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## *In vitro* analysis of 1, 8-cineole in the plasma of domestic fowl by Gas Chromatography–Mass Spectrometry

**Mahesh DM, Juliet S, Suresh N Nair, Nisha AR, Ravindran R, Shijin MS, Suja Rani S, Vanishree H and Ranjith D**

**Abstract**

1,8-cineole is a major monoterpenoid found principally in essential oils of *Eucalyptus globulus*, *Eucalyptus polybractea*, *Helichrysum gymnocephalum*, *Zingiber chrysanthum* and *Rosmarinus officinalis*. It is considered safe and has been used as a flavoring agent in food products, fragrances, cosmetics and phytochemical feed additives (PFAs) in animal health care and livestock production. The present study was undertaken to standardize the protocol for recovery of the phytochemical 1,8-cineole from the blood of fowl. The parameters were optimized for determination of 1,8-cineole by GC-MS using a single quadrupole GC-MS instrument (GCMS-QP2010 Ultra, Shimadzu, Japan) coupled with an auto sampler and Teledyne Tekmar Versa. 1. The retention time was 10.2 min on scan mode and the limit of detection (LOD) of 1,8-cineole was 0.001 µg/mL on SIM mode. Maximum recovery from was obtained with hexane as the solvent and the recovery percentages from plasma fortified with 0.25, 0.5 and 1 ppm was 81.76, 84.23 and 82.13 respectively.

**Keywords:** 1,8-cineole, plasma, recovery, GC-MS

**Introduction**

Phytobiotics, also known as phytochemical feed additives (PFAs) or herbs were used in traditional animal health care and livestock production in Europe (Franz *et al.*, 2010) [6]. It included a wide range of plant-derived products such as essential oils, herbs and oleoresins. Feed additives comprising of whole seeds or extracts of black cumin (*Nigella sativa*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) and chilli (*Capsicum annum*) were used either alone or in combination in commercial poultry nutrition to improve performance (Grashorn, 2010) [7].

1,8-cineole known chemically as eucalyptol is a monocyclic monoterpene ether (oxide) found in essential oils of a number of aromatic plants like *Eucalyptus globulus*, *Eucalyptus polybractea* (95%), *Helichrysum gymnocephalum* (47.4%), *Zingiber chrysanthum* (42%), *Rosmarinus officinalis* (43.7%) (Hans *et al.*, 1992; Tschigger and Bucar, 2010; Iqbal *et al.*, 2011; Afoulous *et al.*, 2011; Elaissi *et al.*, 2012, Bhowal and Gopal, 2015) [8, 12, 9, 1, 4, 2]. It is frequently utilized by the pharmaceutical industry in drug formulations, as a percutaneous penetration enhancer and for its decongestant and antitussive effects and in aromatherapy as a skin stimulant (Santos and Rao, 2000; Lima *et al.*, 2013) [11, 10]. Eucalyptol was given generally recognized as safe (GRAS) status by the Flavor and Extract Manufacturer's Association (FEMA) in 1965 and was also approved by the United States Food and Drug Administration (US FDA) for use in food products. Toxicological data available on eucalyptol are rather inadequate. The European Commission (EC) Opinion of the Scientific Committee on Food on eucalyptol (2002) [5] states that "However, the available animal data do not indicate a cause of concern associated with the daily intake from food." The compound is reported to possess good pharmacological effects which includes gastroprotective, hepatoprotective, anti-inflammatory, antimycotic and antimicrobial activity (Bhowal and Gopal, 2015) [2]. An attempt has been made in the present study to standardize the protocol for analyzing the phytochemical 1,8-cineole by Gas Chromatography Mass Spectrometry (GC/MS) from plasma of domestic fowl.

## Material and Methods

**Chemicals:** 1,8-cineole were purchased from M/s Sigma Aldrich India Ltd., Bengaluru and used without further purification. The purity of the standard was  $\geq 97\%$ . All the solvents used in the study were GCMS grade procured from M/S Merck India Ltd., Mumbai.

**Birds:** The chicken were raised under standard management conditions. The experiment was conducted strictly in accordance to the regulations of Institutional Animal Ethics Committee (IAEC/COVAS/PKD/3/2018) and conformed to the national guidelines.

## Collection of blood and separation of plasma

Blood (approx. 2 ml) was collected from the wing veins of six chicken of ninety days age and weighing an average of  $771 \pm 64.96$  g into tubes added with anti-coagulant (Blood collection tubes K3 EDTA 2ml; double cap). The blood was centrifuged at  $2655 \times g$  for 5 minutes for separation of plasma. The separated plasma was immediately collected into separate cryotubes and stored at  $-20^\circ\text{C}$  until further processing.

## Instrumentation

### GC-MS conditions

A single quadrupole GC-MS instrument (GCMS-QP2010 Ultra, Shimadzu, Japan) coupled with an auto sampler and Teledyne Tekmar Versa was used for the analysis of 1,8 cineole. A RXI 5 SILMS capillary column (30m X 0.25 mm, id. 0.25  $\mu\text{m}$ ) was used. The carrier gas used was high purity helium (99.9999 per cent) with a flow rate of 1 mL/min. The sample volume was 1  $\mu\text{L}$  with a split ratio of 10:1. Oven temperature program was initially set at  $60^\circ\text{C}$  for 1 min, then ramped at  $5^\circ\text{C}/\text{min}$  to  $220^\circ\text{C}$  and held for 0.0 min, then ramped at  $15^\circ\text{C}/\text{min}$  to  $280^\circ\text{C}$  and held for 1.0 min. The total run time was 38 min with a solvent delay of 3.01min. The temperature of injection port and interface were  $250^\circ\text{C}$ , and  $280^\circ\text{C}$ , respectively. Ionization was performed in electron impact ionization (EI) mode at 70 eV. Scan range at start m/z to end m/z was 40: 300. From the mass fragmentation pattern of the compound, selective ion monitoring (SIM) was set for quantitation with dwell time 100ms/ion. The obtained spectrum of the analytical grade compound was matched for similarity using NIST-11 and Wiley 08 software.

## Standardization of analytical procedure

### Preparation of standards

Stock solution of 1,8-cineole was prepared in hexane by dissolving 1.085  $\mu\text{L}$  of 1,8-cineole making volume up to 5mL with hexane to obtain final concentration of 200  $\mu\text{g}/\text{mL}$ . From the master stock, stock solution of the concentration 1  $\mu\text{g}/\text{mL}$  (1 ppm) was prepared. The limit of detection (LOD) of 1,8-cineole was 0.001  $\mu\text{g}/\text{mL}$ . Composite working standards of 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1  $\mu\text{g}/\text{mL}$  were prepared by diluting the stock solution with suitable quantities hexane and stored at  $4^\circ\text{C}$  until analysis by GC-MS. Standard stock solution kept at  $-20^\circ\text{C}$ .

### Calculation of Limit of Detection (LOD)

LOD was calculated using  $\text{LOD} = 3.3 (\text{Sy}/\text{S})$  where Sy is standard deviation of y-intercepts of regression lines and S is slope of the calibration curve (S).

### Linearity

Ascending concentrations of 1,8-cineole viz., 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1  $\mu\text{g}/\text{mL}$  in hexane were analyzed using GC-MS and peaks with area were calculated and plotted in excel sheet/ graph. The reproducibility of results was

verified at least thrice with each concentration of 1,8-cineole. Linear regression analysis was done by plotting concentration versus peak area curve with MS-Excel and regression equation and  $R^2$  values were developed.

## In vitro recovery studies

The extraction efficiency was determined for 1,8-cineole by comparing the peak areas from drug free samples spiked with known quantities of drug in the range of concentration of calibration curves and standard solutions with suitable solvent in which the analytical grade test compound was soluble, injected directly into analytical column. The plasma separated from the blood collected from the birds as described above were used as the matrix. The matrices are spiked with different known concentrations of 1,8 cineole. Recovery was carried out to ascertain the reliability of the method and three replicates were used for each concentration of the test compound. After necessary work up, the concentration of were analyzed by 1,8-cineole by GC-MS. Mean and standard deviation of each concentration were calculated. The relative standard deviation was calculated by using the formula.

$$\text{RSD} = \frac{\text{Standard deviation of replicates of each concentration} \times 100}{\text{Mean of the replicates of each concentration}}$$

## Extraction of 1,8 cineole from plasma

To a sample of plasma (200 $\mu\text{L}$ ), 1,8-cineole was spiked at three different concentrations (0.25, 0.5 and 1  $\mu\text{g}/\text{mL}$ ) separately in 1.5 mL microcentrifuge tubes with three replicates each. The blank comprised of three replicates without the test compound. To all the tubes including blank, saturated ammonium sulphate in 2.5% sulphuric acid (200  $\mu\text{L}$ ) and hexane (500  $\mu\text{L}$ ; Suprasolv<sup>®</sup>) were added and vortexed for 30 seconds. The tubes were centrifuged at  $20817 \times g$  in a refrigerated centrifuge (Model 5430 R, M/s Eppendorf, GmbH Germany) at  $4^\circ\text{C}$  for 20 min. The hexane layer was collected into a 1.5 ml glass vials (Borosil<sup>®</sup>). To the pellet, 500  $\mu\text{L}$  of hexane was added and the above procedure was repeated twice. The collected pooled hexane layer was filtered using 0.22  $\mu\text{m}$  fluoropore filter (M/s Millipore, India) in GCMS auto sampler vials and the volume was made up to 2 mL with hexane (Suprasolv<sup>®</sup>). The samples were analyzed by GC-MS as described previously.

## Statistical analysis

Statistical analysis for the results obtained in the analysis of 1,8-cineole was done by using SPSS software for mean and standard error.

## Results and Discussion

### Optimization of GC-MS parameters for 1,8 cineole

The MS spectral in scan mode was performed from 40-300 m/z and the chromatogram of the 1,8-cineole analytical standard along with the different concentration are presented in figures 1 and 2. 1,8-cineole was detected at the retention time of 10.2 min in scan mode. Then the m/z ions were selected for the 1,8-cineole. The characteristic ions m/z ions of 1,8-cineole were shown in table 1 and figure 3.

**Table 1:** The characteristic ions (m/z) of the (a) 1, 8-cineole

	Channel 1	Channel 2	Channel 3	Channel 4	Channel 5
a	43.00	81.00	84.00	71.00	108.00

Selective ion monitoring was used to increase the sensitivity of the analyte 1,8-cineole. The total ion concentration (TIC)

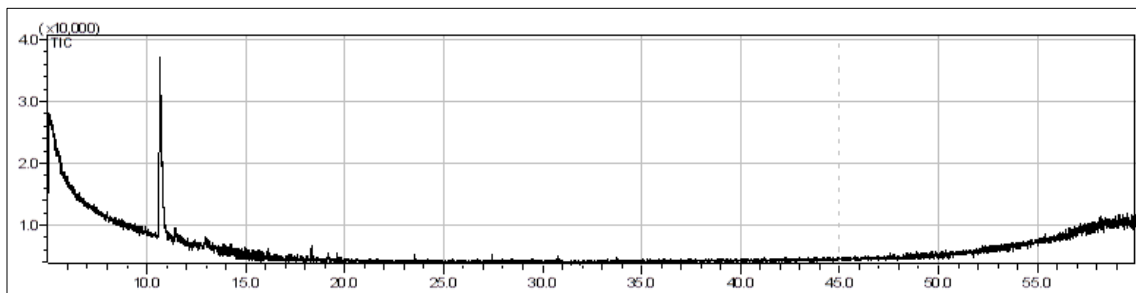
of 1,8-cineole in SIM mode is shown in figure 4. The limit of detection (LOD) of 1,8-cineole was 0.001 µg/mL.

**Response linearity**

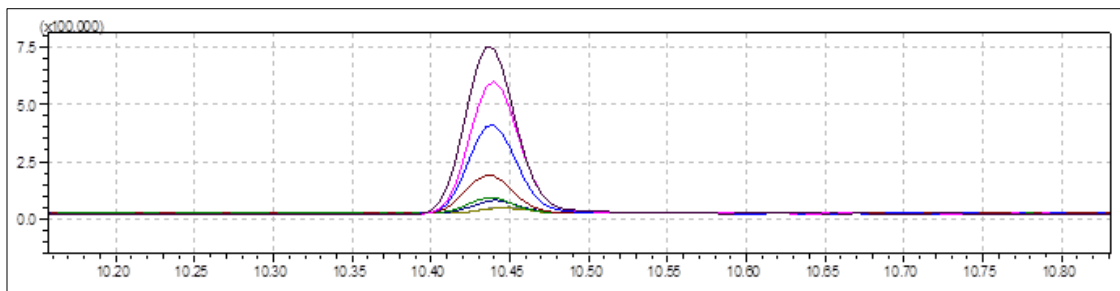
Calibration curve was prepared for 1,8-cineole, at 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 µg/mL in hexane analyzed by GC-MS. A linear regression was carried out on the data set and regression coefficient ( $R^2$ ) of 0.9984 was obtained for 1,8 cineole. The data on peak area against the known concentrations of 1,8-cineole is presented in table 2. Graphical representation of the calibration curve for 1,8-cineole is depicted in figure 5.

**Table 2:** Mean peak area values of known concentration of 1,8-cineole in hexane following estimation by GC-MS. (n=3, Mean ±SD)

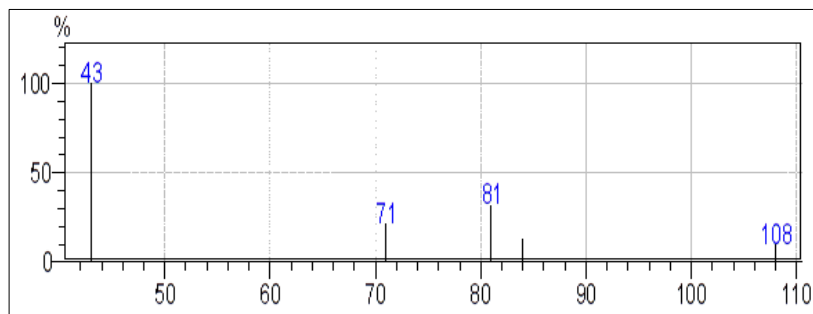
Concentration (µg/ml)	Peak area (Mean ± SD)
0.01	418.75 ± 30.97
0.025	1112.75 ± 99.51
0.05	2041.50 ± 186.72
0.1	3774.25 ± 177.39
0.25	10686.00 ± 863.39
0.5	18316.25 ± 665.14
1	37091.25 ± 1275.91



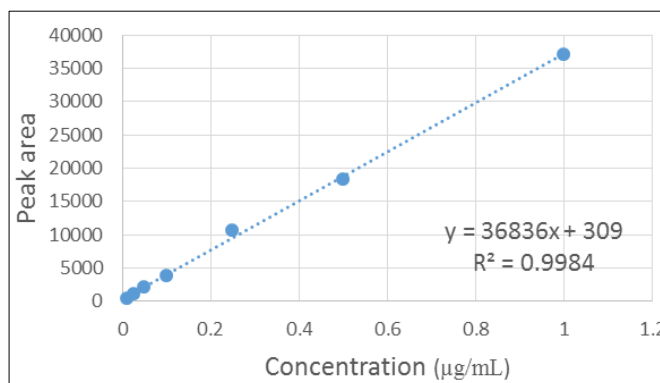
**Fig 1:** Chromatogram of 1,8-cineole (scan mode)



**Fig 2:** Different concentration of 1,8-cineole in Scan mode



**Fig 3:** Full mass spectrum of 1,8-cineole



**Fig 4:** Total Integrated curve (TIC) of 1,8-cineole (SIM mode)

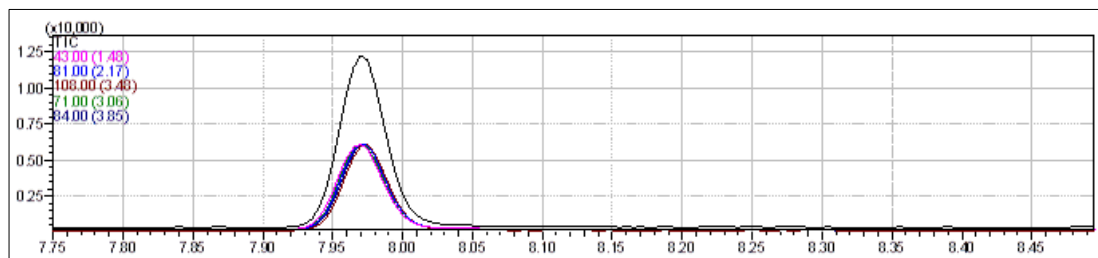


Fig 5: Graphical representation of the calibration curve for 1,8-cineole

### In vitro recovery studies

#### Extraction and recovery of 1,8-cineole from plasma

Maximum recovery of 1,8 cineole after fortifying plasma with different concentrations like 0.25, 0.50 and 1  $\mu\text{g/mL}$  of 1,8 cineole was obtained with hexane as the solvent. Hence hexane was selected as suitable solvent for further experiment. The area under peak was measured to determine the concentration of 1,8-cineole recovery from plasma using hexane as extraction solvent and comparison using the RSD values. Recovery per cent of 1,8-cineole in plasma is shown in table 3. Mean recovery per cent of 1,8-cineole in plasma for 0.25, 0.5 and 1  $\mu\text{g/mL}$  was 81.76, 84.23 and 82.13 respectively.

Analysis of 1,8-cineole by GCMS was carried out using the parameters described by the Boyle *et al.* (2000) [3] with the slight modifications. Response linearity for 1,8-cineole was measured from 0.01 to 1 ppm. There was a significant linear correlation ( $R^2$ ) of 0.998 for the range of concentration tested. Recovery 1,8-cineole from plasma were 81.76, 84.23 and 82.13 per cent respectively for 0.25, 0.5 and 1  $\mu\text{g/mL}$  concentration and the RSD for 1,8-cineole in plasma ranged from 1.89 – 4.28 per cent which were within the criteria of Codex Alimentarius Commission for residue analysis, that is recovery of 70-110% and RSD of <20%. The mass fragment ions selected for 1,8-cineole was  $m/z$  43, 71, 81, 84 and 108.

Table 3: Recovery percent of 1,8-cineole in plasma

Spiked concentration ( $\mu\text{g/mL}$ )	Peak area	Detected concentration ( $\mu\text{g/mL}$ )	Per cent recovery	Mean recovery per cent	Mean concentration	SD	RSD
				1,8-cineole			
0.25	R1	8031	0.209	83.852	81.764	0.204	0.004
	R2	7836	0.204	81.735			
	R3	7649	0.199	79.704			
0.5	R1	15159	0.403	80.627	84.238	0.421	0.014
	R2	15821	0.421	84.221			
	R3	16492	0.439	87.865			
1	R1	30715	0.825	82.544	82.132	0.821	0.012
	R2	29932	0.804	80.418			
	R3	31043	0.834	83.434			

### Conclusion

The procedure standardized can be effectively used for the quantification of 1,8-cineole and metabolites of it in biological samples and other matrices *in-vivo* following administration of 1,8 cineole by different routes.

### Conflict to Interests

The authors declare that there is no conflict of interest regarding the publication of this research paper.

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