

DEVELOPMENT AND VALIDATION OF GC METHOD FOR THE ESTIMATION OF EUGENOL IN CLOVE EXTRACT

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ABSTRACT

Objective: The aim of study was to develop a simple, sensitive and precise gas chromatographic method for the analysis of eugenol in alcoholic and aqueous extract of clove and validate according to current ICH guidelines.

Methods: The eugenol was one chief active constituent of clove buds. The aqueous and ethanolic extract of clove was prepared and further extracted eugenol using methanol as solvent. The GC method was used for the analytical determination of eugenol. The sample was estimated using gas chromatography with flame ionisation as a detector. Nitrogen at a flow rate of 1.18 mL/min was used as a carrier gas and total run time was 10 minutes. The injection port and detector temperature were set to 225°C and 270°C, respectively. The retention time of eugenol was found to be 5.8 minutes.

Results: The linearity of the developed method was tested in the range of 200 ng/mL-1000 ng/mL for eugenol, limit of detection was found to be 64.31 ng/mL and the percentage recovery was 98.1%.

Conclusion: A simple, precise and accurate GC-FID method has been developed for the determination of eugenol in aqueous and ethanolic extracts.

Keywords: Gas chromatography, Eugenol, Flame Ionisation Detector.

INTRODUCTION

Cloves are the aromatic dried flower buds of a tree belonging to the family of Myrtaceae, 'Syzygium aromaticum' (Cloves) is native to the Maluku islands in Indonesia which are used as a spice in cuisines all over the world. Eugenol (Fig. 1) comprises 72-90% of the essential oil extracted from cloves and responsible for the aroma of the clove. Other important essential oil constituents of clove oil include acetyl eugenol, β -caryophyllene, vanillin, cratogeomyc acid, tannins such as bicornin, gallotannic acid, methyl salicylate, the flavanoids like eugenin, kaempferol, rhamnetin, and eugenitin, triterpenoids like oleanolic acid, stigmasterol and campesterol and several sesquiterpenes [1].

Clove oil (*Syzygium aromaticum*) is widely used as a perfume and food flavoring as a medicine for the treatment of asthma and various allergic disorders in Korea and as a general antiseptic in medical dental practices. The essential ingredient responsible for its antifungal activity is eugenol from the clove. Clove oil, applied to a cavity in a decayed tooth, also relieves toothache [2].

Eugenol, the major constituent of clove oil, has been widely used for its anesthetic and analgesic action in dentistry. Eugenol exhibits pharmacological effects on almost all systems. It possesses significant antioxidant, anti-inflammatory and cardiovascular properties, in addition to analgesic and local anesthetic activity [3,4]. Eugenols treat cold, dental abscesses, gum disease, ear ache and arthritis pain. It also act as anti-fungal, anticonvulsant and anticarcinogenic and antimutagenic activities [5]. From the literature review it suggests that there have been some methods developed for clove by gas chromatography [6-9], HPLC [10]. This work describes a simple, sensitive, and precise gas chromatographic method for the analysis of eugenol from clove extract. The method validated according standard ICH guidelines [11] and used for the determination of eugenol in alcoholic and aqueous extract.

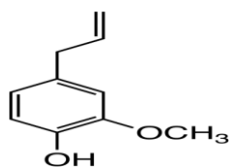


Fig. 1: Chemical structure of eugenol

MATERIALS AND METHODS

Instrumentation and Analytical Conditions

A gas liquid chromatography with flame ionization detector (SHIMADZU, 2014 Shimadzu Technologies, Japan) was used for the determination of eugenol in clove extract. LC solution software was used to analyse the sample. Instrument is coupled with a split/split less injector, operated in a split-mode and FID. The computer with LC solutions software has been used to control the gas chromatograph and Rtx-5 capillary column (cross bond 5% diphenyl/95% dimethyl polysiloxane) with a length of 30 meters and an internal diameter of 0.25 mm was used throughout the study.

The GC-FID parameters used in the method development were based on the boiling point of the drug. Eugenol has a boiling point of about 254°C. The injection port and detector temperature were set to 225°C and 270°C, respectively.

Manual split less injection of approximately 1 μ L sample was performed at an inlet temperature of 225°C. The detector temperature was set to 270°C. After injection, the oven temperature was increased quickly from 110°C and then programmed within 8 min to 220°C at a rate of 15°C per min for 2 min. Nitrogen at a flow rate of 1.18 mL/min was used as a carrier gas. Synthetic air (flow rate of 100 mL/min), hydrogen (25 mL/min) were fed to the FID. All the gases used in these studies were of pharmacopoeial purity.

Procurement of plant materials

The clove buds were collected from local market of Mysore.

Preparation of extract

Collected clove buds were washed with water and dried in shade, then coarsely powdered in a blender. From the coarse powder 50gm was subjected to reflux for 2 hours at temperature 80°C to 90°C. The solvents were decanted and filtered with filter paper and recovered by distillation. The extracts were dried under water bath at 60°C to 70°C respectively.

Preparation of standard Sample

A standard stock solution of eugenol (20mg/mL) was prepared by dissolving 9.3 mL of accurately weighed eugenol in 99.9% ethanol and volume was made up to the 10 mL. From the standard stock

solution, working standard (200-1000 ng/mL) was prepared for the gas chromatography method.

Preparation of Extract Sample

10 mg of ethanolic and aqueous extracts of clove was weighed accurately in 10 mL volumetric flasks and it was dissolved in with 99.9% of methanol followed by sonication for 5 to 10 minutes and volume was made up using the same solvent. Solution was filtered using 2 µm syringe filter. From the stock solution 1 µl was injected on the head of the injector. This concentration was used for the estimation of eugenol from the extract of clove.

RESULTS AND DISCUSSION

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines.

System suitability

After the method conditions were established as described above, method was validated for precision, accuracy, and linearity. Precision was measured as the repeatability of a series of results ($n=6$) and was also checked inter-day. Accuracy was determined as percentage recovery ($n=3$) at three concentrations (80,100 and 120% of the amount expected) achieved by spiking placebo with reference standard. Linearity was established by chromatography of a series of solutions ($n=5$) of decreasing concentrations. The limit of detection (LOD) and quantification (LOQ) were determined. These values are summarized in Table 1

Quantitative analysis of the drugs was performed under the conditions established. The selectivity of the method was evaluated by comparing retention time values in chromatograms obtained from the analyzed product with those in the chromatograms obtained from reference standard.

Table 1: Validation report for gas chromatograph for determination of eugenol

Parameter	Value
Linearity (ng)	200 -1000ng/ mL
Limit of detection (LOD) (ng)	64.310 ng
Limit of Quantification (LOQ) (ng)	194.88 ng
Recovery (%)	98-102%
Regression Coefficient	0.9919
Retention time (min)	5.8

Table 2: Linearity of eugenol by GC-FID method

Concentration (ng/mL)	Peak area
200	16545.2
300	53020.2
400	87515.4
500	127767.6
600	168977
700	209869.4
800	284019.2
900	320433.7
1000	365856.2

Table 3: Intraday Precision study of the eugenol

Concentration	Area	AVG	SD	%RSD
200ng intraday	16325.4	16468.1	134.0868	0.8142
	16647.6			
	16431.3			
600ng intraday	115286.6	115387.6	2178.278	1.887
	112771.7			
	118104.5			
1000ng intraday	284828.7	285508.6	758.0241	0.265
	286566.3			
	285130.7			

Table 4: Interday Precision study of the eugenol

Concentration (ng/ml)	Area	AVG	SD	%RSD
200	10531.7	10563.3	167.5718	1.586
	10782.5			
	10375.7			
600	95760.2	96371.9	448.5879	0.465
	96823.4			
	96532.1			
1000	228440.1	226533.4	1408.717	0.621
	226080			
	225080			

Linearity

Linearity was studied by preparing standard solutions standard eugenol at different concentrations. The linearity of peak area response versus concentration for eugenol was studied between concentration ranges of 200-1000 ng/mL. The calibration curve constructed was evaluated by its correlation coefficient. The calibration equation from six replicate experiments, $y = 445.77x + 85903$ ($r^2 = 0.991$), demonstrated the linearity of the method.

Precision

The precision of the analytical method was determined by repeatability (intra-day) and intermediate precision (inter-day). Three different concentrations were (200, 400, 600 ng/mL) were analyzed six time in one day for intra-day precision and once daily for three days for inter-day precision. The RSD value for intra-day precision and interday was found to be less than 2%. The results are tabulated in table 3 and 4.

Accuracy

Accuracy of the method is determined by performing the recovery studies. Recovery study was performed by addition of known amount of standard drugs to pre analyzed commercial pharmaceutical product sample. Accuracy was performed at three levels 400, 500, 600ng/mL. The experiment was repeated three times. These values are listed in table 5.

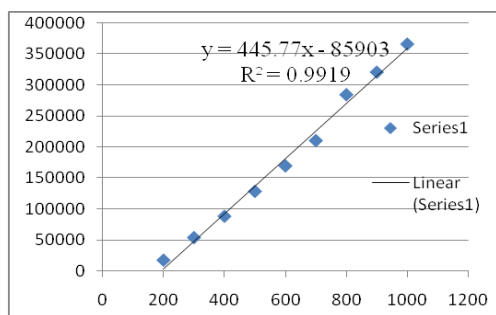


Fig. 1: Linearity curve of eugenol

Table 5: Recovery values of eugenol by GC-FID method in prepared clove extract

Amount of eugenol added (ng/mL)	Amount of extract added (ng/mL)	Recovery (%)	RSD%*
400	500	97.8	1.04
500	500	95.3	0.6
600	500	98.2	0.8

RSD: Relative standard derivation

*: average of six replicate determinations.

Limit of detection and limit of quantitation

According to the ICH recommendation, the approach based on the standard deviation (SD) of the response and slope was use for the determining the LOD and LOQ values.

$$\text{LOD} = 3.3 \sigma/s \quad \text{LOQ} = 10 \sigma/s$$

σ = Standard deviation of response

S = Slope of calibration curve

The LOD & LOQ was found to be 64.310 ng and 194.88 ng for Eugenol.

Analysis of sample

Required quantity of extract was accurately weighed and transferred into 10 mL volumetric flask, dissolved in methanol and sonicated for 5 minutes. The solution was filtered by 2 μ syringe filter and volume made up to the mark with methanol. From the sample solution 1 μ L were injected on the head of the injector.

RESULTS AND DISCUSSION

The eugenol is a volatile product separated at retention time of 5.8 in the chromatogram as shown in Figure 2, It was eluted from a capillary GC column, having a single peak. The reaction was carried out in methanol. A better GC response (average peak height/peak area) was observed. The reaction mixture was sonicated at room temperature (30°C) for 5-10 min and the optimum response was observed within 10 min. Individual chromatograms were recorded for blank chromatogram in Figure 3, chromatograms of ethanolic and aqueous extracts are shown in Figure 4 & Figure 5. The chromatogram of clove oil was shown in Figure 6 On the basis of the chromatograms obtained, characteristic retention times were determined for the drug as the basis for qualitative identification. From the chromatogram of both the extract, it is clear that aqueous extract does not contain eugenol.

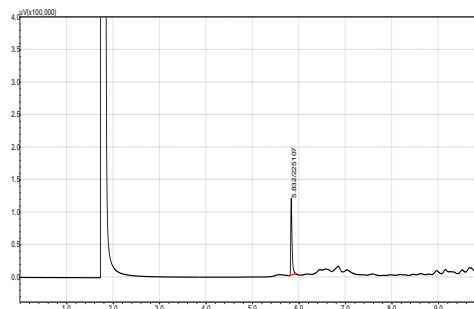


Fig. 2: Chromatogram obtained from pure eugenol solution

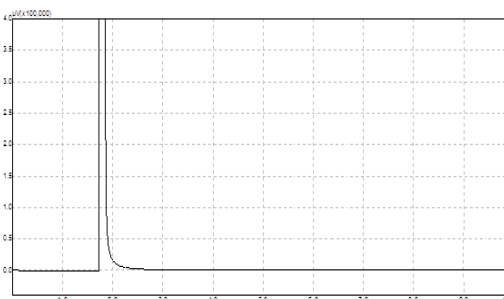


Fig. 3: Blank chromatogram

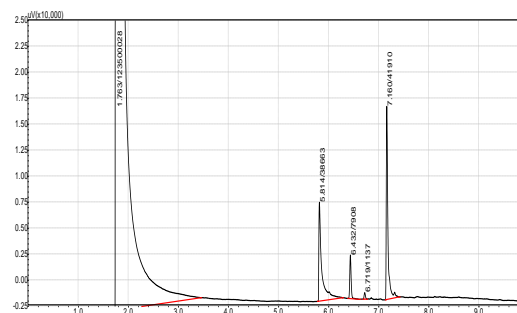


Fig. 4: Chromatogram obtained from ethanolic extract of clove

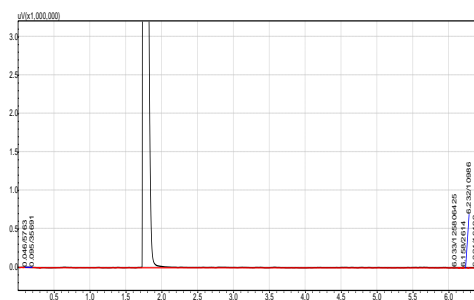


Fig. 5: Chromatogram showing aqueous extract of clove

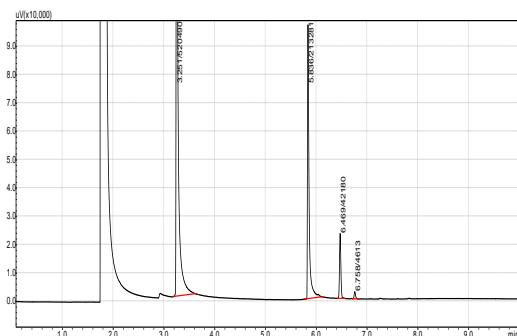


Fig. 6: Chromatogram showing the clove oil

CONCLUSION

The developed method was validated as per ICH guidelines and was found to be within the prescribed limit. It concludes that the developed method is simple, accurate, sensitive and precise. The method is especially suitable for the high throughput analysis of botanical samples and herbal formulations containing eugenol.

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