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Method development and validation of simultaneous determination of Eugenol, Thymoquinone and β-Careophyllene in mixed spice samples

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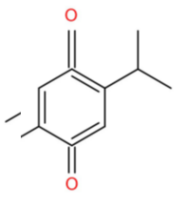
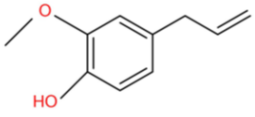
Abstract
A rapid and simple Gas Chromatographic (GC) method has been developed for the simultaneous quantification of eugenol, thymoquinone and β-careophyllene in individual and mixed spice samples. Analysis was performed using capillary column (30 m x 0.25 mm x 0.25 µm) by using Flame ionization detector. Mixed spices were extracted by methanol. The calibration plot was linear over the range studied (2-500 ng for each eugenol, thymoquinone and β-careophyllene standards) with a correlation of 0.99 for eugenol, thymoquinone and β-careophyllene. The method was also validated for the linearity, range, precision, recovery and detection limits. Thus, the method is suitable for routine analysis of eugenol, thymoquinone and β-careophyllene in mixed spices.

Keywords: Eugenol, thymoquinone, β-Careophyllene, GC, validation

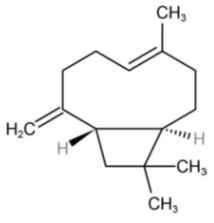
Introduction
Herbs and spices have been used from ancient times for adding flavour, aroma and color to the food and hold a unique role for culinary art [1]. But, the use of spices is not confined to food alone; they are known to have antioxidant, antimicrobial, anti-inflammatory, antidiabetic and antihyperlipidaemic activities and thus used widely in alternative system of medicines such as Ayurvedic, Siddha and Unani systems of medicine [2].
Eugenol (4-allyl-2-methoxyphenol) is the main component of clove and known to be a potent antioxidant, antimicrobial and anti-inflammatory agent. Eugenol, a yellow volatile phenolic compound, from the class of phenylpropanoides is responsible for clove aroma and medicinal properties [3].
The sesquiterpene, β-caryophyllene is present in many spices such as cinnamon, oregano and black pepper. It is known to be a ligand of cannabinoid receptor 2 (CB2), the activation of which decreases pain and promotes faster wound healing [4].
Thymoquinone, a monoterpene, is found in abundance in the seeds of black cumin. It is known to have antioxidant and anti-inflammatory activities. It is also believed to have anticancer activity as well [5].
The aim of the present work is to develop a rapid and simple Gas Chromatographic (GC) method for the simultaneous quantification of eugenol, thymoquinone and β-careophyllene in mixed spice samples. The present work shall be of paramount interest in the field of standardization of spices to ensure the quality and efficacy.



Thymoquinone



Eugenol



β-caryophyllene

Materials & methods

Chemicals and reagents

All the solvents were of GR grade purchased from MERCK India. The standards eugenol, thymoquinone and β-careophyllene were purchased from Sigma Aldrich (India).

Instrumentation and Analytical Conditions: A gas liquid chromatography with flame ionization detector (Thermo Fischer, 1331) was used. The computer with Chromeleon software has been used to control the gas chromatograph and DB5 column (30 m, 0.25 mm ID, 0.25 µm) was used throughout the study. An auto injector was employed for sample injections. All system parameters for are as follows:
Injection Split (20:1), 1 µL
Injector Temperature: 250 °C
Carrier Gas Flow: 40 mL/min
Hydrogen Gas Flow: 30 mL/min
Dry Air Flow: 300 mL/min
FID Temperature: 220 °C
Oven Temperature Programme

Total time (minute)	Rise of °C / minute	Reach value °C	Hold time (minute)
2.5	0	100	2:5
10:00	20	250	0:0
20:00	10	300	5:00

Preparation of standard stock solution

The stock solutions containing 500µg/ml of eugenol, thymoquinone and β-careophyllene were prepared in methanol. Aliquots of eugenol, thymoquinone and β-careophyllene (2.0-500.0 ng) were prepared by serial dilution by methanol.

Preparation of sample solution

The mixed spice samples were extracted by methanol. Briefly, 1 g of each powdered samples were weighed and 80 ml methanol was added and the solutions were sonicated for 30 minutes. After sonication, the solutions were filtered through Whatmann no 1 filter paper in 100 ml volumetric flask. The volume was made by methanol.

Method Validation

Linearity

The calibration curve was linear over the concentration range of 2.0 to 500ng for eugenol, thymoquinone and β-careophyllene.

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of eugenol, thymoquinone and β-careophyllene in sample were confirmed by comparing the retention time.

Precision

Three replicated injections at seven different concentration of eugenol, thymoquinone and β-careophyllene (2.0, 10.0, 20.0, 50.0, 100.0, 200.0 and 500.0 ng) were made and analyzed to examine the precision of the method.

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at three levels by adding a known quantity of eugenol, thymoquinone and β-careophyllene to the pre analyzed samples and the mixtures were analyzed according to the proposed method.

Sensitivity

The sensitivity of measurement of eugenol, thymoquinone and β-careophyllene by the use of proposed method were estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). 5 replicates at lowest level was carried out and from the standard deviation (SD) Limit of Detection was calculated as 3 x SD and Limit of quantitation was calculated as 10 x SD. The limit of detection is the smallest concentration of the analyte that gives a measurable response. The limit of quantitation is the smallest concentration of the analyte which gives a response that can be accurately quantified.

Results and discussion

Method Development

The GC condition was developed and optimized using trial and error method. Various flow rates and oven temperatures were tried to get resolution of eugenol, thymoquinone and β-careophyllene. The optimized GC method could resolve eugenol, thymoquinone and β-careophyllene apart from each other and the peaks obtained were compact too. The optimized chromatographic condition yielded a symmetrical peak for eugenol, thymoquinone and β-careophyllene with Retention Time (RT) 7.19 minutes (for eugenol), 6.34 minutes (for thymoquinone) and 7.78 minutes (for β-careophyllene). The GC chromatogram of eugenol, thymoquinone and β-careophyllene is shown in Figure 1.
The developed method was then validated and successfully applied for quantitation of eugenol, thymoquinone and β-careophyllene from the samples. Regression analysis data is shown in Table 1.
The calibration curve of eugenol, thymoquinone and β-careophyllene were linear in the range of 2.0 to 500.0 ng. Precision, expressed in terms of % RSD, analyzing the substances at five different concentrations, in triplicate, summarized in Table 2.
The specificity of the method was assessed evaluating retention times of triplicated injections of five different concentrations of eugenol, thymoquinone and β-careophyllene as standards and in sample. The results were summarized in Table 3, 4 and 5.
To ensure the accuracy of the method, recovery studies were performed by standard addition method at three different levels, to the pre-analyzed samples and the subsequent solutions were re-analyzed. At each level, three determinations were performed and the results obtained are shown in Table 6.
The sensitivity of measurement of eugenol, thymoquinone and β-careophyllene by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were given in Table 7.
The method was applied to the mixed spice samples. When the formulation was analyzed in GC, eugenol, thymoquinone and β-careophyllene gave sharp and well defined peaks at specific RT. The chromatograms are shown in Figure 2, 3 and 4.
The estimation of eugenol, thymoquinone and β-careophyllene were carried out in three marketed mixed spice samples. The results were incorporated in Table 9, Figure 5.

Conclusion

In this proposed method the linearity was observed in the concentration range of 2.0-500.0 ng for eugenol, thymoquinone and β-careophyllene with co-efficient of correlation, r^2 = 0.992, 0.997 and 0.996 for eugenol, thymoquinone and β-careophyllene respectively. The result of the analysis of mixed spice samples by the method was found to be highly reproducible and reliable. The matrix and other ingredients present in the samples did not interfere with determination of eugenol, thymoquinone and β-careophyllene. So, the developed GC method is simple, precise and accurate and can be used for simultaneous determination of eugenol, thymoquinone and β-careophyllene in mixed spice samples.

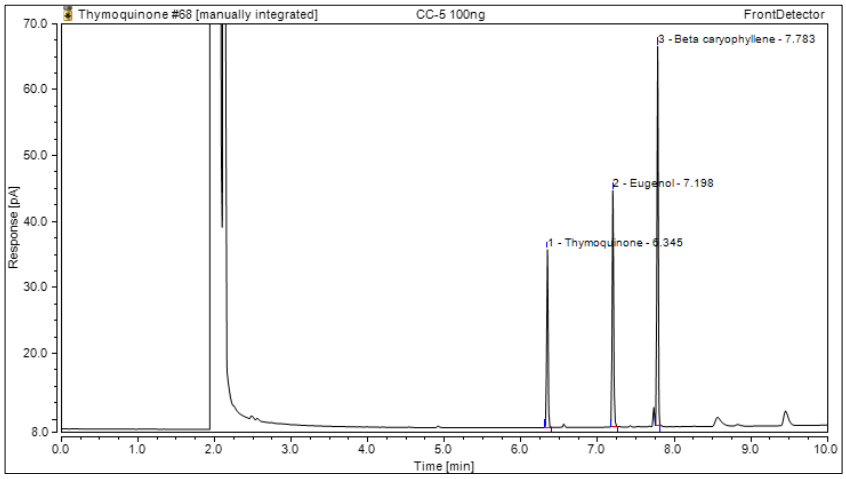


Fig 1: A Typical Chromatogram of eugenol, thymoquinone and β-careophyllene

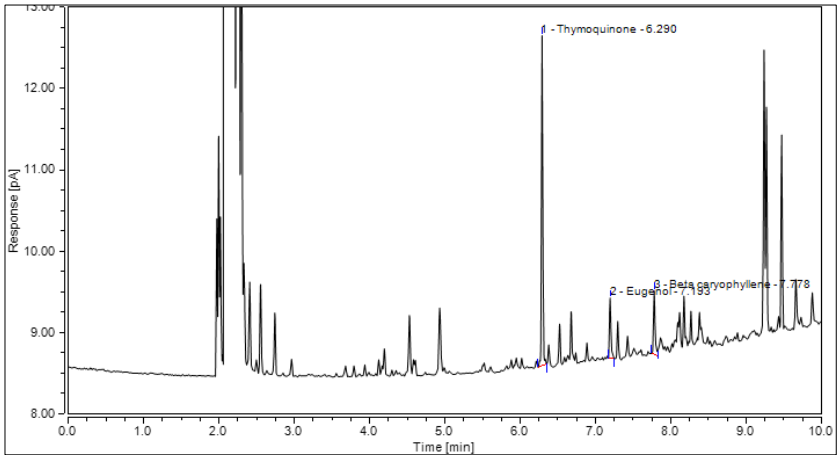


Fig 2: A Typical Chromatogram of Mixed Spice sample (Sample A)

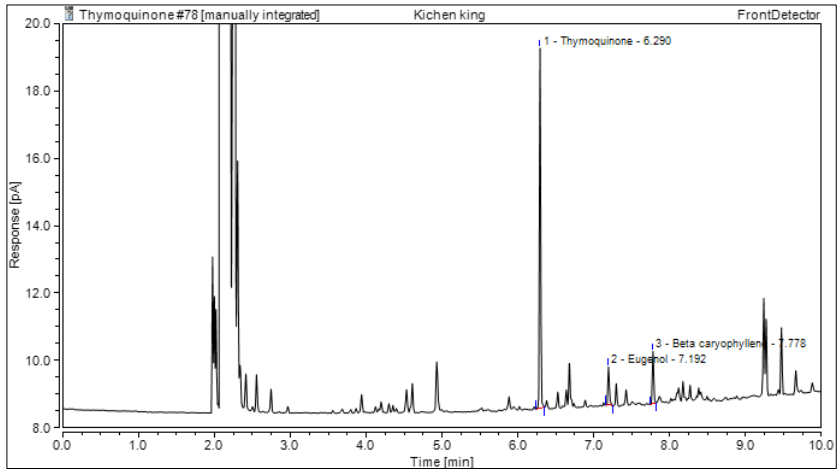


Fig 3: A Typical Chromatogram of Mixed Spice sample (Sample B)

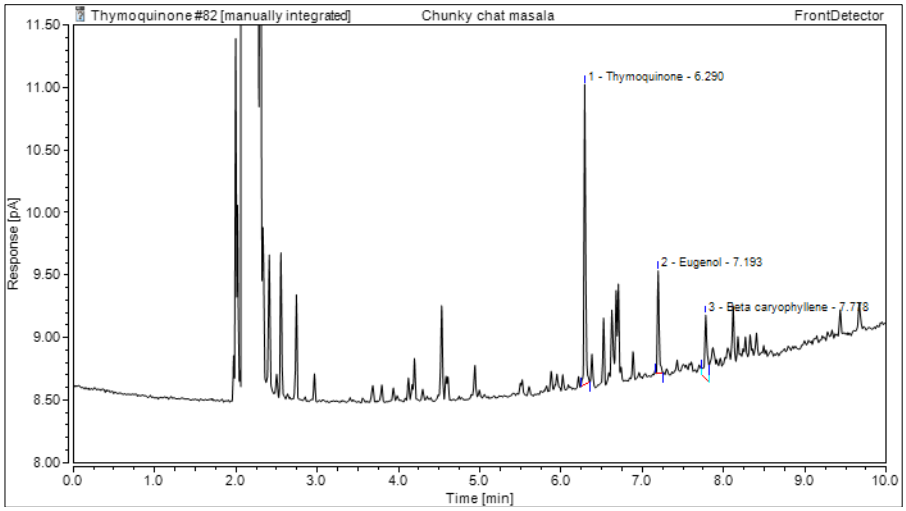


Fig 4: A Typical Chromatogram of Mixed Spice sample (Sample C)

Table 1: Statistical Data of eugenol, thymoquinone and β-careophyllene

Parameters	Results		
	Eugenol	Thymoquinone	β-careophyllene
Linearity range	2.0-500.0 ng	2.0-500.0 ng	2.0-500.0 ng
Slope	0.005	0.004	0.009
SD of slope	0.0001	0.0001	0.006
Intercept	0.056	0.019	0.037
SD of intercept	0.034	0.014	0.041
Regression Equation	y = 0.005x+0.056	y = 0.004x+0.019	y = 0.009x+0.037
Correlation Coefficient	0.992	0.997	0.996

Table 2: Precision Data for Eugenol, Thymoquinone and β-careophyllene

Eugenol			Thymoquinone			β-careophyllene		
Content in ng	Mean peak area (n=3)	SD	Content in ng	Mean peak area (n=3)	SD	Content in ng	Mean peak area (n=3)	SD
2.0	0.016	0.001	2.0	0.011	0.000	2.0	0.024	0.001
10.0	0.062	0.004	10.0	0.050	0.001	10.0	0.094	0.004
20.0	0.130	0.001	20.0	0.090	0.0004	20.0	0.196	0.001
50.0	0.285	0.030	50.0	0.220	0.008	50.0	0.393	0.111
100.0	0.740	0.100	100.0	0.550	0.050	100.0	1.098	0.104
200.0	1.270	0.170	200.0	1.000	0.086	200.0	1.975	0.158
500.0	2.730	0.180	500.0	2.310	0.100	500.0	4.550	0.240

Table 3: Specificity data of Eugenol

Sl No	Eugenol (Content in ng)						
	2.0	10.0	20.0	50.0	100.0	200.0	500.0
1	7.198	7.205	7.197	7.195	7.197	7.195	7.197
2	7.197	7.198	7.195	7.195	7.198	7.197	7.197
3	7.198	7.198	7.197	7.197	7.198	7.197	7.197
Mean	7.198	7.200	7.196	7.196	7.198	7.196	7.197
SD	0.001	0.004	0.001	0.001	0.001	0.001	0
%RSD	0.008	0.056	0.016	0.016	0.008	0.016	0

Table 4: Specificity data of Thymoquinone

Sl No	Thymoquinone (Content in ng)						
	2.0	10.0	20.0	50.0	100.0	200.0	500.0
1	6.342	6.352	6.340	6.338	6.340	6.340	6.340
2	6.340	6.342	6.338	6.338	6.345	6.341	6.340
3	6.342	6.342	6.340	6.342	6.343	6.340	6.340
Mean	6.340	6.350	6.340	6.340	6.340	6.340	6.340
SD	0.001	0.006	0.001	0.002	0.003	0.001	0
%RSD	0.020	0.091	0.020	0.036	0.040	0.018	0

Table 5: Specificity data of β-careophyllene

Sl No	β-careophyllene (Content in ng)						
	2.0	10.0	20.0	50.0	100.0	200.0	500.0
1	7.782	7.790	7.782	7.780	7.782	7.782	7.782
2	7.780	7.783	7.780	7.782	7.783	7.782	7.782
3	7.782	7.783	7.782	7.782	7.783	7.782	7.782
Mean	7.781	7.785	7.781	7.781	7.783	7.782	7.782
SD	0.001	0.004	0.001	0.001	0.001	0	0
%RSD	0.015	0.052	0.015	0.015	0.007	0	0

Table 6: Specificity data of eugenol, thymoquinone and β-careophyllene in Sample

Sl No	Mixed spice sample		
	Eugenol	Thymoquinone	β-careophyllene
1	7.203	6.303	7.788
2	7.193	6.292	7.778
3	7.192	6.290	7.778
Mean	7.196	6.295	7.781
SD	0.006	0.007	0.006
%RSD	0.085	0.111	0.074

Table 7: Recovery data for determination of Eugenol, Thymoquinone and β-careophyllene

Eugenol			Thymoquinone			β-careophyllene		
Amount present in sample (ng±RSD)	Amount added (ng)	% Recovery± RSD	Amount present in sample (ng±RSD)	Amount added (ng)	% Recovery± RSD	Amount present in sample (ng±RSD)	Amount added (ng)	% Recovery± RSD
2.48	4.64	93.77±2.15	6.72	12.10	96.82±1.34	2.21	3.98	98.96±1.39
	4.96	96.03±2.26		13.44	101.11±0.75		4.42	100.35±0.60
	5.46	95.42±2.63		14.78	104.43±1.10		4.86	101.29±0.29

Table 8: Results for Sensitivity data for determination of Eugenol, Thymoquinone and β-careophyllene

Parameters	Results		
	Eugenol	Thymoquinone	β-careophyllene
LOD (ng)	0.002	0.003	0.003
LOQ (ng)	0.006	0.010	0.010

Table 9: Results of Eugenol, Thymoquinone and β-careophyllene in Mixed Spice samples

Parameters	Results		
	Eugenol	Thymoquinone	β-careophyllene
Mixed Spice sample (Sample A)	0.034	0.41	0.036
Mixed Spice sample (Sample B)	0.018	0.082	0.019
Mixed Spice sample (Sample C)	0.019	0.12	0.020

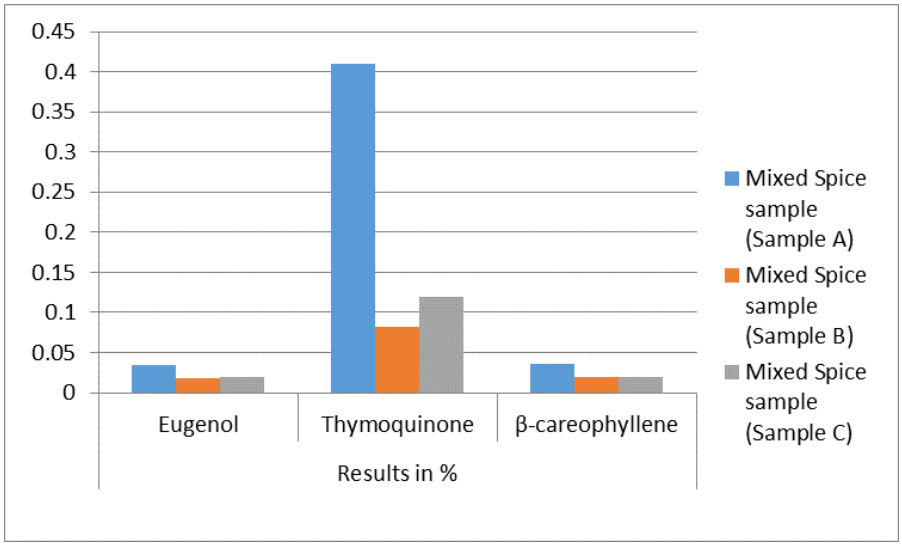


Fig 5: Results of Eugenol, Thymoquinone and β-careophyllene in Mixed Spice samples

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