays like determination of specificity, limits of detection and quantification (LOD and LOQ), linearity, precision (intra-day and inter-day), reproducibility, stability, ruggedness and accuracy were performed following the International Conference on Harmonisation (ICH) guidelines.^[13] All analyses were performed in triplicate and the variations were evaluated in terms of mean, standard deviation (SD), relative standard deviation (% CV) and % mean difference.

Optimised Extraction Condition

All the raw materials were shade dried, oven-dried at 45°C, powdered and sieved through 85- μm mesh sieve. Different extraction factors, including volume of solvent, sample-solvent ratio and extraction time were optimised. Finally, each dried sample (1.0 g powder) was extracted in 10.0 mL of petroleum ether with continuous shaking on vertical shaker for 240 min. Resultant mixture was filtered through Whatmann filter paper no. 41 and the filtrate was dried under vacuum. The dried extracts were reconstituted

with equal volume of methanol and filtered through nylon microfilter (0.45 mm, Millipore) prior to chromatographic analysis. The injection volume was 20.0 μ L.

Method Validation

Selectivity and specificity

During the experiments an UV scan ranging from 200 to 400 nm in the time window of the analytes using PDA detector was performed with the aim of revealing eventual interfering compounds and evaluating the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds.

System suitability

System suitability experiment was performed by injecting five consecutive injections of each bioactive marker, namely lupeol (2.0 μ g/mL), stigmaterol (4.0 μ g/mL) and β -sitosterol (4.0 μ g/mL) during the start of the method validation. Values with % CV of $\leq 2\%$ were accepted.

Calibration curves

Linearity of LSB was determined at seven different concentrations in triplicate and plotted using linear regression of the mean peak area versus concentration. The linear regression equation was obtained using a least-square method and used to estimate the concentration of LSB in the analysed samples.

Limits of detection and quantification

Sensitivity of the method was evaluated by determining the values of LOD and LOQ. Stock solution of each standard was serially diluted with methanol to prepare the series of samples with least concentration and injected into the HPLC system. The limit of detection (LOD) and quantification (LOQ) for LSB were determined by measuring the signal to noise ratio (S/N). LOD and LOQ was considered at S/N of 3:1 and 10:1, respectively.

Precision

Intra-day precision was evaluated from replicate analysis ($n=3$) of quality control samples on the same day while inter-day precision was assessed by analysing them on three consecutive days in triplicate ($n=3$). The % CV was taken as a measure of precision. Accuracy values within the range of 85–115% and % CV of $\leq 2\%$ were accepted.

Stability

Stock solution stability of LSB was tested at room temperature and samples were analysed in triplicate after 15 days. Same samples in triplicate were also subjected to bench top stability testing at 0.0 h and 6.0 h, respectively. Values within a difference range of $\pm 5\%$ were accepted.

Recovery

Recovery of the method was evaluated using standard addition method. Quality control samples of LSB were added to the known amount of *C. carandas* fruit, extracted and analysed using HPLC. The mean percent recovery was calculated using the formula: Recovery (%) = [(amount found – original amount)/amount added] \times 100. Values within the range of 85–115% were accepted.

Ruggedness

Ruggedness of the method was assessed as per the published reports.^[14] In brief four factors which could influence the result were chosen. The factors were HPLC column [columns of two different lot/batch from same manufacturer (Cosmosil, K64003 and K65110)], analyst (analyst 1 and 2), flow rate (1.00 \pm 0.05 mL/min) and mobile phase composition [acetonitrile (40 \pm 1): ethanol (60 \pm 1), v/v]. Effect of these deliberate changes on the response (area) and retention time of QC samples of LSB was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of $\pm 5\%$ were accepted.

Estimation of LSB from the Samples of *Carissa*

Relative retention time and relative peak area of each characteristic peak from the samples of *Carissa* related to the peak from LSB were calculated for quantitative expression of the chemical properties in the chromatographic pattern of *C. carandas* using regression equation.

Statistical Evaluation

Microsoft Excel 2007 was used to determine mean, standard deviation, relative standard deviation and mean difference during the analysis.

RESULTS

Optimisation of Extraction

To obtain satisfactory extraction efficiency of triterpenoids from samples; solvents of different polarities like toluene, *n*-hexane, petroleum ether, chloroform, methanol and water were used. As triterpenoids are generally soluble in petroleum ether four different sample-solvent ratios (1:10, 1:25, 1:50, 1:80 and 1:100, w/v) were tested, compared and the ratio of 1:10 was selected. Different extraction time (10, 30, 60, 120, 240, 360, 720 min) were also tested and efficient extraction was obtained at 240 min. *C. carandas* possess natural complicated matrices of a wide range of phytochemical constituents. Among them, triterpenoids are more soluble in petroleum ether. Hence, a pre-extraction stage was utilised to remove the interfering components and extract the target molecules using it.

Method Development

Triterpenoids represent a large class of secondary metabolites found in the terrestrial/marine flora and fauna. Their presence especially in the plant kingdom has aroused interest from both an evolutionary and a functional perspective.^[15]

Over the past few years, beneficial health effects of triterpenoids resulted in a great interest in quantifying them in different food.^[15,16] Different analytical methods have been developed for separation of triterpenoids in various food stuffs.^[16-19] LSB are most abundantly found triterpenoids with potential to decrease the level of serum low-density lipoprotein and protective action for wide range of hepatic disorders.^[20]

In HPTLC experiment, separation of LSB was tried on HPTLC plate along with *C. carandas*. Among the mixture of LSB, lupeol ($R_f=0.51$) got easily resolved from stigmasterol and β -sitosterol because of its peculiar chemical nature. Stigmasterol ($R_f=0.41$) and β -sitosterol ($R_f=0.41$) were not separated from each other in the plant sample [Figure 1]. Results obtained were in compliance with the earlier published reports.^[21] Hence, separation of LSB was tried and achieved using HPLC.

In the HPLC experiment, acetonitrile:ethanol resulted in good resolution and symmetrical peak shape of LSB in the chromatograms. All the analytes exhibited the UV maximum absorption at 210 nm. HPLC chromatograms corresponding to LSB, *C. carandas* fruits, *C. densiflora* L. fruits and *C. carandas* flowers are represented in Figure 2. All the reference compounds were easily resolved in the optimised HPLC condition and eluted within 10 min [Table 1]. Hence, the developed method was found to be time effective when compared with the other published report.^[22]

Method Validation

Selectivity and specificity

During the UV scan no appreciable difference was found in the spectra of reference standards (LSB) and the analysed samples. Hence, the method demonstrated a high degree of selectivity.

System suitability

System suitability tests are an integral part of liquid chromatographic method. They are used to verify whether the resolution and reproducibility of the chromatographic system are adequate for the analysis. For LSB, the % CV was

1.18%, 0.95% and 0.74% for retention time and 1.02%, 0.62% and 0.64% for response respectively during the system suitability study.

Calibration curves and linearity

Seven point standard curves were obtained for LSB by plotting the peak area versus concentration for each standard. Calibration curves were linear over the concentration range studied. Mean correlation coefficient (coefficient of determination, r^2) for LSB was found to be more than 0.996 during the course of validation indicating positive linear relationship between peak area and concentration. Results of regression analysis are summarised in Table 1.

Sensitivity

Sensitivity of the method was affirmed in terms of LOD and LOQ for LSB and the results are represented in Table 1. The

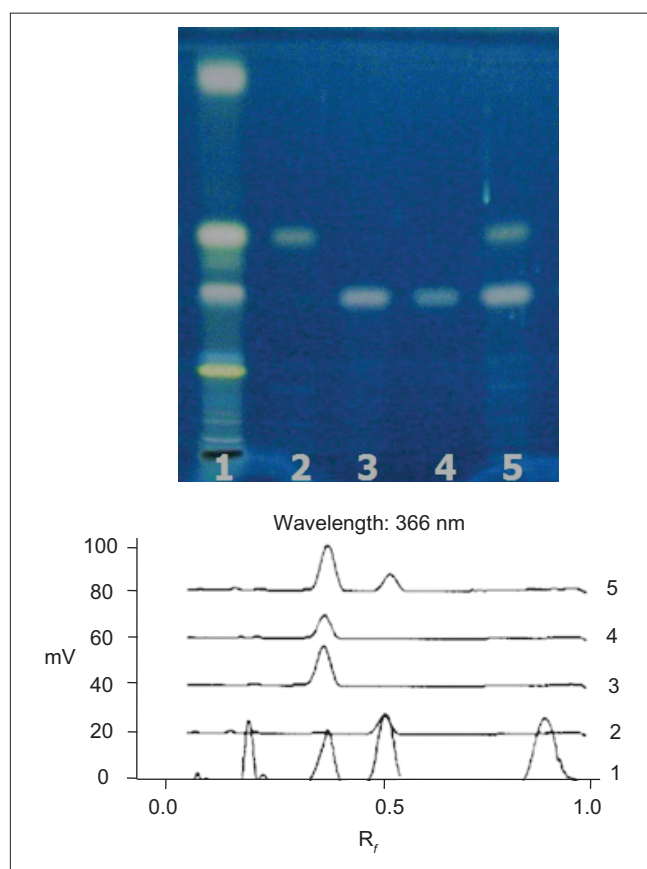


Figure 1: Chromatographic plate and overlay of *Carissa carandas* sample with Lupeol, Stigmasterol and β -sitosterol. Track details, 1: *Carissa carandas* sample; 2: Lupeol; 3: Stigmasterol; 4: β -sitosterol, 5: Mixture of Lupeol, Stigmasterol and β -sitosterol

Table 1: The regression data, linearity, LOD and LOQ and retention times for three bioactive compounds by HPLC

Compounds	Retention time	Regression equation	r^2	Linear range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Lupeol	5.67	$y=3966x-14188$	0.999	2.0-350.0	0.7	1.0
Stigmasterol	6.28	$y=2984x-3494$	0.999	5.0-250.0	2.0	5.0
β -sitosterol	6.84	$y=3570x-7704$	0.996	5.0-200.0	2.0	4.0

LOD – Limits of detection; LOQ – Limits of quantification; HPLC – High performance liquid chromatography

values for both LOD and LOQ were low when compared to other published works,^[16,18,19] which indicated that the method is capable of detecting and quantifying trace amount of LSB in plant samples.

Precision

In the repeatability study intra-day and inter-day precision of the HPLC method were investigated using replicate injection ($n=3$) of quality control samples of LSB. The

developed method was found precise with % CV < 2% and % accuracy of 88.61–103.90% [Table 2].

Stability

Stock solution stability study of LSB samples stored for the period of 15 days at 4°C showed % CV < 2% with the % mean difference within $\pm 5\%$. During bench top stability study, similar results were obtained. Stability studies showed that LSB samples were found stable for at least

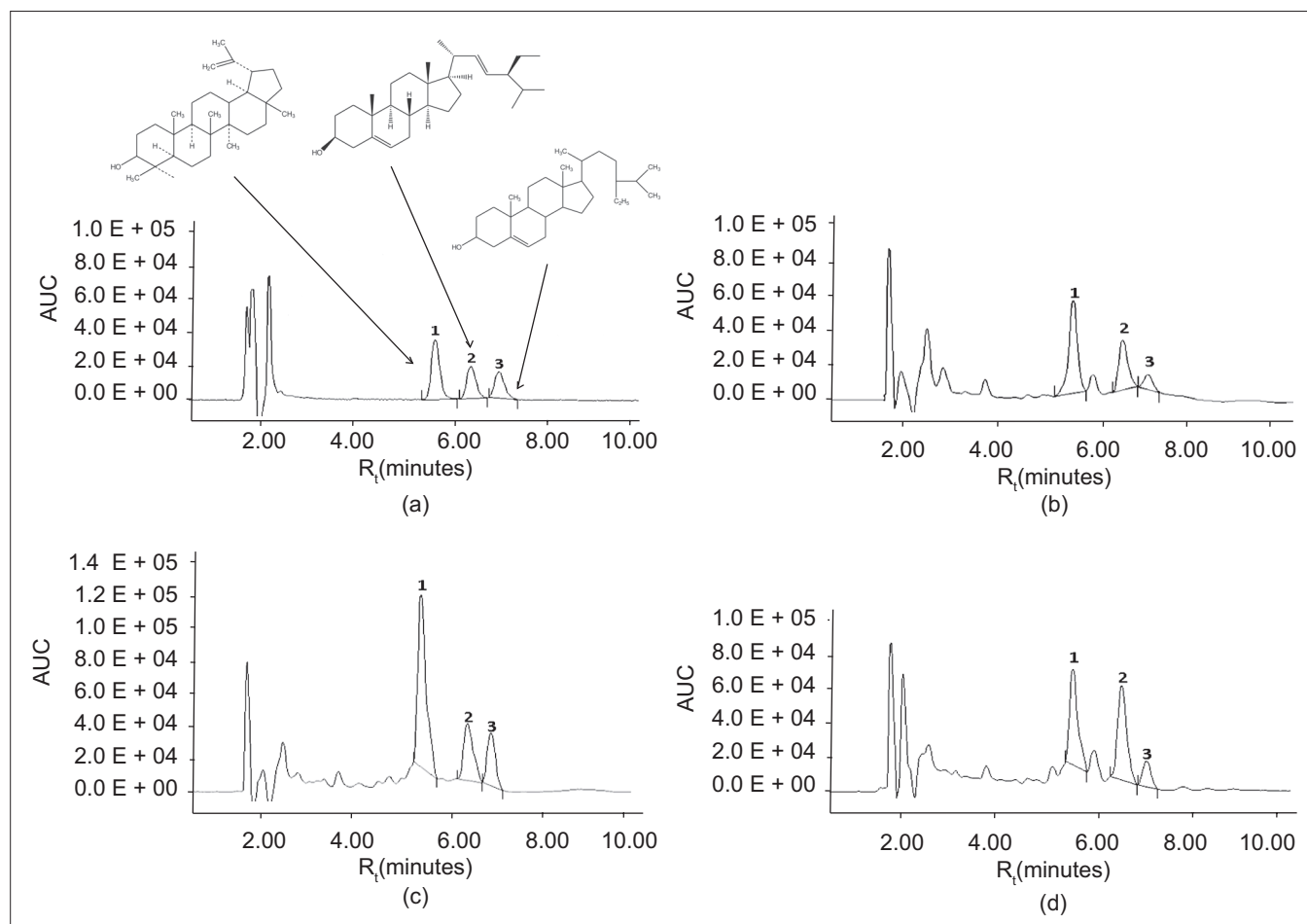


Figure 2: HPLC profiles of standards (a) *Carissa carandas* fruit sample collected from Kalyan; (b) *Carissa densiflora* closely related species of *Carissa carandas*; (c) and Flower of *Carissa carandas*; (d) Peaks represented: 1: Lupeol; 2: Stigmasterol; 3: β -sitosterol

Table 2: Intra- and Inter-day precision of three bioactive compounds

Compounds	Concentration ($\mu\text{g/mL}$)	Intra-day			Inter-day		
		Observed value ($\mu\text{g/mL}$) (Mean \pm SD, $n=3$)	% CV	Accuracy (%)	Observed value ($\mu\text{g/mL}$) (Mean \pm SD, $n=3$)	% CV	Accuracy (%)
Lupeol	3.5	3.29 \pm 0.061	1.855	94.10	3.43 \pm 0.040	1.177	98.10
	30.0	29.27 \pm 0.524	1.789	97.57	27.32 \pm 0.319	1.167	91.08
	280.0	279.95 \pm 1.248	0.442	99.98	278.80 \pm 0.820	0.294	99.57
Stigmasterol	7.5	6.87 \pm 0.087	1.272	91.56	6.62 \pm 0.072	1.089	88.27
	40.0	40.87 \pm 0.488	1.193	102.18	36.43 \pm 0.445	1.222	91.08
	200.0	187.55 \pm 2.152	1.147	93.78	188.08 \pm 2.222	1.181	94.04
β -sitosterol	7.5	6.76 \pm 0.085	1.264	90.13	7.16 \pm 0.111	1.555	95.47
	35.0	34.22 \pm 0.671	1.962	97.77	31.01 \pm 0.546	1.760	88.61
	160.0	166.23 \pm 2.437	1.466	103.90	146.88 \pm 2.279	1.551	91.80

6.0 h at room temperature and 15 days at 4°C of storage condition.

Recovery

The results of recovery study are summarised in Table 3. Recovery experiment showed that 94.51–102.69%, 93.77–99.16%, 95.60–101.23% of LSB respectively spiked into the plant matrix was recovered following the extraction procedure.

Ruggedness

Proposed method was not influenced by the four factors considered for ruggedness study. Small but deliberate changes in the method parameters like change in column and analyst did not affect the retention time and response of LSB samples since % CV ($\leq 1.5\%$) and % mean difference ($\leq 3\%$) were found within the acceptance limits. Change in flow rate and mobile phase composition affected the retention time of LSB but the results were satisfactory since % CV was $< 2\%$ with % mean difference $< 5\%$.

Table 3: Recovery of three bioactive compounds

Compounds	Concentrations		Accuracy (%)	% CV
	Spiked ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$) (Mean \pm SD, n=3)		
Lupeol	3.5	3.347 \pm 0.049	95.62	1.474
	30	28.353 \pm 0.355	94.51	1.252
	280	287.537 \pm 1.829	102.69	0.636
Stigmasterol	7.5	7.190 \pm 0.132	95.87	1.840
	40.0	37.507 \pm 0.393	93.77	1.047
	200.0	198.327 \pm 1.035	99.16	0.522
β -sitosterol	7.5	7.170 \pm 0.098	95.60	1.374
	35.0	35.430 \pm 0.663	101.23	1.872
	160.0	159.957 \pm 0.907	99.97	0.567

Table 4: Content of three bioactive compounds in different samples of *Carissa*

Samples	Assay results (mg/g) (Mean \pm SD, n=3)					
	Lupeol		Stigmasterol		β -sitosterol	
	Content	% CV	Content	% CV	Content	% CV
Regional variation (<i>Carissa carandas</i> ripe fruits)						
Igatpuri (hilly region)*	0.563 \pm 0.010	1.776	0.176 \pm 0.001	0.356	0.502 \pm 0.002	0.430
Malshej (hilly region)*	0.758 \pm 0.015	1.959	ND	ND	0.783 \pm 0.007	0.947
Ratnagiri (coastal region)*	0.432 \pm 0.005	1.221	0.312 \pm 0.002	0.629	0.087 \pm 0.001	1.715
Matheran (hilly region)*	0.835 \pm 0.013	1.519	1.007 \pm 0.016	1.583	0.183 \pm 0.002	0.918
Kalyan (plains)*	1.615 \pm 0.023	1.421	1.206 \pm 0.019	1.556	0.303 \pm 0.002	0.801
Variation in vegetative parts (<i>Carissa carandas</i>)						
Stem	0.590 \pm 0.011	1.853	0.552 \pm 0.003	0.542	0.167 \pm 0.002	1.470
Leaves	0.356 \pm 0.006	1.701	0.061 \pm 0.001	1.769	0.591 \pm 0.008	1.398
Flowers	1.660 \pm 0.029	1.730	2.462 \pm 0.006	0.257	0.519 \pm 0.002	0.463
Unripe fruits	0.819 \pm 0.006	0.703	0.553 \pm 0.003	0.512	0.084 \pm 0.001	1.605
Seeds	0.277 \pm 0.002	0.575	0.317 \pm 0.003	0.942	0.063 \pm 0.001	0.825
Pulp	1.609 \pm 0.017	1.029	2.423 \pm 0.033	1.358	0.541 \pm 0.006	1.174
Species variation						
<i>Carissa densiflora</i>	3.360 \pm 0.055	1.652	1.758 \pm 0.006	0.339	1.152 \pm 0.017	1.438

*Different regions of Maharashtra

Estimation of LSB from the samples of *Carissa*

The method was finally tested to evaluate the content of LSB in different parts of *C. carandas*, ripe fruits collected from different provinces of India and *C. densiflora* using regression equation. Results are summarised in Table 4.

DISCUSSION

C. carandas fruits have been used as a medicinal food or dietary supplement for centuries and are of increasing interest to consumers.^[23,24] At present, many commercial *C. carandas* fruit products are available in the Asian market hence there is a concern about authenticity and quality of *C. carandas* fruit. In the current work, quality of *Carissa* samples was evaluated on the basis of LSB simultaneously using the validated HPLC method. A phytochemical comparison of these components was performed on HPTLC and finally on HPLC. A good separation of analytes was obtained using C_{18} -column with solvent system acetonitrile:ethanol (40:60, v/v).

The effect of geographical conditions on the content of these bioactive markers in the fruits collected from different provinces of India was also investigated. The method was further applied to estimate the LSB content in different parts of *C. carandas* and from a closely related species of *Carissa* [Figure 2].

The experimental results indicated that *C. carandas* fruits collected from different regions exhibit different chemical profiles in terms of LSB content (Contents ranging from 0.432 to 1.615, 0.176 to 1.206 and 0.087 to 0.783 mg/g for LSB, respectively [Table 4]). Such variation may be caused by significant impact of geographical and environmental

factors such as soil, sunlight, temperature, moisture, air, nutrients, etc.^[8]

C. carandas fruits collected from different geographical location and its different part were not identical in terms of their phytochemical components although all the analytes were detected in each sample (except fruits collected from Malshej [hilly region of Maharashtra] where stigmasterol was not detected). The content of lupeol and stigmasterol was high in the fruits collected from Kalyan (plains of Maharashtra), while sample from Malshej was rich in β -sitosterol. Flowers of *C. carandas* showed high LSB content in comparison with the other parts.

Species confusion may exist during harvesting of food crop. Availability of fourteen different species of *Carissa*^[25] creates confusion or misuse of other species with *C. carandas* may cause misidentification during harvesting which may directly affect the quality of finished product. Hence, fruits of two different closely related species of *Carissa* (*C. carandas* and *C. densiflora*) were compared on the basis of their LSB content. LSB content was found to be extremely high in *C. densiflora*. Such variations in the phytochemical content of these fruits may suggest different toxicological and pharmacological properties. Together these characteristic components can be used as reference markers for identification and authentication of *C. carandas* fruit and its commercial products.

CONCLUSION

Proposed HPLC method can be used as an analytical tool for quality evaluation of plants and formulations containing LSB as chemical markers. It is an efficient method to screen *C. carandas* fruit samples in order to assess its quality and authenticity. Hence, it can be demonstrated that HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

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