HPLC –UV Method Development and Quantification of Eugenol from Methanolic Extracts of Some Spices.

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Abstract

Eugenol is one of the important constituents in various spices such as clove, cinnamon, nutmeg, tulsi oils which are widely used as flavouring agents in foods and beaverages. In this study, a simple, sensitive and precise reversed- phase high performance liquid chromatography (HPLC) method has been developed, validated and used for quantitative determination of eugenol from the methanolic extracts of spices viz, dried bud powder of clove, bark powder of Cinnamomum zeylanicum, leaves powder of Cinnamomum tamala, seed powder of Myristica fragrans and leaves powder of Ocimum sanctum. The soxhlet extraction method was used for the extraction of these compounds. The reverse – phase HPLC analysis was carried out using C_{18} (150mm x 4.6mm, 5 µm) column and a mobile phase comprising of methanol : acetonitrile : water in the volume ratio of (10 : 50 : 40) which was obtained by trial and error method at a flow rate of 0.7 cm^3/min in an isocratic system. This method was developed for both standard eugenol and eugenol present in methanolic extracts of various spices. The detection and quantitation of both compounds was carried out at 280 nm.

Keywords- Eugenol , Quantitation , HPLC

Introduction

In last few decades , several studies have been carried out by Indian Scientists and researchers to suggest the role of essential oils and eugenol in therapeutic potential of various spices. Eugenol , a phenolic flavouring compound is an important constituent of essential oils extracted from the spices taken for the present study.

Materials And Methods

Collection of Plant Samples

Scientific and common names of the spices studied

The experimental material consists of 5 spices: cloves, cinnamom , nutmeg , tejpat and tulsi. All the spices except tulsi herb were purchased from the local market in Nagpur, Maharashtra , India. Tulsi plants were grown in nagpur farm itself , then the leaves were collected, cleaned and dried in the shade. All the spices also were cleaned, shade dried and powdered . Their herbarium was prepared and authenticated from Department of Botany RTMNU, Nagpur. The powders were stored separately in an air– tight labelled containers at room – temp (28±2^{0}c) They were classified according to their English name , scientific name and the used part of the plant.(Table 1).

Table 1 : Scientific and common names of spices to be studied

Common Name	Scientific Name	Family	Used Parts
Cloves	Syzygium aromaticum	Myrtaceae	Bud
Dalchini	Cinnamomum zeylanicum	Lauraceae	Bark
Tejpat	Cinnamomum tamala	Lauraceae	Leaves
Nutmeg	Myristica fragrans	Myristicaceae	Seed
Tulsi	Ocimum sanctum	Labiateae	leaves

Proximate analysis of raw materials

To assess the quality of raw materials, proximate parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter were determined using standard pharmacopoeial methods as per WHO guidelines .(Data not mentioned)

Preliminary phytochemical evaluation

Phytoconstituents in above mentioned five spices clove, Dalchini , Nutmeg, Tejpatta and Tulsi were evaluated by performing preliminary phytochemical tests for flavonoids, essential oils, tannins, glycosides, alkaloids and resins as per standard methods[11].(Data not mentioned)

Chemical reagents and standard Material

Methanol (HPLC grade), Acetonitrile (HPLC grade) , water for HPLC, 99% Pure Eugenol (Sigma – Aldrich, Bangluru), and other chemicals and reagents were procured from authorized suppliers in Nagpur. The buds of Syzygium aromaticum , barks of Cinnamomum zeylanicum , leaves of Cinnamomum tamala seeds of Myristica fragrans and leaves of Ocimum sanctum were purchased from the local wholesale market of Nagpur (M.S.), India . Its herbarium was prepared and authenticated from Dept. of Botany RTMNU, Nagpur. All the spices were cleaned, shade dried and powdered . The powders were stored separately in an – tight labeled containers at room – temp (28 ± 2^0c)

Preparation of extracts

The powdered spices each 50 gms. were extracted with methanol by using soxhlet extraction apparatus separately. The extracts were then filtered and concentrated by distilling off the solvent to obtain the crude extract which was then dried by rotary evaporator and stored separately in 5 glass bottles with proper labelling for further study.

Preparation of stock solution of eugenol

A stock solution of Eugenol (1000μg/ml) was prepared by dissolving 10 mg accurately weighed Eugenol in methanol and diluting it to 10ml of methanol .From this stock solution , solutions of 0.2μg/ml - 10μg/ml were prepared for calibration curve of Eugenol. The three quality control samples of 2, 6 and 8μg/ml were prepared for studying precision, accuracy and ruggedness.

Preparation of working standard solution of eugenol

Intermediate solution of 100 µg/ml was prepared from stock solution of 1000 µg/ml. The concentration range of eugenol selected for linearity was 0.2µg/ml-10 µg/ml. The aliquots of 0.02, 0.04, 0.07, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml taken of the intermediate stock solution (100 µg/ml) were transferred to different 10.0 ml standard volume flasks and each flask was diluted to mark with methanol to obtained working standard solutions of eugenol with concentration 0.2 to 10 µg/ml. Working standard solution of eugenol in the concentration range of 0.2 µg/ml to 10 µg/ml were preparation by diluting the aliquots of 0.02-1.0 cm^3 of stock solution of eugenol (1000 µg/ml) to 100 cm^3 with mobile phase.

Preparation of sample solution

All the spices were dried properly in the shade , powdered and seived through a mesh .They were stored in an air tight container in the refrigerator till further use.

The powdered sample of 50 g clove was placed in a soxhlet extraction setup using methanol solvent for about 10 -12 hours. The extract was then concentrated by distillation process .In the same way extracts of all other samples as dalchini , tejpatta ,nutmeg and tulsi were obtained.

5 mg of each methanolic extract was taken in 10 ml volumetric flask and the solution was made in methanol upto 10 ml .This solution was ready for the assay experiment. Same procedure was done for the rest of the sample extracts.

Chromatographic condition

HPLC analysis was conducted using Zorbax C_{18} column , (150 mm * 4.6mm, 5µm) with Chemito (Kanmer) Isocratic Type pump, autosampler and 20 µl loop. The instrument was equipped with UV visible detector. Wincats 3 Software was used for data acquisition. The mobile phase comprising a mix of methanol- acetonitrile- water in volume ratio of 10 : 50 : 40 was delivered at flow rate of 0.7cm^3/min and the detection was done at 280 nm.

Method Validation

Validation of the developed HPLC method was carried out as per the International Conference of Harmonization (ICH) guidelines for specificity, sensitivity, linearity, accuracy, precision, repeatability, and robustness.

Linearity

Ten working standard solutions of eugenol in concentration range of 2 µg/ml- 10 µg/ml were prepared . Each solution was injected in triplicate in the chromatographic system under optimized condition. The calibration plot for standard was obtained by plotting a graph of mean peak areas of standard against its injected concentration. The results are listed in table 2.

Limits of detection and limits of quantification

Limit of Detection and Limit of Quantitation: Limit of detection (LOD) and limit of quantitation (LOQ) of the developed method was affirmed by analyzing progressively low concentrations of eugenol along with methanol as blank. Limit of detection (LOD) and limit of quantitation (LOQ) were established at a signal to noise ratio of 3:1 and 10:1 respectively. The results are presented in table 2.

Precision

Inter-day, Intra day and precision were studied for eugenol. Intra day precision was evaluated by having a single operator analysis, 3 quality – control replicate samples within a day. Inter day and instrumental precision was assessed by replicating the analysis of quality samples for 3 days.

The instrumental precision was studied by repetitive injections (n=6) of standard solution of eugenol (5µg/ml). The values of % RSD of Peak area of eugenol for interday, Intra day and instrumental were determined and results are tabulated in table 2.

System suitability

System Suitability of eugenol was carried by injecting standard solution of eugenol (5µg/ml) 5 times in the chromatographic system under optimized chromatographic conditions [9]. The values and retention times of Eugenol were noted for each injection concentration of standard. As the % RSD was less than 2%, the system was found to be suitable.

Accuracy :-

The accuracy of the method was established by performing a recovery experiment using standard addition method. For zero level, only sample solution was analyse by HPLC in 7 replicates. The average values of % recoveries of Eugenol was determined and found to be 99.9%. The result is given in table 4 .

Quantitaion of Eugenol in all Spices

Under the optimized Chromatographic condition, the 20 µl of sample solution was injected into system. The identities of peaks of eugenol were determined by comparing the chromatogram of each sample solution with that of standard eugenol .The amount of eugenol present in the samples were calculated which is listed in table 3. A typical chromatogram of standard eugenol and the chromatograms of the different samples are shown in Figures 1-5 given below.

Results and discussion

The results for the proximate analysis of raw materials for some parameter like ash values (total soluble ash), loss on drying, moisture content and extraction values were calculated and they were found to be quiet in compliance with the pharmocopeial limits(data not mentioned).

The phytochemical evaluations of above mentioned spice extracts show the presence of different phyto constituents.

Table 2. Method validation data for the quantitation of eugenol

Linearity	0.4-10.0 µg/ml
Correlation coefficient	0.999
LOD	0.05 µg/ml
LOQ	0.2
Intrumental Precision(%RSD) n=6	1.01
Intra-day Precision (% RSD)	0.03
Inter-day Precision (%RSD)	1.08

Linearity and Calibration Curve

The linearity was tested at ten concentration levels i.e. 0.2 – 10 µg/ml. A calibration plot was constructed by plotting peak area against the concentration (µg /ml) with the help of the WIN-CATS software. The linear regression value indicated a linear relationship over a concentration range of 0.4 – 10 µg/ml and its linear equation was y =2.563x + 0.027 the slope, intercept, and correlation co-efficient were also determined. The correlation co-efficient of the calibration plot was 0.99 (Table 2), indicating a good linear relationship between peak area and concentration (Figure 1).

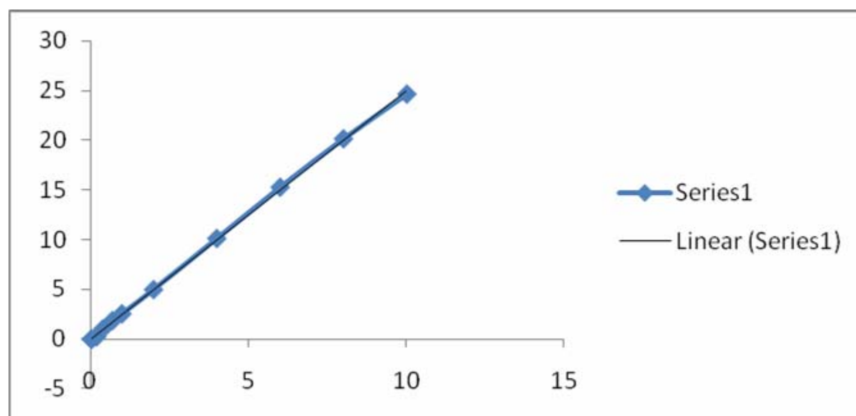

Figure : Graph showing calibration curve of standard eugenol

Table 3 : Amounts of Eugenol found in methanolic extracts of five taken spices .

Sr.No.	Name of spice	Amount of Eugenol (µg/ml)
1	Syzygium aromaticum	21.91
2	Cinnamomum zeylanicum	6.53
3	Cinnamomum tamala	5.37
4	Ocimum sanctum	5.93
5	Myristica fragrans	5.64

The mobile phase of methanol : acetonitrile : water (10 : 50 : 40) in the present work shows a better resolution of various components present in different spices. The use of only acetonitrile and water or methanol and water were found not suitable for separating the chemical constituents. The wavelength at which analysis yielded better sensivity was found to be at 280 nm.

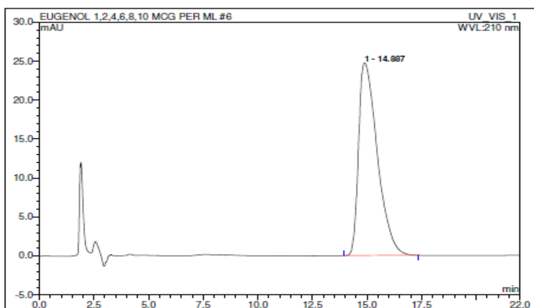

Figure 1 : A typical of standard eugenol solution.

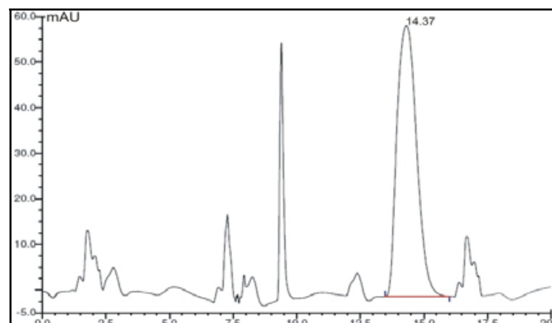

Figure 2 : A typical chromatogram of Methanolic extract of Clove

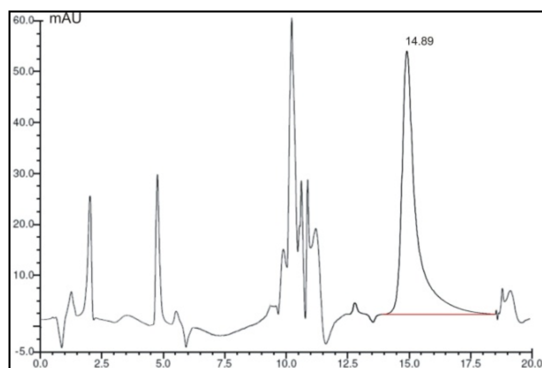

Figure 3 : A typical chromatogram of Tejpatta

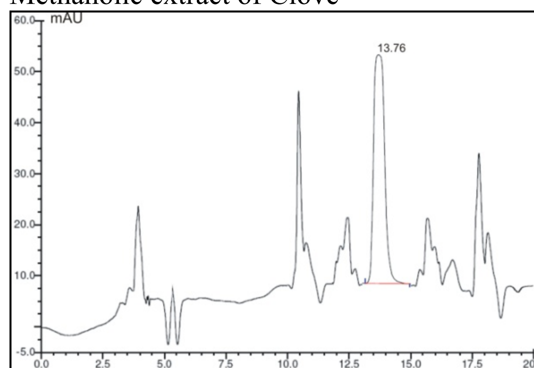

Figure 4 : A typical chromatogram of Cinnamomum zeylanicum

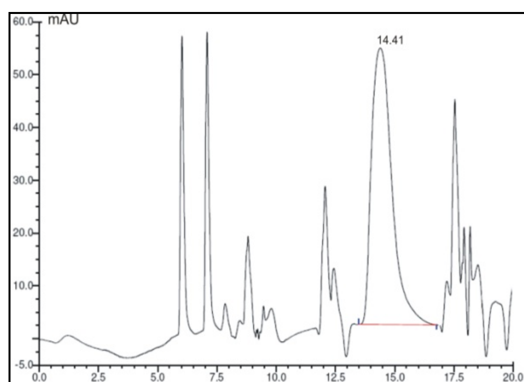

Figure 4 : A typical chromatogram of Tulsi

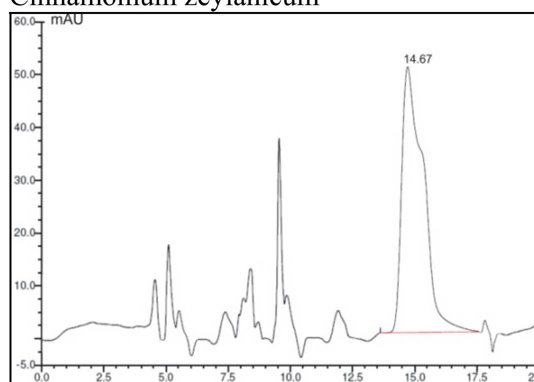

Figure 5 : A typical chromatogram of Nutmeg

The values of percent relative standard deviation for inter-day, intra-day and instrumental precision were less than 2% for the standard , leading to conclude that the method was found to be precise.

The percentage recovery for Eugenol was found to be 99.8%, which is maximum in Syzygium aromaticum. Almost all the five taken spices showed the recovery between the range of 99% to 99.8%

indicating that the pure Eugenol peak only is obtained. No other chemical constituents is interfering in the process.

Table 4. Results of recovery of quantitation of Eugenol from 5 spices methanolic extracts.

Sr.No.	Name of Spice	% Recovery
1	Syzygium aromaticum	99.8
2	Cinnamomum zeylanicum	99.2
3	Cinnamomum tamala	99.7
4	Ocimum sanctum	99.4
5	Myristica fragrans	99.5

A HPLC method has been reported in literature for simultaneous determination of quercetin , eugenol, myriticin and safrole [9] from , nutmeg, fruit and mace there, the column used was RP – 250 *4.6mm , 5-Aum, Zorbax C- 18 column. Various chromatographic methods have been reported in literature for determination of Eugenol alone or in combination with other components in clove or cinnamon , or nutmeg . or Tulsi individually or in some formulations.

Conclusion

In the present work, a simple, precise HPLC method has been developed for quantification of Eugenol from 5 spices mentioned above. This method will be of great use as a quality control method. The HPLC method showed good linearity, precision and accuracy and so to quantify eugenol was easier.

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