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A validated RP-HPLC-UV/DAD method for simultaneous quantitative determination of rosmarinic acid and eugenol in *Ocimum sanctum* L.

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ABSTRACT

Background: *Ocimum sanctum* L. is present as an ingredient in many herbal formulations and hence standardization of its raw materials and extracts is significant. The study was aimed at developing and validating a RP-HPLC method for the standardization of *O. sanctum* L. raw material and extracts by selecting rosmarinic acid and eugenol as markers.
Methods: The developed method used a Phenomenex Luna C_{18} column (250 × 4.6 mm; 5 μm) using a gradient elution of 1 mM ammonium acetate buffer and acetonitrile as the mobile phase with a flow rate of 1.5 mL/min. The analytes were monitored at 278 nm. The method was validated according to the ICH guidelines.
Results: The calibration curves showed good linear regression ($r^2 > 0.999$) within test ranges. Specificity of the method was assessed using PDA profile and the peak purity of all the analytes was greater than 99%. This method showed good reproducibility for the quantification of both the compounds in samples with an RSD of less than 2.5% respectively. Percentage recovery exceeded 90% for all analytes.
Conclusion: The validated method was successfully applied to quantify rosmarinic acid and eugenol in *O. sanctum* raw material and extracts which provides a new basis for overall assessment on quality.
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1. Introduction

Ocimum sanctum L. (Family *Lamiaceae*) has been extensively used in traditional systems of medicine like Ayurveda for its diverse medicinal properties. It is described in ayurvedic texts as 'an elixir of life'. *O. sanctum* is commonly known in India as Tulsi, "the incomparable one". It is widely distributed, spanning the entire Indian subcontinent, ranging from the Himalayas to the Andaman and Nicobar islands. It is a 30–75 cm high, erect, softly haired, biennial or triennial herb which is much branched. It has acute or obtuse, elliptic to oblong, serrate and pubescent on both sides. The flowers are closely whorled, purplish to crimson racemes.[1]

Tulsi is famous for its religious sanctity, aromatic properties, culinary uses and its varied healing properties. Traditionally it has been used in the treatment of cold, cough, bronchitis, malaria, stomach disorders, inflammation, heart disease and various forms of poisoning[2] and also as an antifertility agent.[3] Scientific research shows that *O. sanctum* possesses many pharmacological activities. It shows anti-inflammatory, anti-stress, anti-diabetic, antioxidant, gastroprotective, hypolipidemic, antimicrobial, wound healing, analgesic anthelmintic, anticancer, radioprotective, antifertility and immunomodulatory activity.[2]

The main chemical constituents of this plant are fixed oils and fatty acids, volatile oils, flavonoids, phenolic acids, triterpenes and steroids and tannins. This paper is concerned with the analysis of compounds belonging to class of volatile oils and phenolic acids and hence we concentrate on their content only. The phenolic acids consist mainly of rosmarinic acid and caffeic acid.[4] Volatile oils in this plant are mainly characterized by methyl cinnamate, methyl chavicol (estragol), eugenol and methyl eugenol.[3] Rosmarinic acid shows anti-inflammatory, antiviral, antimicrobial, antioxidant, anti tumour, anti-depressive and immunosuppressive activity.[5–9] It has also been proven effective in treating Alzheimer's disease.[10] Eugenol has anti-inflammatory, antibacterial, antifungal, antimicrobial and antioxidant properties.[11–14]

Various other techniques like HPTLC,[15] Gas chromatography–Mass spectroscopy[16] and Liquid chromatography–Mass spectroscopy[17] have been employed to standardize *O. sanctum*. However, literature survey shows that a HPLC method for the simultaneous

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determination of rosmarinic acid (**1**), eugenol (**2**) (Fig. 1), in *O. sanctum* has not yet been reported to the best of our knowledge. Hence this work attempts to develop and validate a HPLC method for the simultaneous determination of rosmarinic acid and eugenol which can be used for regular quality control of *O. sanctum* raw materials and extracts.

2. Experimental

2.1. Chemicals and reagents

Reference standards for rosmarinic acid (**1)** (batch no T11K109) and Eugenol (**2)** (batch no T11K109) were isolated at the Natural Remedies Pvt. Ltd. The identity and purity were confirmed by chromatographic methods, spectroscopic data (1D-NMR, 2D-NMR and HR-ESI-MS) and by comparison with published spectral data.[18,19] The purity of these standard compounds was calculated to be 95%. HPLC grade methanol, acetonitrile (ACN) and ammonium acetate used in the study were obtained from Rankem. HPLC grade water was obtained from Sartorius Arium 611 water purification system. 0.45μ PES membrane filter was obtained from Rankem.

2.1.1. Instrumentation

Separation was performed on a HPLC system (Shimadzu LC2010 CHT) equipped with quaternary pump, DAD (Photo Diode Array Detector) detector, autosampler, thermostated column oven, degasser and LC Solutions software. A Phenomenex luna C_{18} column (250 × 4.6 mm; 5 μm) was used for separation.

2.1.2. Chromatographic conditions

Separation was performed by following a gradient time program. The mobile phase consisted of solvent A (ammonium acetate in water at pH = 6.2) and solvent B (acetonitrile (HPLC grade)) with the elution profile as follows: 0–5 min, 5%B (v/v, isocratic elution); 5–15 min, 5–25%B (v/v, linear gradient); 15–25 min, 25–70%B (v/v, linear gradient); 25–30 min, 70%B (v/v, isocratic elution); 30–35 min, 70–5%B (v/v, linear gradient); 35–40 min, 5%B (v/v, equilibration). Elution was performed at a flow rate of 1.5 mL/min. The C_{18} column was maintained at 30 °C throughout the analysis. The detection wavelength was set at 278 nm. The injection volume was set at 20 μl. The run time was set at 40 min. The peaks were identified by comparing the retention times of the reference standards with the extract.

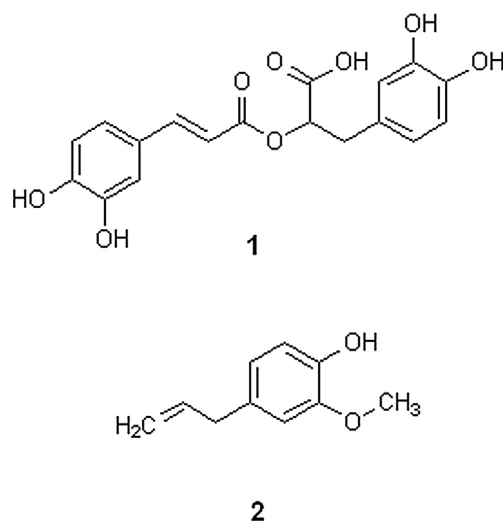

Fig. 1. Structures of the standard compounds – Rosmarinic acid (1) and Eugenol (2).

2.2. Plant material

Total five raw materials of *O. sanctum* and one raw material each of *Ocimum americanum* L., *Ocimum basilicum* L. and *Ocimum kilimandscharicum* Gürke were collected in June and August in the year 2012. All plant materials were authenticated by Dr. P. Santhan, Taxonomist, Natural Remedies Pvt. Ltd, Bangalore, India. Three different extracts of *O. sanctum* were obtained from Natural Remedies Pvt. Ltd., Bangalore, India.

2.3. Preparation of sample solutions

Approximately three to five grams of accurately weighed, powdered plant material was refluxed thrice with 50 mL of methanol for 10 min. The combined extracts were reduced to less than 100 mL, transferred quantitatively into 100 mL volumetric flask and volume was made up to 100 mL with methanol. An accurately weighed sample of the commercial extracts (about 250 mg) was taken in a 50 mL volumetric flask, boiled with a little methanol, sonicated and then the volume was made up to 50 mL with methanol. The raw materials and extracts solutions so obtained were filtered through a 0.45μ PES membrane filter (Rankem) and filled in HPLC vials. These vials were injected in triplicate the HPLC system for determination of content of compounds **1** and **2**.

2.4. Preparation of standard solutions

For calibration, standard stock solutions of **1** and **2** having a concentration of around 1000 ppm were prepared separately in methanol. Aliquots of the individual stock solutions were prepared in the concentration range of around 250 ppm–16 ppm. The obtained dilutions were injected five times into the HPLC system. The linearity of the individual standards was plotted by taking mean peak area versus concentration.

3. Results and discussion

Literature review revealed that the quantification of constituents in *O. sanctum* by HPLC is available for rosmarinic acid and eugenol individually. GC methods are also available for eugenol. A variety of mobile phases have been used in these methods. Majority of the methods use water, methanol, acetonitrile in which organic modifiers like formic acid, acetic acid, trifluoroacetic acid have been added. The wavelength of detection used in these methods ranges from 260 nm to 330 nm. No previous reports have been found for the simultaneous estimation of **1** and **2** in *O. sanctum* to the best of our knowledge.

3.1. Optimisation of the chromatographic conditions

Preliminary separation studies were performed using a binary mobile phase consisting of acetonitrile and phosphate buffer (pH = 3.2) in a gradient elution pattern. Various trials were carried using different gradient elution patterns but satisfactory resolution was not achieved. Therefore trials were made at a higher pH (weakly acidic pH). Finally, acetonitrile: ammonium acetate buffer system in a gradient elution pattern with a flow rate of 1.5 mL/min was found to be optimum. The total run time of the method was 40 min (Fig. 2).

Methanol was chosen as the solvent because of the high solubility of the extracts of *O. sanctum* in methanol and due to its compatibility with the C_{18} column.

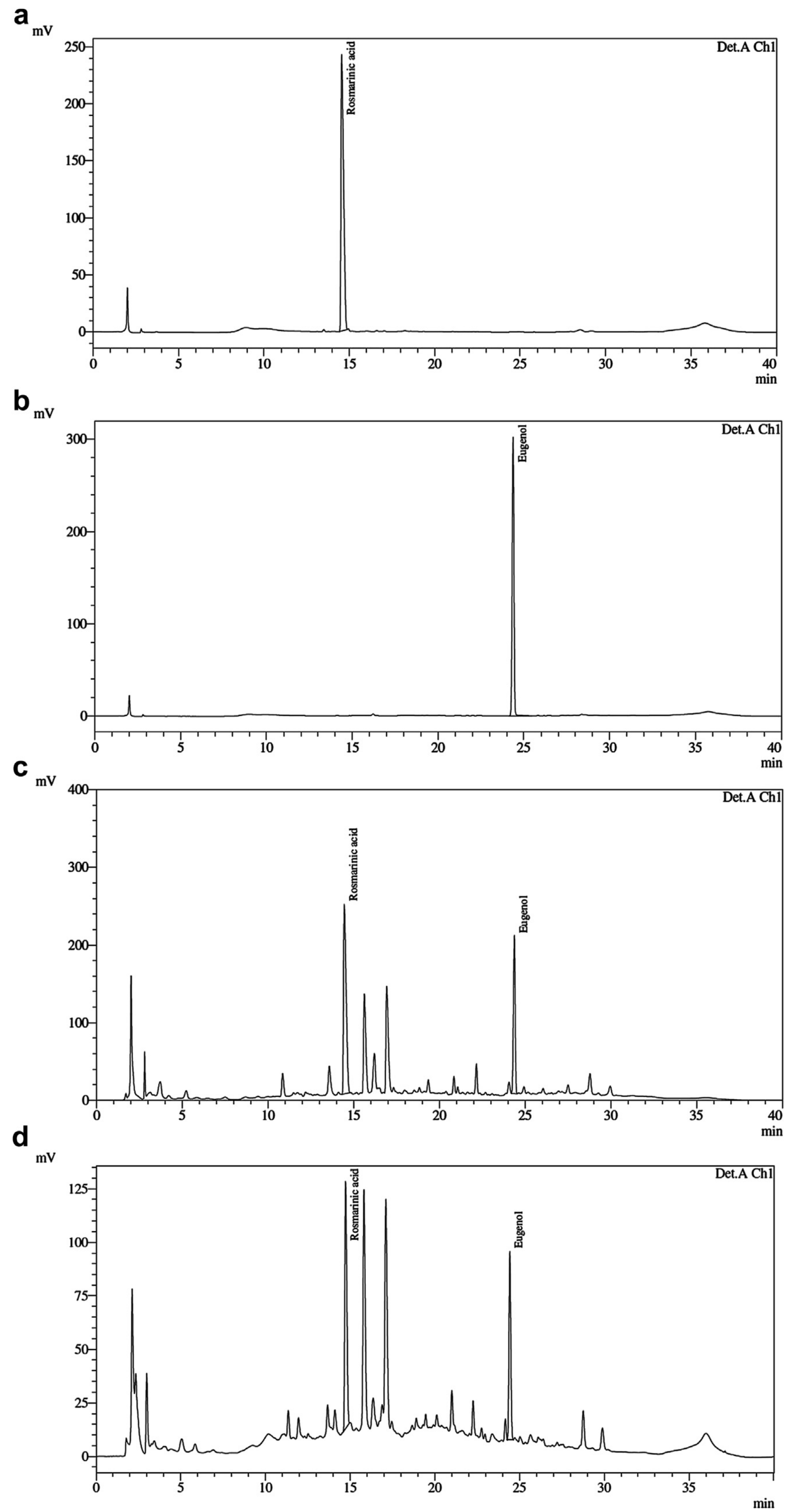

Fig. 2. HPLC Chromatogram of (a) Standards of Rosmarinic acid (**1**) and (b) Eugenol (**2**) (c) Raw material of *Ocimum sanctum* (PCN\OS\156), (d) Extract (OS – 01).

Table 1
System suitability for the determination of the two analytes.[a]

Analytes	Retention time (Rt)	Relative retention time (RRt)	Tailing factor (T)	Theoretical plates (N)	Capacity factor (k')
Rosmarinic acid	14.729	1.000	1.348	63,375	7.276
Eugenol	24.335	1.658	1.157	211,528	12.722

[a] The values for system suitability are an average of n replicates ($n = 5$).

Table 2
Statistical analysis for the calibration curves of the two analytes.

Analyte	Calibration equation[a] (linear model)	Correlation[a] factor, r^2	LOD (ng/mL)[b]	LOQ (ng/mL)[b]
Rosmarinic acid	$y = 23{,}212x + 56{,}288$	1	17.35	57.85
Eugenol	$y = 11{,}873x + 76{,}050$	0.9997	7.24	24.12

[a] Calculated from calibration curve for n concentrations of the individual standards ($n = 6$).
[b] LOD and LOQ are calculated for signal to noise ratio of 3 and 10 respectively.

3.1.1. Method Validation

Method Validation was performed in accordance with ICH guidelines.[20]

3.2. System suitability

System suitability was evaluated by injecting five replicates of the standard mix solution. The acceptance criterion for percentage relative standard deviation (%RSD) for retention time and area was set at 2.5%. The capacity factor (k'), relative retention times (RRT) and tailing factors were obtained from the peak tables of the resultant chromatograms. The column performance was measured by the number of theoretical plates. The results obtained are presented in Table 1.

3.3. Linearity

Calibration curves were plotted for 5 different concentrations of the individual standards ranging from 257 to 16 ppm for **1** and from 377 to 23 ppm for **2**. Linearity was established by calculating the slope, y intercepts and correlation coefficients (r^2) using a least squares regression equation. The details of the regression curves are presented in Table 3. The regression equation and correlation coefficients showed good linearity response for the method developed. The limit of detection and limit of quantification were also calculated using the signal to noise ratio method. A signal to noise ratio of 3 and 10 were considered as the limits for detection and quantitation respectively. The values for LOD and LOQ are presented in Table 2. Range of quantification was established using the mean response of linearity data. The concentrations which showed a response of ±5% of the mean response were considered to be in the range. The results obtained are presented in Table 2.

Table 3
Results of recovery studies for the two analytes.[a]

	Theoretical result (%w/w)	Observed result (%w/w)	Recovery (%w/w)
Rosmarinic acid	1.61	1.54	95.18
	2.00	1.93	96.82
	4.36	4.12	94.34
Eugenol	1.14	1.15	100.85
	1.63	1.73	106.56
	5.10	4.93	96.63

[a] The results presented in the above table are the average values for percentage recovery of 'n' concentrations of sample solutions ($n = 3$) spiked with known quantities of standard.

Table 4
Results of precision for the two analytes.[a]

	%RSD	
	1	2
Retention time	±0.22%	±0.02%
Area	±1.93%	±1.58%

[a] The %RSD is calculated for six concentrations of standards ($n = 5$) as well as two concentrations of sample solutions ($n = 3$).

3.4. Accuracy

Three concentrations of preanalysed sample solutions were spiked with known quantities of the standards and injected in triplicate to perform recovery studies. The percentage recovery was calculated from the data obtained. The percentage recovery obtained confirmed the accuracy of the proposed method. The results of the recovery studies are presented in Table 3.

3.4.1. Precision

Five replicates of six concentrations of the standard solutions as well as three replicates of two concentrations of sample solutions were used for the determination of injection precision and method precision. The %RSD values of the retention time and area under the curve for the above were calculated. The results obtained from this study are presented in Table 4.

3.4.2. Specificity

The specificity of the method for both the analytes was determined by using peak purity studies carried out using a photodiode array detector (PDA). The peak purity index was found to be >0.999 for both the analytes. This shows that the analyte peak has no interference from other matrix components and is well resolved from them. The obtained results are presented in Table 5.

3.4.3. Stability

Sample and standard mix solutions were injected at an interval of 7 h for 49 h. The solutions were found to be stable for a period of 28 h after which there was a significant variation in the %results that were obtained for the standard and sample solutions. Extra peaks were also noticed in the chromatograms after 28 h.

3.5. Quantification of extracts and raw materials

The developed HPLC method facilitates the simultaneous determination of **1** and **2**. The quantification of both the analytes in three extracts, five raw materials of *O. sanctum* and three raw materials of different species of *Ocimum* were carried out using the developed and validated method. The results are summarized in Table 6.

Table 5
Results of specificity.

Analytes	Peak purity index[a]			
	Individual standards	Standard mix	Extract	Raw material
Rosmarinic acid	0.999999	0.999998	0.998922	0.999999
Eugenol	0.99996	0.999985	0.996942	0.999928

[a] Peak purity data is obtained from the PDA (photo diode array) detector.

Table 6
Results of quantification of raw materials of *Ocimum sanctum*, other species of *Ocimum* and commercial extracts.

	Batch no	%Results (w/w)[a]	
		1	2
Ocimum sanctum L.	PCN/OS/156	0.084	0.116
	PCN/OS/164	0.080	0.069
	PCN/OS/175	0.670	1.230
	PCN/OS/176	0.379	0.359
	PCN/OS/179	0.426	0.818
O. americanum L.	PCN/OS/177	0.501	0.005
O. basilicum L.	PCN/OB/178	0.023	0.016
O. kilimandscharicum Gürke	PCN/OK/180	0.048	0.120
Extracts	OS – 01	0.455	0.533
	OS – 02	0.388	0.270
	OS – 03	0.578	0.422

[a] The results (%w/w) presented in the above table are the average values of 'n' concentrations of sample solutions ($n = 2$).

4. Conclusion

A simple and convenient method was developed for the simultaneous estimation of rosmarinic acid and eugenol in *O. sanctum* raw materials and extracts. The proposed method was validated according to the ICH guidelines (Q2 [R1], 2005) to reveal that it was specific, accurate and precise. The validated method was successfully applied to quantify rosmarinic acid and eugenol in *O. sanctum* extracts and raw materials to provide a new basis for standardization. The validated method was also successfully applied for the quantification of rosmarinic acid and eugenol in three other species of *Ocimum*.

Conflicts of interest

All authors have none to declare.

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