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# Analysis and Estimation of Eugenol Content in Microemulsion Formulation Containing Clove Oil (*Syzigium aromaticum*)

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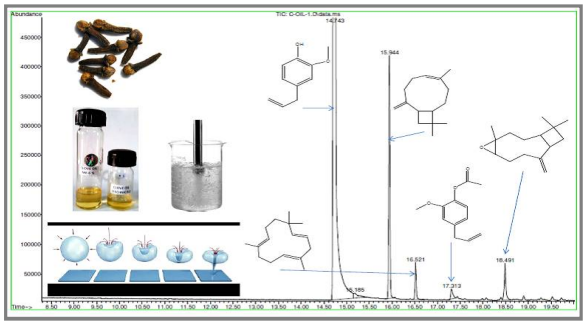
## ABSTRACT

*Oil in water based Microemulsion formulation (ME) containing Clove oil was analyzed and quantitated for its Active Ingredient (A.I.) by Gas chromatography Mass Spectrometry (GC-MS). Clove oil based microemulsion formulation (6%) was developed in-house and tested for its active ingredients. Standard sample preparation methods for testing the Emulsifiable Concentrate (EC) formulations were found ineffective for the estimation of A.I. (i.e. Eugenol) in ME formulation. A sonication based extraction method was developed and optimized to extract and quantify the Eugenol (Clove oil) and its other components from the micro-emulsion (6%, ME). The main five components analyzed in clove oil technical by GC-MS were: Eugenol (92.11%), Eugenol acetate (0.43%), alpha-Caryophyllene (0.90%), Caryophyllene (5.47%) and Caryophyllene oxide(1.09%). Probe based Sonication was found to be the effective approach in breaking the micelle and extracting the A.I. into solution in less time as compare to the bath sonication. Amongst five different extracting solvents (viz. Acetonitrile, Acetone, n-Hexane, Ethyl acetate and Dichloromethane) Ethyl acetate showed better extraction. The same solvent also showed less time for complete A.I. extraction. GC-MS instrument showed linear response to A.I., with $r^2$ = 0.988, measured at 0.1, 0.5, 1.0, 10 and 50 µg/ml. concentrations. Mean recovery of method is more than 95 % with R.S.D below 20%.*

## Graphical Abstract



Extraction of clove oil from its ME formulation and GC-MS, TIC of clove oil technical

**Keywords:** Ultra-Sonication, Microemulsion, Clove-Oil, Gas chromatography mass spectrometry.

## INTRODUCTION

Microemulsion (ME) is a clear, optically isotropic and thermodynamically stable solution of two immiscible liquids, stabilized by amphiphillic surface active agents (surfactants) [**1**]. In comparison to opaque emulsions, ME are transparent due to smaller micellar size, varying from 10 nm to 100 nm [**2**]. They can be classified as, oil in water (O/W), water in oil (W/O) or bicontinuous system wherein, dispersing phase and dispersing media are carefully selected as per requirement. Microemulsions have wide application in drug delivery [**3, 4**], agriculture, food, fuel additives [**5**], cosmetics, paints, coating [**6**], textiles, and analytical science [**7**]. Clove oil from the buds of clove plant (*Syzigium aromaticum*) is well known for its biological activities against microbes like fungi, bacteria [**8, 9**] and also on insects [**10**]. Owing to its biological activity, Clove oil is used in various medicines [**11-12**], food items (as preservative and flavouring agent), anticorrosion coating (Japanese swords/blades), disinfecting/cleansing products [**6**]. A Clove oil based microemulsion formulation (O/W) was developed in-house to employ its insecticidal activity against stored grain pests.

Review of literature revealed that clove oil can be analysed by Thermogravimetry, GC-FID, GC-MS [**6, 13**]. Clove oil from different plant species contains18-38 chemical constituents, belonging to various chemical classes (viz. phenyl propenoid, bicyclic sesquiterpene, monoterpene etc.) have been identified and reported [**6, 8, 14**]. The constituents of clove oil and their relative composition vary from one variety of cloves to other [**15, 16**]. Among various components Eugenol (4-allyl-2-methoxyphenol) is the major and main active component which is responsible for its bioactivity [**17**].

Analysis of Microemulsions for quality assurance is a challenging analytical task as simple method of dilution of formulation sample with a suitable extracting solvent does not give correct and precise results. This paper presents the development of ultra-sonication based extraction method of Eugenol, from Clove oil based ME formulation and its quantification by instrument Gas chromatography Mass Spectroscopy (GC-MS).

## MATERIALS AND METHODS

**Chemicals and Instrumentation:** Microemulsion Sample: Clove oil containing Microemulsion formulation transparent yellow, slight viscous in nature, prepared as in-house shown in figure 1.Clove oil technical, as shown in figure 1, is transparent yellow coloured liquid. Technical purchased was analysed in GC-MS for identification of its composition. HPLC grade solvents: Acetonitrile, Acetone, n-Hexane, Methanol, Ethyl Acetate, (from Thermo Fischer). Glassware: All glass wares sample bottles, pipettes, volumetric flask etc. (Borosil).


**Figure 1.** Vials containing clove oil ME 6% formulation and clove oil technical.

**Equipments:** Two sonicators one bath type and second probe type, Bath type sonicator is of Sonorex Bandelin® Super rk102p, while probe type sonicator is of PCI. Former sonicator delivers constant energy while in later energy can be controlled and modulated by selecting appropriate wavelength, However time adjustment setting for sonication is available in both sonicator types.

Chromatographic Instrument: Agilent Technologies make GC system, 7890A, fitted with Autosampler 7683B series. Instrument equipped with DB-5(5% Phenylated methyl siloxane) fused silica capillary column (J and W Scientific Co., 30 m length × 0.25 mm i.d. × 0.25 µm film thickness). Helium gas was used as carrier gas at constant flow of 1 mL $min^{-1}$. (with gas saver on mode). All injections were done in split less mode both for total identification of components in technical and quantitation of Eugenol from ME formulation. Temperature of Injector and Auxiliary port were set to 250°C and 280°C respectively. Oven was subjected to ramp programming beginning from 170°C, holds for 2 min and then ramped upto 280°C at rate of 10°C $min^{-1}$, a final temperature hold of 4 min was set to remove any high boiler present in technical or sample.

**Mass spectrometry Instrument:** Agilent Technologies make 5975C inert XL EI/CI MS detector with triple axis. A solvent delay time of 2 min opted for solvent phase. Ionization mode was Electron Impact (EI) in positive polarity, with 70 eV energy, mass scan range 29-450 m/z in 0.56s.

**Method:** Standard solution preparation: Clove oil technical of 10 mg ± 1mg was weighed in 100 ml volumetric flask and volume make up by HPLC grade solvent Ethyl acetate. The solution prepared was injected to GC-MS for the compositional analysis. Standard solution prepared was used for preparation of serial dilutions of concentration, 50, 10, 1, 0.5 and 0.1 $\mu$g $mL^{-1}$ to plot the linearity curve.

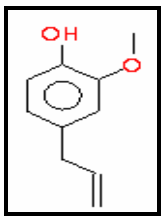
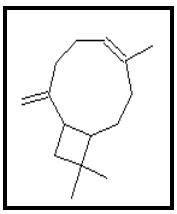
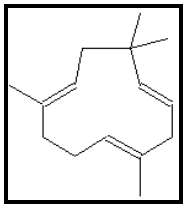
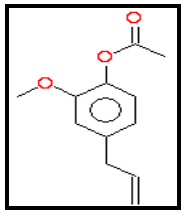
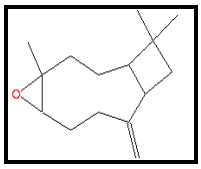
**Sample preparation:** Five solvents viz. n-Hexane, Ethyl acetate, Acetonitrile, Methanol and Acetone were chosen, covering the non-polar, mid-polar and polar phases of solvents to check and select the appropriate solvent for maximum extraction of clove oil (for Eugenol only) from ME formulation. A known amount of ME formulation was added to each solvent of same volume in stoppered 250 mL conical flask. They are put on rotary shaker for 5 min at 70 rpm. After shaking 1 mL supernatant from each solvent was injected to GC-MS and analyzed for the peak under area of each component.

**Sonication:** Simple solvent extraction of ME formulation, for Eugenol content analysis was found ineffective as the technical/active ingredient is encapsulated in micelle and did not gave desired results. In order to break the micelle, sonication was considered as the technique of sample preparation. Two different types of sonication one bath type and second probe type were used and compared for their extraction efficiency. Sample solutions prepared in ethyl acetate were subjected to sonication for different time period (5, 10, 15, 20 and 25 min) at their maximum energy setting. One mL of sample was taken out after each interval and injected to GC-MS instrument.

## RESULTS AND DISCUSSION

**Technical Analysis:** Technical solution prepared was analyzed by GC-MS and identification of each peak was made by comparing spectra with NIST library. Percentage area of each peak is considered as its percentage composition. A total of 5 main components were identified in the technical:- Eugenol (92.11%), Eugenol acetate (0.43%), Caryophyllene (5.47%), alpha Caryophyllene (0.90%)and Caryophyllene oxide (1.09%). Table 1 presents the structure, molecular weight, formula of each compound with their respective CAS No. Each peak was sharp and chromatographically well separated figure 2 presents the Total Ion Chromatograph (TIC) of the Clove oil technical. Percent purity of Eugenol in clove oil obtained as above was in close agreement with the purity declared by supplier and considered as true value to evaluate the recovery experiments.

**Table 1.** Percentage composition of clove oil technical

| GC-MS Analysis of Clove oil Technical | | | | | |
|---|---|---|---|---|---|
| **Peak** | **R.T.** | **Compound** | **Structure** | **Cas No.** | **Relative Percentage Area** |
| 1 | 14.73 | Eugenol ($C_{10}H_{12}O_2$) Mol wt. 164 |  | 97-53-0 | 92.11 |
| 2 | 15.94 | Caryophyllene ($C_{15}H_{24}$) Mol. Wt. 204 |  | 87-44-5 | 5.47 |
| 3 | 16.52 | Alpha Caryophyllene ($C_{15}H_{24}$) Mol wt. 204 |  | 6753-98-6 | 0.90 |
| 4 | 17.31 | Eugenol Acetate ($C_{12}H_{14}O_3$) Mol. Wt. 206 |  | 93-28-7 | 0.43 |
| 5 | 18.49 | Caryophyllene Oxide ($C_{15}H_{24}O$) Mol. Wt. 220 |  | 1139-30-6 | 1.09 |

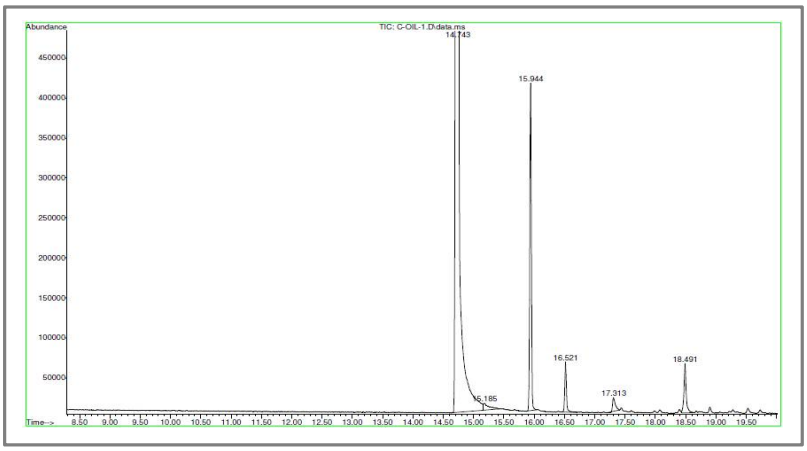
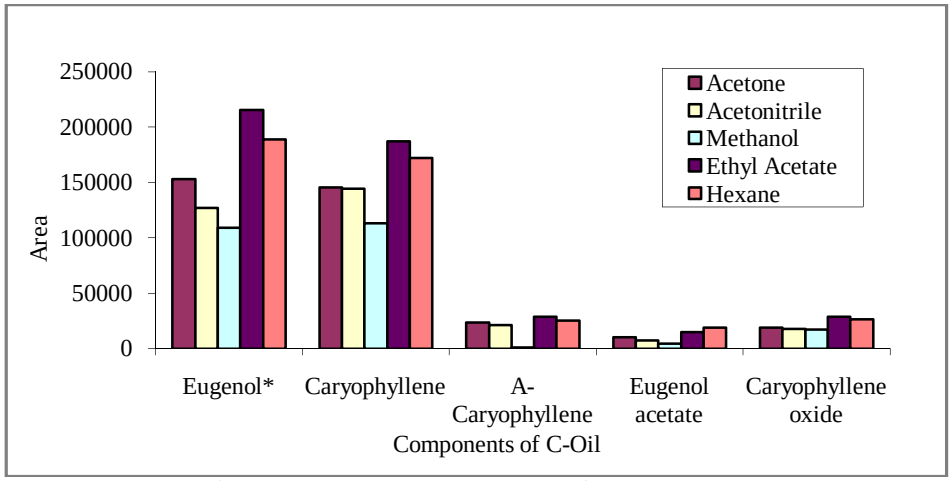



**Figure 2.** Total ion chromatograph (TIC) of clove oil technical by GC-MS instrument.

**Solvent Extraction Efficiency:** In solvent extraction efficiency ethyl acetate was found to be a better solvent for extraction of all components followed by hexane. Methanol and acetonitrile showed the poor extraction ability. Figure 3 shows the comparison of solvents for their extraction ability.




***Area of Eugenol is scaled down by 10 times for comparative analysis

**Figure 3.** Solvent comparison for the extraction of clove oil components from ME formulation.

**Instrument linearity:** Instrument linearity for each component of clove oil was checked by preparing the technical solution of 50, 10, 1, 0.5 and 0.1 $\mu$g mL$^{-1}$ concentrations. Correlation coefficient of all components was found fairly well near to 1 (Figure 4). Table 2 presents the $r^2$ values obtained for each component of technical.

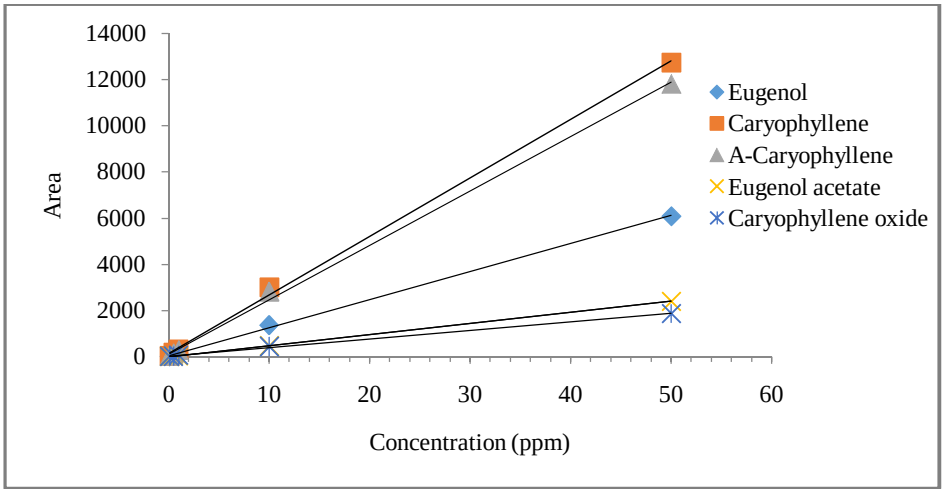



**Figure 4.** Calibration curve (5 points) of components of clove oil technical.

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** LOD values of the all five components was calculated from standard deviation (SD) of the GC response at 0.1 $\mu$g mL$^{-1}$ concentration (lowest concentration level of calibration curve) and slope (m) of calibration curve, using formula; LOD = 3.3(SD m$^{-1}$). In Calculation of LOQ the formula used is; LOQ = 10(SD m$^{-1}$). The LOD and LOQ values of clove oil components are presented in table 2.

**Repeatability and Precision:** Repeatability or Precision of the method was checked by processing three samples of same A.I. Content simultaneously using a common method and compared by Percentage Relative Standard Deviation (% RSD) of their A.I. Content, which is 10.28 %.

**Table 2.** Correlation coefficient ($r^2$), LOD and LOQ values of each component

| Components | Correlation coefficient | $Y = mx + c$ | | | LOD ($\mu$g mL$^{-1}$) | LOQ ($\mu$g mL$^{-1}$) |
|---|---|---|---|---|---|---|
| | $r^2$ | $m$ | $c$ | SD | 3.3 (SD m$^{-1}$) | 10 (SD m$^{-1}$) |
| Eugenol | 0.9993 | 121.5 | 33.22 | 1.62 | 0.044 | 0.133 |
| Caryophyllene | 0.9988 | 253.6 | 118.49 | 3.9 | 0.051 | 0.154 |
| A-Caryophyllene | 0.9985 | 235.49 | 108.07 | 4.9 | 0.069 | 0.208 |
| Eugenol Acetate | 0.9999 | 47.86 | 4.34 | 1.2 | 0.083 | 0.251 |
| Caryophyllene oxide | 0.9989 | 36.952 | 0.586 | 1.8 | 0.161 | 0.487 |

SD = Standard Deviation, m = Slope, c = Intercept

**Recovery:** Recovery Studies were done by assuming the 6% ME as the spiked Sample, at 6% spiking level and calculating the recovered percentage of active ingredient as net recovery. Average Recovery percentage was calculated observed to be 99.6%.

**Calculation of Active Ingredient (Eugenol content):** Eugenol content of ME formulation was comparatively calculated from clove oil technical whose percentage of Eugenol was previously estimated by GC-MS. Equi-molar concentrations of Technical and ME formulation were prepared separately in Ethyl acetate. Comparison of Probe and bath type of sonication indicated that probe sonication is fast and rapid in breaking the micelle and extracting the Clove oil content (Figure 4). Hence, ME sample formulation was processed by probe sonication for 10 minutes and finally injected and analysed on GC-MS. The formula used for calculation of A.I (i.e. Eugenol) is;

$$\text{Active Ingredient (\%)} = \frac{\text{Peak under Area of Sample}}{\text{Peak under area of Technical}} \text{ X } \frac{\text{Weight of Technial}}{\text{Weight of sample}} \text{ X } \frac{\text{Purity of Technical (\%)}}{1} \quad .. 1$$

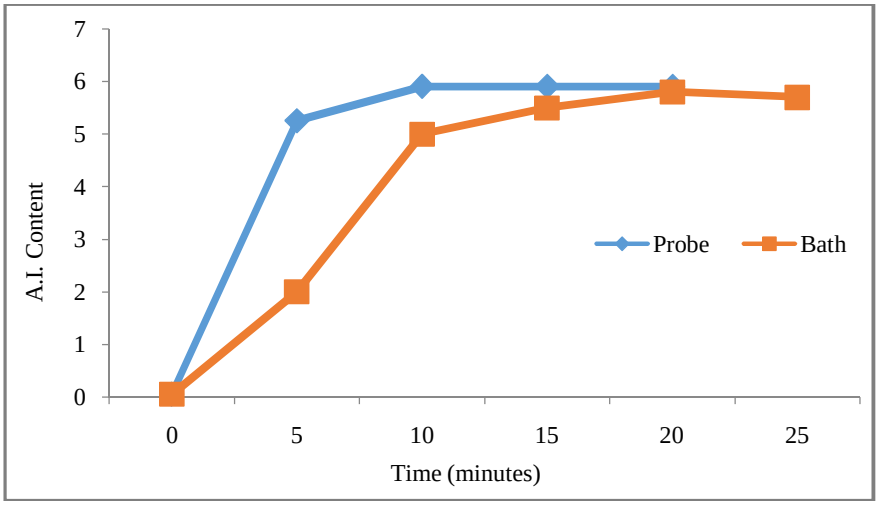



**Figure 4.** Graph of calculated A.I. content vs time, by two different sonication methods at different time intervals.

## APPLICATION

The analytical method presented in this paper provides the insight for developing and validating the single laboratory method for the estimation of active ingredient content of any pharmaceutical or natural product from its microemulsion formulation. Sonication based extraction with a suitable organic solvent has been found as a fast and effective extraction technique.

## CONCLUSION

Major five components viz. Eugenol (92.11%), Caryophyllene (5.47), alpha Caryophyllene(0.90%), Caryophyllene oxide (1.09%)& Eugenol acetate (0.43%) were observed in the clove oil technical used in ME formulation by GC-MS instrument. Instrument's optimized parameters showed good linearity (5 point calibration) in the range of 0.1–50 $\mu$g $mL^{-1}$ concentration. Ethyl acetate and n-Hexane both were found to be the most suitable extracting solvents for the most of the components from the formulation. In order to break the micelle of ME formulation and extract the A.I. (Eugenol) into extracting solution, two sonication technique (bath and probe type) was used as for better recovery. The probe sonication exhibits the better results in comparatively lesser time (10 min) duration than the bath sonication method. Method developed and presented in this paper was found satisfactory for the analysis and estimation of Eugenol content in ME formulation containing Clove oil.

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