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RESEARCH ARTICLE

GC-FID METHOD DEVELOPMENT AND METHOD VALIDATION OF EUGENOL IN CLOVE OIL EXTRACTED BY HYDRODISTILLATION

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Abstract

Eugenol is the essential component of many spice oils such as clove, cinnamon leaf, basil, nutmeg, etc. The determination of the authenticity of the essential oil is pivotal for estimating the purity of any essential oil. Hence, there is a high time to identify the marker compounds present in a particular essential oil. From ancient times, clove oil is known for many physiological properties. It is also widely used in dentistry and has potent antioxidant property. In this study, eugenol content in clove oil was evaluated by using GC FID. The ICH guideline was used to determine the suitability of the analytical method developed and validated.

Introduction:-

Cloves(Syzygiumaromaticum) are aromatic dried flower buds of a tree belonging to family Myrtaceae (M. Hakki Alma et al, 2007). Clove tree is native to the small islands of Maluku in Eastern Indonesia, which is also known as the "Spice Islands" (IwanSafrudin et al, 2015). Clove have been used as a culinary spice in India from ancient times. The dried flower buds are most commonly used as a spice because its fine aromatic flavor blends well with sweet and savory dishes (T. Thangaselvabai et al, 2010). Clove oil is extracted from the flower buds, stems and leaves of the clove tree (Syzygiumaromaticum or Eugenia aromatica or Eugenia caryophyllata). Clove oil has been listed as a "Generally Regarded as Safe" substance by the United States Food and Drug Administration when administered not more than 1500ppm (İlhamiGülçin et al, 2010). Recently many researchers confirm the antibacterial, antifungal, antiviral and anticarcinogenic properties of clove oil (Esha Tambe and SulekhaGotmare, 2020); clove oil, in particular, has attracted the attention due to the potent antioxidant, anti-inflammatory, cytotoxic, insect repellent, anaestheticand antimicrobial activities (Diego Francisco Cortés-Rojas et al, 2014, Kamel Chaieb et al, 2007). Eugenol is the primary or main component of clove oil (B. Pavithra, 2014; Kamel Chaieb et al, 2007; Esha Tambe and SulekhaGotmare, 2020). Eugenol (4-allyl-2-methoxyphenol) is an aromatic compound of clove, cinnamon and other spices (B. Pavithra, 2014).

While developing an analytical method by using GC, there are several steps which needs to be taken in consideration such as column selection (chemistry and dimensions of stationary phase: column id, length and film thickness), carrier gas selection, flow rate of carrier gas, temperature programming (initial temperature, ramp rate, final temperature and hold times), injector temperature and detector temperature (Santosh Kumar Bhardwaj et al, 2016). The steps involved in the analytical method development is more clearly explained in the figure 1.

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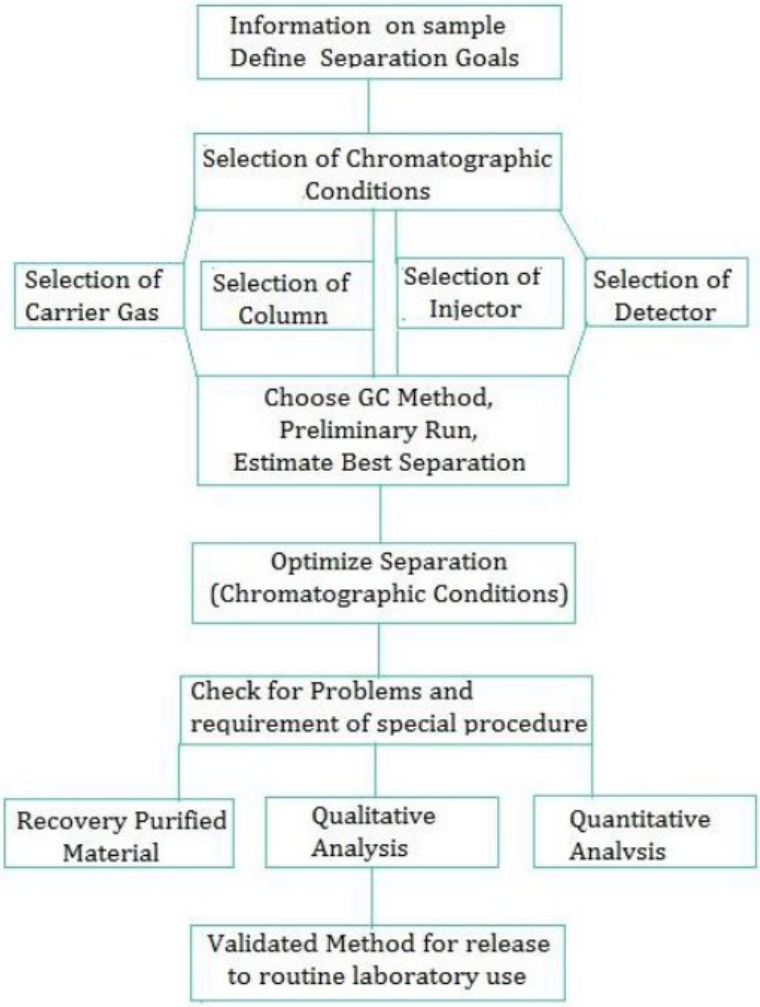

Figure 1:- Steps involved while performing method development using GC. Source: - Santosh Kumar Bhardwaj et al, 2016.

The process of analytical method validation is adopted to assure that the employed analytical procedure for specific tests meets the intended requirements. Certain guidelines such as USP, ICH, FDA, etc. provide the necessary framework to perform validations (Panchumarthy Ravisankar et al, 2015).

Clove essential oil is a complex matrix that need to be analyzed by different techniques to ensure quality, consumer safety and fair trade (ThiKieuTiên Do et al, 2015). The literature survey provides evidence that there are various analytical methods for the determination of eugenol by UV-Visible spectrophotometer, high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) using various detectors (B. Y. K. Sruthi et al, 2014; Sagar Saran et al 2013; Mohamad Taleuzzamana et al, 2017). Amongst these methods, gas chromatography coupled with mass spectrometer is mostly used for separation and identification of volatile compounds present in essential oils. In this study, an analytical method was developed for determining the eugenol content in clove oil by using GC FID instrument and the method developed was a simple, sensitive, accurate, precise and fast method with no sample preparation step required. The method developed was validated by using International Council for Harmonization (ICH) guidelines.

Materials and Methods:-

Sample collection and authentication of sample

The dried clove buds were purchased from D Mart, Thane, Maharashtra, India. Clove bud samples were sent to Agharkar Research Institute, Pune for authentication purpose. The authentication was done to ensure the exact

spices and botanical name of the clove buds. The authentication report stated that the clove buds belonged to Myrtaceae family and the botanical name was Syzygiumaromaticum (L.) Merr. & L. M. Perry.

Reagents, standards and solvents

The whole clove oil extracted by hydrodistillation was used as sample. Standards used for the study were eugenol from Yasho Industries with 99.64% purity, eugenyl acetate from Yasho Industries with 99.42% purity and β-caryophyllene from Aramacs Essential oil & Extracts with 98% purity. Methanol from S D Fine Chemicals with HPLC Grade was used as a diluent or solvent of clove oil sample and standards.

Extraction method

The whole and dried clove buds were subjected to hydrodistillation for extracting the clove oil from it. The extraction process was performed by using conventional distillation apparatus. In this study, for the extraction procedure, 125g of whole and dried clove buds was added to a1000ml round bottom flask and to this 500ml of distilled water was added. The flask was electrically heated by placing it in a heating mantle. Initially, the flask was heated to a temperature of about 80°C and then gradually increased to 100°C. The extraction continued at this temperature till no more drops of oil was coming out of the condenser.

Optimization of GC FID method

For chromatographic separation, sample preparation is a crucial step because if this step is not performed properly then the system will be unable to detect the analyte. Moreover, the sample should be clear and must not contain any fine solid or precipitated particles as it may clog the column. In case of essential oils, the oils may be either diluted with a suitable solvent or injected onto the column in neat condition. In this study, the clove oil was diluted in methanol and then it was injected on the capillary column. Though the sample was diluted with methanol, split mode of injection was employed to avoid overloading of sample onto the column, poor peak shape, for example, tailing or fronting, and poor resolution. The literature survey and GC column's manufacturer recommendations suggested to use capillary column for better resolution and peak shape. Moreover, the stationary phase consisting of 5% - diphenyl and 95% - dimethylpolysiloxane is more preferred for separating molecules of clove oil. The dimensions also play a critical role in separation and the use of 25 – 50m long column, inner diameter of about 0.2 – 0.3mm and film thickness of 0.25µm are almost industry standard column conditions. Hence, in this study the column considered for method validation was RTx-5ms 30m x 0.25mm ID x 0.25µm with stationary phase composed of 5% - diphenyl and 95% - dimethylpolysiloxane. The detector used was flame ionization detector as the clove oil consists of molecules with carbon, hydrogen and oxygen atoms. Initially, the clove oil was subjected to an isothermal column temperature of about 250°C, but this resulted into a poorly resolved chromatogram which had overlapped and broadened peaks. Hence, gradient temperature was considered in further method development stages. Furthermore, a gradient oven temperature,with 100:1 split injection mode for 1µl of sample injection, analysis was conducted and the chromatogram of this 43 minutes run time analysis showed 5 resolved peaks. The first peak showed tailing which might be because of high concentration of the analyte due to high injection volume. In addition to this, no peaks were observed after 22 minutes. By this time, it was clear that injection volume needs to be reduced and the gradient oven temperature was further improved to decrease the analysis time from 43 minutes to 30 minutes.

GC FID final method

The quantification of eugenol content in clove oil was carried out on Shimadzu GC FID (GC 2014). For the method validation the GC column used was RTx-5ms 30m x 0.25mm ID x 0.25µm with the temperature range of -60°C to 350°C. This is a low bleed column. Nitrogen was used as the carrier gas and the flow rate was 1 ml/min. The injection volume of clove oil samples and standards namely, eugenol, eugenyl acetate and β-caryophyllene was 0.2µl with the split ratio of 100:1. The injector was subjected to an isothermal temperature of 220°C while the detector was maintained at the temperature of 250°C. For better resolution, gradient oven temperature programming was selected with the initial column temperature of 70°C with 2 minutes hold, followed by increase in temperature till 270°C with the ramp rate of 10°C/min and 0 min hold. The total run time was 30 minutes. For the detector, the flow rate of air was maintained at 400ml/min and hydrogen was maintained at 40ml/min.

Results And Discussions:-

In this study, the analytical method validation was performed by using the guidelines stated in ICH Harmonised Tripartite Guideline, 2005. As per the ICH Harmonised Tripartite Guideline, there are different characteristics for impurity and assay testing and for the determination of eugenol content in clove oil, the characteristics applicable to

assay testing were employed in the validation. The figure 2 illustrates the various characteristics depicted in ICH guideline for testing of impurities and assay.

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitat.	 limit	ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

Figure 2:- Different parameters or characteristics for method validation laid down by ICH- signifies that this characteristic is not applicable and + signifies that this characteristicis normally evaluated.

(1) denotes that in cases where reproducibility has been performed, intermediateprecision is not needed, (2) denotes that the lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s) and (3) denotes that the detection limit may be needed in some cases

According to the figure 2, limit of detection and limit of quantification is not required for assay analysis. The method for determination of eugenol in clove oil was validated using the characteristic such as accuracy, precision, repeatability, intermediate precision, specificity, linearity, robustness and range. As this is a chromatographic method, system suitability was performed. The acceptance limit for %RSD (relative standard deviation) or %CV (coefficient of variation) is that the RSD should not be more than 15% and recovery should be within the range of 90 – 110%.

The various parameters of method validation evaluated are described as follows: -

1)System suitability:-

Prior to the validation experiments, it is paramount to establish that the chromatographic systemand the procedure is capable to provide data of acceptable quality. Hence, the tests which verifies the resolution and repeatability of the analytical method needs to be performed. System suitability tests are based on the concept which helps to evaluate the equipment, electronics, analytical operations and samples. System suitability is, basically, to check and ensure that the chromatographic system is performing properly before or during the analysis (Ghulam A. Shabir, 2003). In this study, to evaluate the performance of gas chromatographic system, the system suitability was conducted by injecting six replicates of 10% Eugenol standard solution onto the column. The solution was prepared in methanol. The acceptance criterion for system suitability to pass is that the %RSD should not be more than 2%. The below table 1 illustrates the results of system suitability.

Sr. no.	Retention time of eugenol	Peak area of eugenol standard
1	11.641	1779067

2	11.641	1808769
3	11.642	1831536
4	11.641	1875326
5	11.640	1827183
6	11.641	1853339
Average	**11.641**	**1829203**
Std dev	**0.000632**	**33617.16**
%RSD/%CV	**0.00543**	**1.84**

Table 1:- System suitability result summary.

The RSD of system suitability tests is less than 2% and hence, the system is suitable to perform the method validation.

2) Specificity:-

Specificity of a chromatographic method means the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in the test mixture containing the analyte and all potential sample components is compared with the response of a solution containing only the analyte (Ghulam A. Shabir, 2003). The specificity tests, in this study, was performed by injecting 0.2µl of blank (placebo), 10% eugenol standard solution, 10% □-caryophyllene standard solution, 10% eugenyl acetate standard solution and 10% clove oil sample solution. From the chromatograms, the retention times of the three standards and components present in clove oil were recorded in the below table 2 and 3. No interferences were observed in blank, standards and clove sample. Hence, the method is specific.

Sr. no.	Name of the compounds present in clove oil	Retention time
1	Eugenol	11.672
2	□-caryophyllene	12.633
3	Eugenyl acetate	13.918

Table 2:- Retention time of the compounds present in the clove oil.

Sr. no.	Name of the standards	Retention time
1	Eugenol	11.654
2	□-caryophyllene	12.664
3	Eugenyl acetate	13.966

Table 3:- Retention times of the standards run individually.

3) Linearity and range:-

To execute linearity, ICH guidelinesrecommended to evaluate a minimum of five concentration points to establish linearity and wider range of concentrations, while other approach to linearity can also be adopted with a justifiable reason. The linearity standard solution may be prepared by serial dilutions of a single stock standard solution and the response of the analyte is plotted as a linear graph against the theoretical concentration. The statistical parameters required for the linearity are Y-intercept, slope of the regression line and residual sum of square (Ghulam A. Shabir, 2003; G. Geetha et al, 2012; Shashi Daksh et al, 2015). The linear relationship is established by determining the response of standard solutions in the range of 50 to 150% (S. Chandran and R.S.P. Singh, 2007).

Practically, the range of an analytical method is determined from thedata of linearity and accuracy studies. The range is the interval between the upper and lower levels (S. Chandran and R.S.P. Singh, 2007). In this study, linear was established by plotting the graph of the response of five different concentrations of eugenol standard against the theoretical concentration. For a linear acceptable graph, R^2 should be more than 0.995. The table 4 provides complete linearity data and the figure 3 illustrates a good relationship between the eugenol standard responses and theoretical concentrations.

Sr. no.	Linearity levels	Eugenol standard and sample concentration	Area of eugenol standard and sample	Correlation coefficient, y-intercept, slope of regression
1	50%	4.0%	319085	Y = 8E+06x + 8674.8, R^2 = 0.9985
2	80%	6.4%	489674	
3	100%	8.0%	612093	

4	120%	9.6%	724512	
5	150%	12.0%	928140	
6	NA	10% clove oil sample	631544	NA

Table 4:- Consolidated data of linearity, NA – Not applicable.

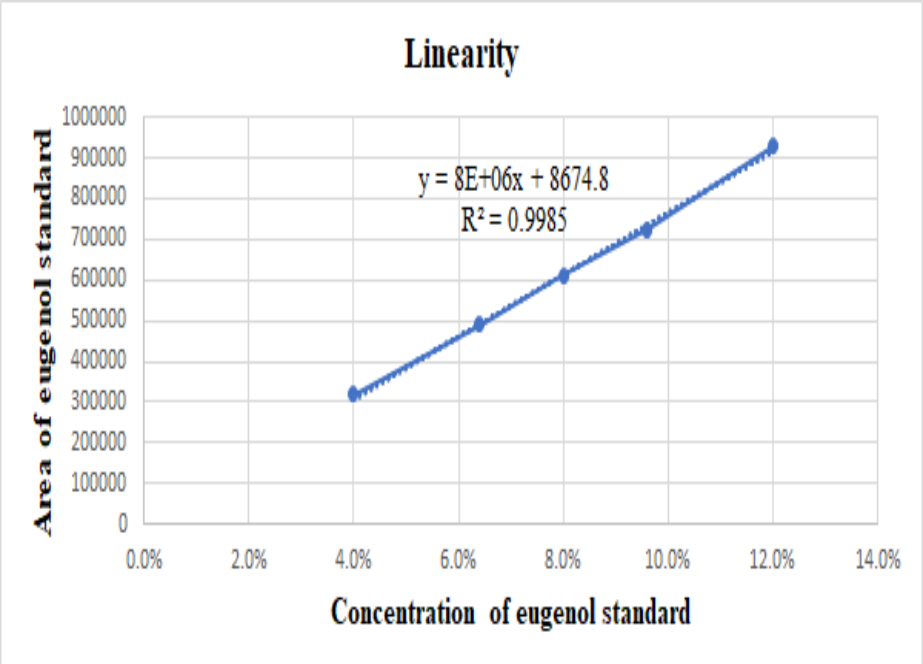

Figure 3:- Calibration curve of eugenol standard.

The analytical method passes the linearity tests as the R^2 value is greater than 0.995.
The eugenol content in 10% clove oil is calculated by using y = mx + c equation. As per this equation, y is area of eugenol, m is the slope of the equation, x is the unknown concentration of eugenol and c is the y-intercept. By substituting the values in this equation and applying the dilution factor, the percent concentration of the eugenol found was 77.86%. This value was considered as the 100% concentration and will be used for calculating the %recovery in accuracy and recovery tests.

4) Accuracy:-
The accuracy of an analytical method is defined as the closeness of the result obtained to the true value (Ghulam A. Shabir, 2003; S. Chandran and R.S.P. Singh, 2007). There are four different approaches to determine the accuracy of analytical method, out of which, a single certified reference material or test material solution were used to compare its results with the true value for estimating the accuracy of the method (S. Chandran and R.S.P. Singh, 2007). In this study, clove oil samples were injected at three different levels of concentration and %recovery was calculated. The three different levels of concentration chosen were 80%, 100% and 120%. For calculating the %recovery, the true value considered was 77.86%. The 80% of clove oil solution was prepared by weighing 0.8023g of clove oil in a 10ml standard volumetric flask and then diluted up to the mark with methanol. Similarly, for 100%, 1.0003g of clove oil and for 120%, 1.2001g of clove oil sample was weighed in 10ml of standard volumetric flask and diluted with methanol to the mark.The %RSD should be less than 15% and %recovery should fall with the range of 90 - 110%, for the accuracy test to pass. The table 5 shows a consolidated result data of accuracy test.

Sr. no.	RT of eugenol in clove oil			Peak area of eugenol in clove oil			Conc. of eugenol in clove oil (%)			%Recovery of eugenol in clove oil		
	80%	100%	120%	80%	100%	120%	80%	100 %	120 %	80%	100%	120%
1	11.668	11.678	11.684	482796	605870	790662	73.87	74.65	81.45	94.86	95.88	104.61
2	11.659	11.665	11.681	509713	639013	748956	78.06	78.79	77.11	100.26	101.19	99.04

3	11.666	11.675	11.677	488534	619808	756845	74.76	76.39	77.93	96.02	98.11	100.09
Avg.	11.664	11.673	11.681	493681	621563.7	765487.7	75.56	76.61	78.83	NA	NA	NA
Std dev	0.0047	0.0068	0.0035	14177.44	16641.11	22155.57	2.21	2.08	2.31	NA		
% RSD	0.0405	0.0583	0.0301	2.87	2.68	2.89	2.92	2.72	2.93	NA		

Table 5:- Consolidated result data of accuracy tests comprising %RSD and %recovery values, NA – Not applicable.

The %RSD for retention time, peak area and concentration of eugenol is less than 15% and recovery of all the nine determinations are within the range of 90 - 110%. Hence, the method passes the accuracy tests.

5) Precision:-

As a part of the method validation, precision is defined as the degree of the closeness or the scatter of individual tests results of multiple measurements. Precision can be categorized in four different types namely, instrument precision or injection repeatability, repeatability or intra-assay precision, intermediate precision and reproducibility (S. Chandran and R.S.P. Singh, 2007). However, the ICH states that precision may considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability may be assessed by using a minimum of nine determinations at three different concentration levels or a minimum of six determinations at 100% of the test concentration. The intermediate precision may be evaluated by including variations in the experiment such as days, analysts, equipment, etc (ICH Q2(R1), 1996). In this study, repeatability was established by injecting eugenol standard and 10% clove oil sample at the 100% concentration level, and intermediate precision was performed by recording response of six measurements of eugenol standard and 10% clove oil sample at the 100% concentration level by two different analysts on different days. For both the precision tests to pass, the %RSD should not be more than 15%. The table 6 and 7 provides an amalgamated view of the result data of repeatability and intermediate precision.

Sr. no.	RT of eugenol in standard	Area of eugenol in standard	RT of eugenol in clove oil	Area of eugenol in clove oil	Conc. of eugenol in clove oil
1	11.659	594087	11.673	609849	75.15
2	11.658	587093	11.674	633334	78.08
3	11.659	586059	11.675	659735	81.38
4	11.660	615543	11.675	676954	83.53
5	11.659	603178	11.677	600864	74.02
6	11.659	631035	11.675	617513	76.10
Average	11.659	602832.5	11.675	633041.5	78.05
Std dev	0.00063	17678.37	0.00133	29850.81	3.73
%RSD	0.00542	2.93	0.0114	4.72	4.78

Table 6:- Consolidated result data of repeatability.

Sr. no.	RT of eugenol in standard	Area of eugenol in standard	RT of eugenol in clove oil	Area of eugenol in clove oil	Conc. of eugenol in clove oil
1	11.656	576827	11.677	626731	77.26
2	11.659	601244	11.676	637020	78.54
3	11.659	602548	11.677	648865	80.02
4	11.660	610845	11.677	576447	70.97
5	11.659	606753	11.678	598235	73.70
6	11.644	600299	11.678	606049	74.67
Average	11.656	599752.7	11.677	615557.8	75.86
Std dev	0.00611	11899.3	0.00075	26888.61	3.36
%RSD	0.0524	1.98	0.0064	4.37	4.43

Table 7:-Consolidated result data of intermediate precision.

As per the table 6 and 7, the %RSD for eugenol and standard and clove oil sample is less than 15% and hence, repeatability and intermediate precision tests is passed.

6) Robustness (Ruggedness):-

Robustness of an analytical method determines whether the method remains unaffected by some small changes in the experimental parameters which are carried out deliberately. These change in method parameters may be determined, in two ways, either by one factor at a time or simultaneously as part of a factorial experiment (S. Chandran and R.S.P. Singh, 2007). According to ICH guideline, the typical variation in the gas chromatographic method includes variation in temperature, carrier gas flow rate and difference in the columns such as lots or suppliers. In this study, robustness of the method was established by varying the oven temperature and carrier gas flow rate (ICH Q2(R1), 1996). Different lot number or supplier GC capillary column was available and hence, this factor was not evaluated. The acceptable limit of %RSD for robustness is that it should not be more than 15%. To perform robustness, triplicate measurements of clove oil sample at 100% concentration level were determined. The oven temperature was increased by 0.2°C and the flow rate was changed from 1.0ml/min to 1.2ml/min. The table 8 furnishes the result summary of both the factors of robustness.

Sr. no.	RT of eugenol in clove oil (variation in oven temp.)	PA of eugenol in clove oil (variation in oven temp.)	Conc. of eugenol in clove oil (%)(variation in oven temp.)	RT of eugenol in clove oil (variation in oven temp.)	PA of eugenol in clove oil (variation in oven temp.)	Conc. of eugenol in clove oil (%)(variation in oven temp.)
1	11.652	606175	74.69	11.155	579880	71.40
2	11.654	647913	79.90	11.155	585686	72.13
3	11.654	577488	71.10	11.155	593454	73.10
Avg.	11.653	610525.3	75.23	11.155	586340	72.21
Std dev	0.00116	35413.5	4.43	0.00	6810.59	0.85
%RSD	0.0099	5.80	5.88	0.00	1.16	1.18

Table 8:- Robustness data depicting the %RSD values for variation in oven temperature and variation in carrier gas flow rate, RT – Retention time, PA – Peak Area.

The %RSD of both the method parameters (oven temperature and flow rate) is less than 15% and hence, the robustness tests were passed.

The typical chromatogram of the eugenol standard, □-caryophyllene, eugenyl acetate and clove oil sample is shown in the below figure 4, 5, 6 and 7 respectively.

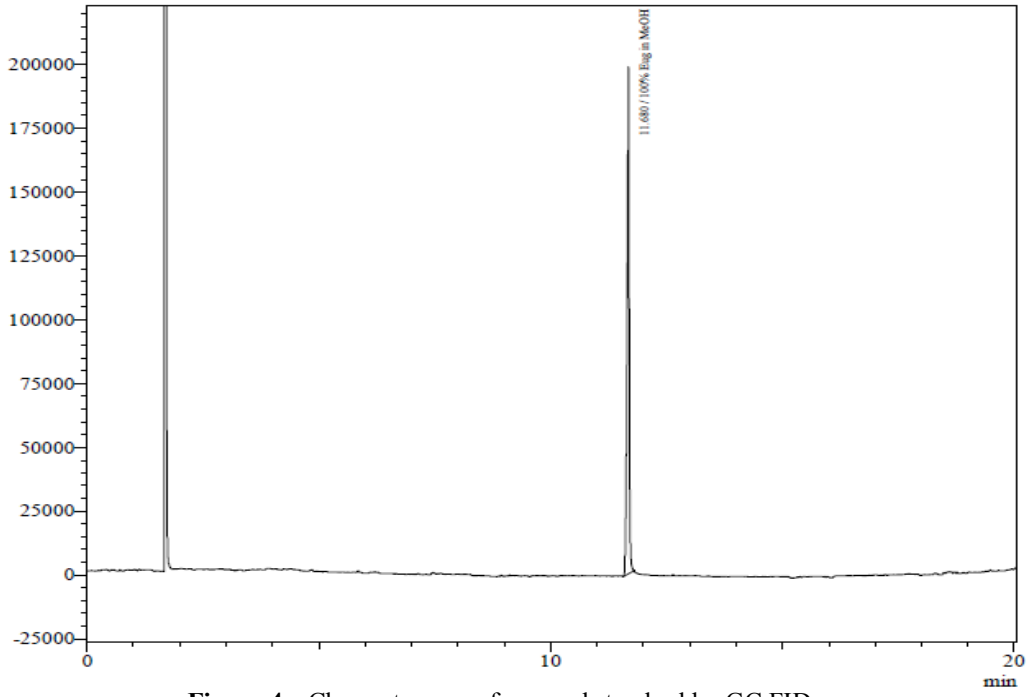

Figure 4:- Chromatogram of eugenol standard by GC FID.

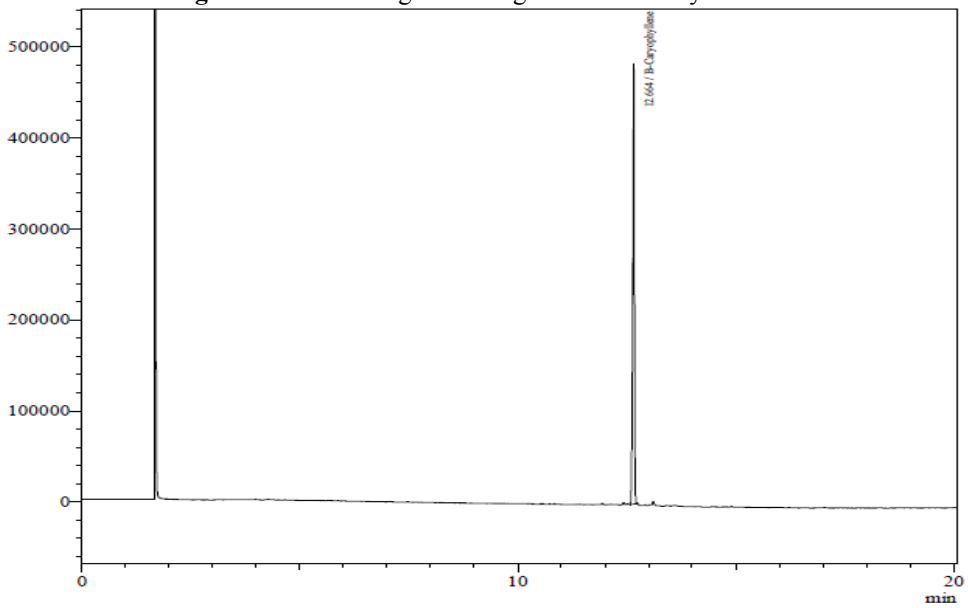

Figure 5:- Chromatogram of □-caryophyllene standard by GC FID.

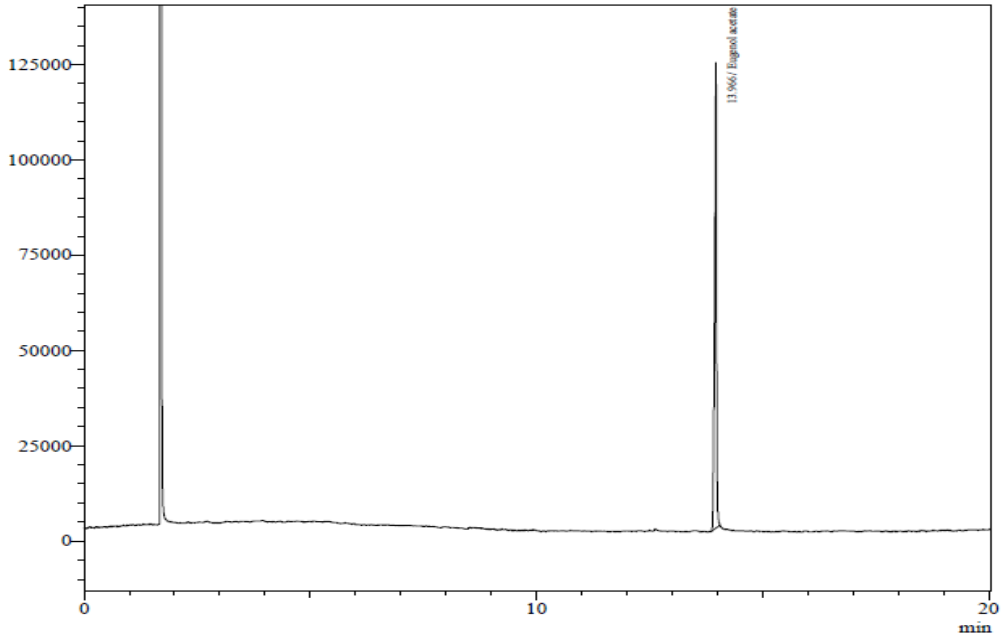

Figure 6:- Chromatogram of eugenyl acetate standard by GC FID.

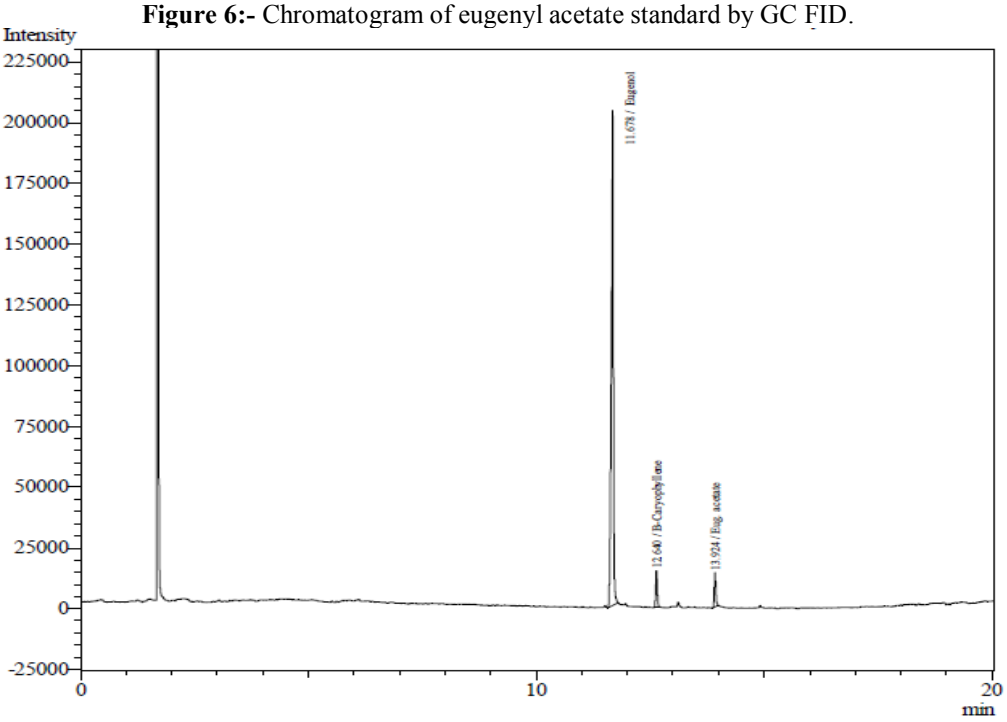

Figure 7:-Chromatogram of clove oil sample by GC FID.

Conclusion:-

In this study, a simple, precise and robust GC method was developed to determine the eugenol content in clove oil. All of the validation parameters such as system suitability, specificity, accuracy, precision- repeatability & intermediate precision, linearity, range and robustness are well within the acceptable criteria and hence, this analytical method successfully passes the ICH method validation. Eugenol is the prime component of clove oil and it is one of the marker compounds as well (Esha Tambe and SulekhaGotmare, 2020). To determine the authenticity of clove oil, it is crucial to estimate the amount of eugenol present in clove oil and this method will, certainly, serve as the rapidquality control method as it does not contain any complex and time-consuming sample preparation steps.

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