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Analytical Method Validation of an Herbal Formulation by Headspace Gas Chromatography Using QbD

Joydeep Mazumder[1*], Devender Pathak[1], Rachna Kumria[2]

[1]*Rajiv Academy for Pharmacy, Delhi-Mathura Highway, P.O Chhatikara, Mathura-281 001, Uttar Pradesh, India*
[2]*Swift School of Pharmacy, Village- Ghaggar Sarai, Rajpura, Patiala, Punjab, India*

ABSTRACT

Essential oils are rich sources of biologically active compounds possessing diverse medicinal properties. These form integral part of a number of herbal formulations. The most challenging part of commercialization of herbal formulation is to ensure consistency in quality from batch to batch. The aim of present study was to develop a validated gas chromatographic method based on quality by design (QbD) for routine quality control purpose.

A blend of essential oils possessing synergistic carminative properties was formulated in oily base. Ajowan oil, cardamom oil, caraway oil, coriander and fennel oil were selected for development of formulation. A gas chromatography method was developed for routine quality control purpose of the developed formulation by quality by design techniques (QbD). Each oil was characterized making use of a marker compound which was linalool for coriander oil, cineol for cardamom oil, anethol for fennel oil, carvone for caraway oil, thymol for ajowan oil and menthol for peppermint oil. Marker compound was characterized using mass spectroscopy. Chromatography method was established by quality by design approach and validated based on ICH guidelines.

Keywords: Essential oil, gas chromatography, headspace analysis, marker compound, quality by design (QbD), Design of experiment (DoE).

INTRODUCTION

In medical sciences there is a continuous quest to discover new chemical entities to fight new and emerging diseases. These chemical entities with diverse chemical structures act at the receptors sights using varied mechanisms of action. As such chemical entities from plant origin often act as lead molecules for such discoveries. Products from plant origin however, contain a complex mix of chemical compounds of which some may show pharmacological activity while the rest may be inactive. Traditionally plants have been used in folklore medicines for common ailments. As per WHO, a majority of world's population depends upon traditional medicines for primary healthcare needs. Modern science struggles to isolate the active compounds from plant extract, a holistic approach of preparing and administered complete extracts has been successfully used traditionally.

Recent years have seen a revival of interest in the use of plant products as these have been reported to be safe and devoid of any side effects. These are in fact being preferred over the synthetic counterparts owing to the GRAS status. Essential oils are natural, complex, lipophilic compounds, volatile in nature possessing a characteristic pleasant aroma. These are obtained from

***Corresponding author: Mr. Joydeep Mazumder,**
Rajiv Academy for Pharmacy, Delhi-Mathura Highway, P.O Chhatikara, Mathura-281 001, Uttar Pradesh, India; **Tel.:** +91-9704577208;
E-mail: joydeeppharma@gmail.com
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plant parts such as fruits, flowers, seeds, buds, leaves, bark, wood, twigs or roots. They comprise of a terpene component as well as a non-terpene component. Essential oils are known to possess a number of medicinal properties like antimicrobial, antioxidant, antifungal, analgesic, anti-inflammatory activity. [1-6] These are very good carminatives and are used in a number of gastrointestinal ailments.

Being sourced from plants, essential oils may vary in composition depending upon species of the plant used, location where plant is grown, condition of the soil, weather conditions, level of experience of the cultivator, etc. Due to its complex chemical composition, as such the characterization and estimation of purity of individual essential oils is quite challenging. Another challenge lies in authenticating and quality control of herbal formulation with varied ingredients. An herbal formulation comprising of essential oil combination with a proposed syngeristic carminative activity was developed for gastrointestinal disorders. Analytical method development together validation was carried out for routine analysis of the herbal essential oil formulation. Gas chromatography is an important tool of analytical techniques for identification and characterization of actives present in essential oils. [7-8] In the light of the above, it was proposed to develop an analytical method for identification and estimation of purity individual and blend of essential oil.

In the present study the primary objective is,

- To develop a sequential technique to identify the key drug characteristic of essential oil.
- Develop a suitable, precise analytical gas chromatographic headspace method by QbD approach to quantify the active present in the blend of essential oil.
- To get document of assurance on reliable result and method is suitable for its intended use, validation of the method had established as per ICH guideline.

In the present study a blend of oil was prepared by combination ajowan, cardamom, caraway, coriander, fennel oil and peppermint oil which individually possess medicinal properties such as antacid, carminative, anti-flatulent, antimicrobial etc. [9-13] The marker Linalool is a major compound present in coriander oil; cineol in cardamom oil, anethol in fennel oil, carvone in caraway oil, thymol in ajowan oil and menthol in peppermint oil (Fig. 1) used as a standard to identify and estimation of purity of the individual oil. [14-15] The all marker compound was characterized by mass spectroscopy. [16]

CTD format emphasizes the requirement of QbD. Through application of QbD a design space is created and working within the design space is not considered as change. Working on similar lines, QbD approach including DoE concept into analytical method development is picking up momentum. In analytical method development, the performance of the analytical method within the allowable change needs to be demonstrated. ICH Q8 defines the critical quality attributes (CQA) as physical chemical, biological, or microbiological property or characteristic that should be within the appropriate limit, range or distribution to be sure of the desired product quality. The performance of the analytical method as given by precision, accuracy, specificity, linearity, range and limit of quantitation of impurities, should be targeted. The developed method should be capable of providing a measurable control of the CQA during manufacturing and stability testing. For instance, the acceptance criteria for method accuracy and precision will he required to be tighter for a product with a narrower assay limit of ± 5% as compared to a product with an assay limit of ± 10%. Analytical gas chromatography using head space method [17-19] has been developed by quality by design (QbD) approach [20-22] which has become an important concept for the pharmaceutical industry to find out and measure the CQA and its design space. In order to confirm the assay method are accurate, method validation has been carried out according to current ICH guidelines. [23-26]

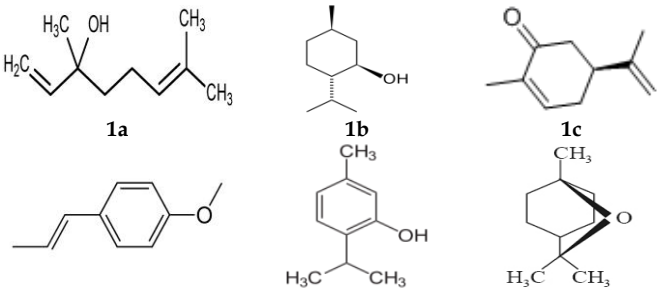

Fig. 1: Chemical structure of Linalool (1a), Menthol (1b), D-carvone (1c) Anethol (1d), Thymol (1e), and Cineol (1f)

MATERIALS AND METHODS

Material and Reagent

Cardamom, Coriander, Fennel, Carawa, Ajowan oil and peppermint oil were purchased from Ultra International Ltd., Uttar Pradesh, India. Markers compounds namely linalool, cineol, anethol, carvone, thymol and menthol were also procured from Ultra International Limited, Uttar Pradesh, India. Methanol, Hydrochloric Acid, Sodium Hydroxide and other reagents used for the analysis were of Analytical grade. The purities of all the standards were not less than 98%.

Instrumentation and software

Gas chromatography fitted with flame ionization detector equipped with Headspace auto sampler analyzer (Agilent, Gas chromatograph with Headspace auto sampler, Model-7890A with Headspace autosampler-G1888) connected with Empower software was used for carrying out analysis. DB-624 Capillary column with 30 meters length, 0.32 mm ID and 1.8 µm (PerkinElmer Part no.-125-1334, Sl. No.-USB1564H), Design-expert software 8.0.7.1 work station.

Physical characterizations of oils

The physico-chemical properties were measured according to Russian Pharmacopoeia (1990). The color of the individual oils was checked by visual observation. 5 ml of the individual oil in was poured into a standard test tube and the color was noted. Refractive index and optical rotation of the oils has been checked using Abbe's Refractometer and polarimeter. Specific gravity test has been performed using Borosil Specific Gravity Bottle and calculate by the flowing formula:

$$d^{25}_{25} = \frac{m_3 - m_1}{m_2 - m_1}$$

Where, m_1=Mass in grams of empty gravity bottle, m_2=Mass in grams of gravity bottle with distilled water at 25°C, m_3=Mass in grams of gravity bottle with test sample at 25°C, d=Relative density.

Characterization of marker compounds by Mass spectroscopy

The individual marker compound was characterized making use of Atmospheric-Pressure Chemical Ionization (APCI) mass spectroscopic method to determine the molecular mass of the marker compound. This study was carried out at Radiant Research Center, Bangalore, India.

Identification and estimation of purity

GC-Headspace method was developed to identify & estimate the purity of individual oil based on marker compound present in each oil where respective marker compound was used as standard. The gas chromatographic system included a Gas chromatograph with Headspace auto sampler and a flame ionization detector. A DB-624 Capillary column with 30 meters length, 0.32 mm ID and 1.8µm film thickness was use as a stationary phase. Initial oven temperature was programmed at 60°C with a hold for 2 minutes, with a 15°C/ minutes rate. The temperature was raised to 180°C and hold for 10 minutes. The final temperature was elevated to 240°C at a rate of 15°C/ minutes. Nitrogen was used as a carrier gas at flow of 1.2 ml/min. Detector parameter were programmed as: temperature 270°C, range 1 and attenuation - 4. Split ratio selected 5:1. The total run time is 20 minutes. In the head-space the oven temperature was kept at 85°C , Needle temperature 95°C, Transfer line temperature 100°C, GC cycle time 22 minutes, thermostat time 30 minutes, pressurization time 2 minutes, injection time 0.5 minutes, withdrawal time 0.5 minutes. Headspace mode was kept constant and Headspace carrier pressure was fixed to 15 psi. Each individual oil has considered as a sample & their respective marker compound considered as standard which were dissolved in methanol in a separate volumetric flask and the final concentration was 0.5 mg/ml. 1ml of sample solution and their respective each marker standard stock solution was transferred to different headspace vial and immediately crimped. The sample was identified against the active present in the each oil and purity was calculated against the marker compound on consideration of the purity of each marker compound as 100%. The column was equilibrated for up to 1 h before stating the experiment. After equilibration, six injections of solution/each marker compound of respective oil with the test sample of each oil were injected.

Preparation of blend of sample and standard:

A blend of stock sample had prepared by transferred 1g of each oil in 200 ml volumetric flask. Added approximately 500 mg of peppermint oil (used as soothening agent) into made up the volume with sunflower oil used as a base. The sample was stored in a close tight amber color container. From the above stock, 1 ml of sample had transferred into 10 ml volumetric flask and made up the volume with methanol. A blend of standard was prepared by transferring accurately weighed 5 mg of each marker compound into 10 ml of volumetric flask and made up the volume with methanol.

QbD approach and Identify the CQA

The experimental design along with statistical analysis of data was performed by Design-Expert 8.0 software, Full Version (Stat Ease Stat-Ease, Inc., Minneapolis, MN, USA). An experimental design is an experimental set-up to simultaneously evaluate several factors at a given numbers of levels in a predefined number of experiments. Several types of experimental designs (Two level full factorial, two level fractional factorial, Placket-Burman, mixed level designs) are available and these designs allow the simultaneous examination of qualitative, quantitative and mixture related factors. A two level full factorial design is selected for the present study to determine the main effects and all interactions between the factors.

To make systemic experimental design, it is necessary to identify the CQA of the method. The design comprised with 21 robustness experiment trial by changed the chromatographic condition of each parameter like gas flow rate, detector temperature, split ratio and head-space oven temperature, Needle temperature, transfer line temperature, thermostat time, headspace carrier pressure (Table 1). Among all the chromatographic parameters gas flow, head space oven temperature and head space thermostat time were identified as CQA of the method which could alter the two critical system suitability parameter resolution (RS1) between D-carvone- anethol and resolution (RS2) between thymol and cineol peak.

A two level full factorial design (2^3) is selected for the present study to determine the interaction of the three variables (CQA) and their effects. Minimum ±10% variation was considered from the actual chromatographic condition of those three variables and the design set as a standard set of gas flow 1.0 ml /minutes to 1.4 ml/minutes, thermostat time from 27 to 33 min and thermostat oven temperature from 77°C to 94°C. This led a total 20 chromatographic condition including 4 duplicate center points (Table 2).

Table 1: Robustness experiment

Gas Chromatograph Parameters	(-)ve control condition	Control condition	(+)ve control condition	Number of experiment
Carrier gas flow(N_2)	1 mL/min.	1.2 mL/min.	1.4 mL/min.	3
Detector temperature	250°C	270°C	290°C	3
Injector temperature	210°C	220°C	240°C	3
Split ratio	1:1	5:1	10:1	3
Oven temperature	75°C	85°C	95°C	3
Needle temperature	90°C	95°C	100°C	3
Transfer line temperature	90°C	100°C	110°C	3
Thermostat time	20 minutes	30 minutes	40 minutes	3

Table 2: DoE experiment Study design

Std.	Run	Factor-1 Flow/Min.	Factor-2 Oven temperature(°C)	Factor-3 Thermostat time (min.)
13	1	1.00	94.00	33.00
8	2	1.40	94.00	27.00
20	3	1.20	85.50	30.00
12	4	1.40	77.00	33.00
6	5	1.00	94.00	27.00
10	6	1.00	77.00	33.00
11	7	1.40	77.00	33.00
14	8	1.00	94.00	33.00
19	9	1.20	85.50	30.00
2	10	1.00	77.00	27.00
17	11	1.20	85.50	30.00
15	12	1.40	94.00	33.00
7	13	1.40	94.00	27.00
3	14	1.40	77.00	27.00
9	15	1.00	77.00	33.00
1	16	1.00	77.00	27.00
16	17	1.40	94.00	33.00
18	18	1.20	85.50	30.00
5	19	1.00	94.00	27.00
4	20	1.40	77.00	27.00

Table 3: Result of system suitability parameter in DoE experiment

Std.	Run	Factor-1 Flow/Min.	Factor-2 Oven temperature(°C)	Factor-3 Thermostat time (min.)	RS1*	RS2**
13	1	1.00	94.00	33.00	5.6	5.1
8	2	1.40	94.00	27.00	3.7	3.2
20	3	1.20	85.50	30.00	4.8	4.2
12	4	1.40	77.00	33.00	3.8	3.5
6	5	1.00	94.00	27.00	6.1	5.2
10	6	1.00	77.00:	33.00	5.9	5.4
11	7	1.40	77.00	33.00	3.8	3.2
14	8	1.00	94.00	33.00	6.2	5.2
19	9	1.20	85.50	30.00	5.2	4.4
2	10	1.00	77.00	27.00	6.2	5.3
17	11	1.20	85.50	30.00	4.9	4.3
15	12	1.40	94.00	33.00	3.9	3.8
7	13	1.40	94.00	27.00	4.1	3.6
3	14	1.40	77.00	27.00	4.1	3.6
9	15	1.00	77.00	33.00	5.8	5.6
1	16	1.00	77.00	27.00	5.8	5.4
16	17	1.40	94.00	33.00	3.8	3.2
18	18	1.20	85.50	30.00	4.9	4.4
5	19	1.00	94.00	27.00	5.6	5.8
4	20	1.40	77.00	27.00	3.9	2.9

* RS1 Resolution between D-carvone- Anethol and resolution

** RS2 Resolution between Thymol – Cineol peak

Table 4: The table lists the physical properties of the oils

Essential oil	Color	Odor	Relative density	Refractive index	Optical rotation
Coriander	Colorless to pale yellow	Characteristic	0.867	1.465	+ 9.004°
Caraway	Colorless to pale yellow	Characteristic	0.915	1.487	+ 71.01°
Fennel	Colorless to pale yellow	Characteristic	0.962	1.541	+ 12.182°
Ajowan	Colorless to pale yellow	Characteristic	0.924	1.501	+ 0.447°
Cardamom	Colorless to pale yellow	Characteristic	0.920	1.463	+ 26.709°

Evaluation of result and desirable space: The results (Table 3) obtained from 20 experiments were analyzed through Design Expert ® software. The effect on the three dependent variables with the independent

variables was explained by using Pareto chart (Figures 2 and 3).

It was observed that the system suitability parameter, resolution (RS1) between D-carvone and Anethol and resolution (RS2) between Thymol and Cineol was affected by either individual three variable or due to interaction between two or three variables.

The 1st pareto chart (Fig. 2) demonstrate resolution (RS1) between D-carvone and Anethol was effected due to dual as well as mixed interaction between the three variable. A positive effect was found due to interaction between flow rates with oven temperature or flow rate with thermostat temperature and even due to mixed interaction between three variables but individually has no independent effect on this system suitability parameter. Hence negative effect was found which has demonstrated in the pareto chart in the blue histogram. Interaction between oven temperatures with thermostat temperature also showed negative effect in the chart.

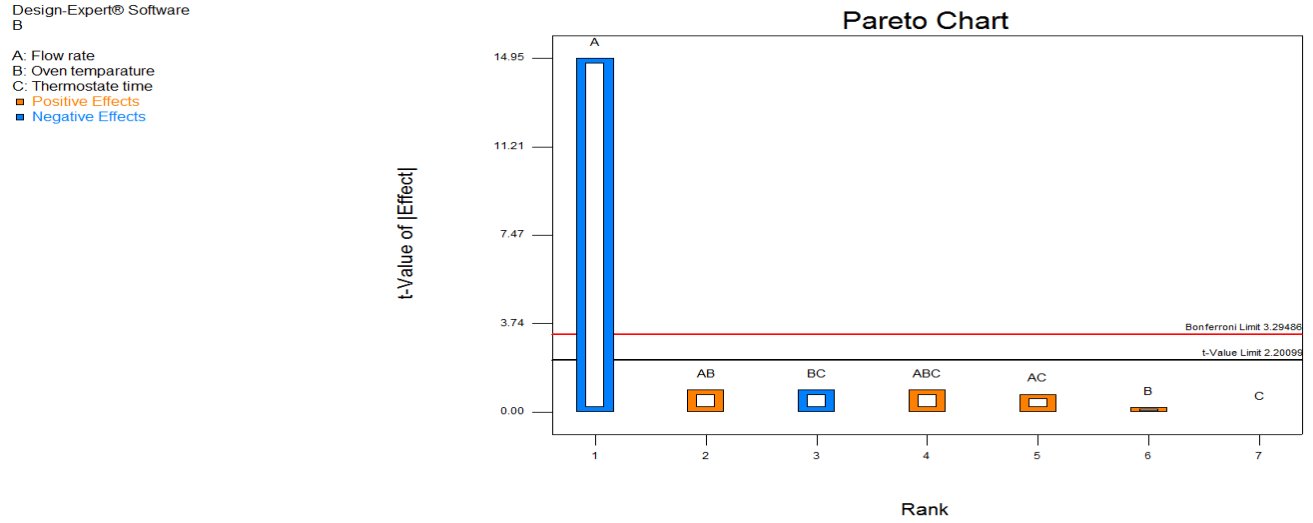

Fig. 2: Pareto chart of resolution between D-carvone and Anethol

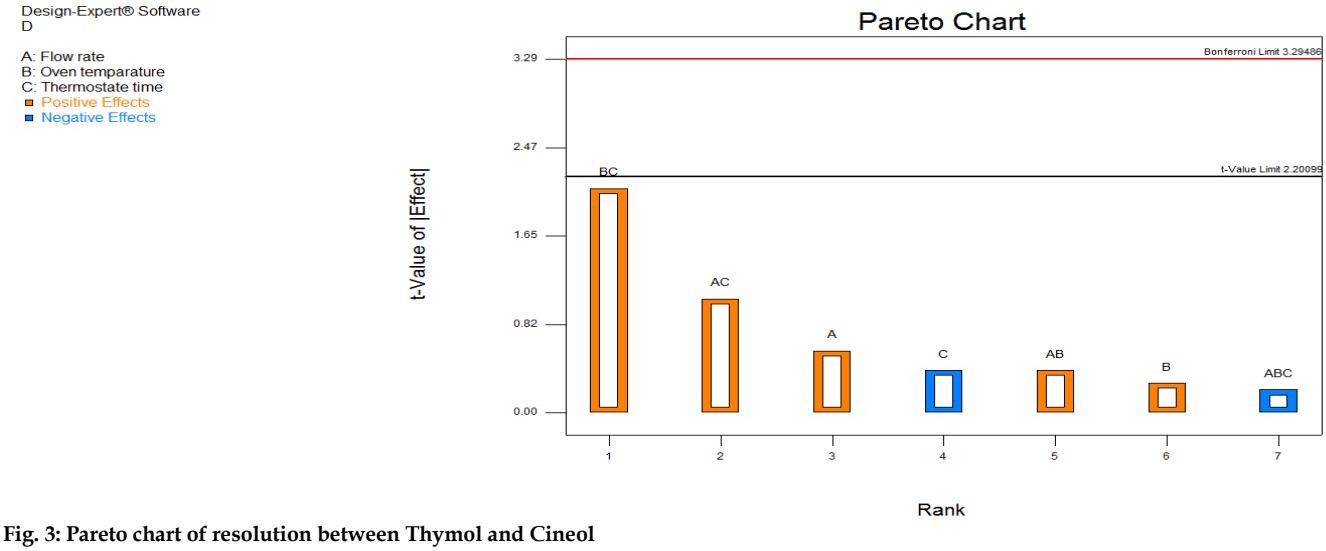

Fig. 3: Pareto chart of resolution between Thymol and Cineol

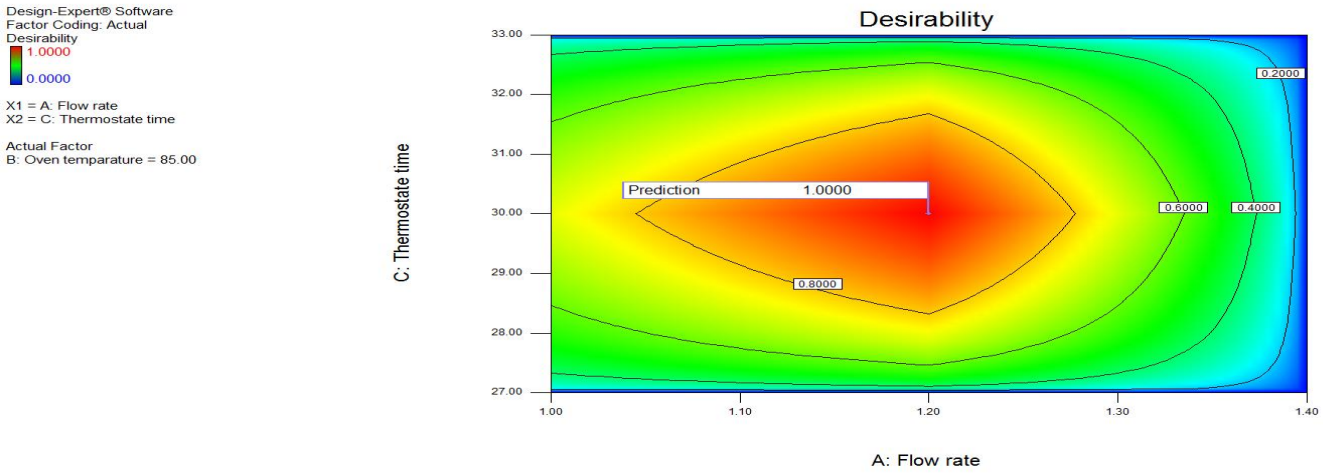

Fig. 4: Counter desirability graph on CQA of Flow rate vs Thermostat time

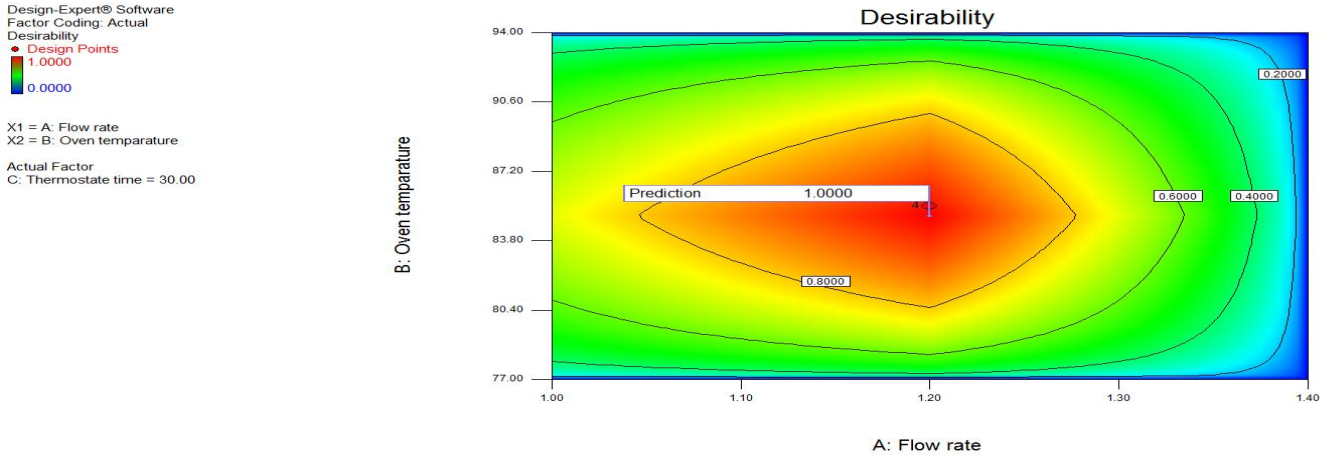

Fig. 5: Counter desirability graph on CQA of oven Flow rate vs oven temperature

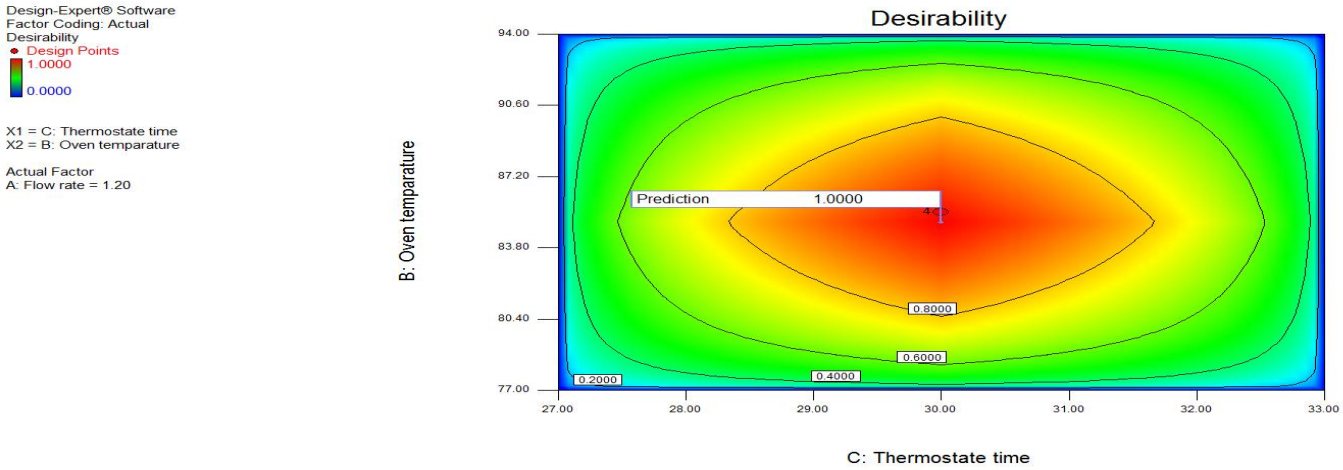

Fig. 6: Counter desirability graph on CQA of Thermostat time vs oven temperature

Table 5: The table lists the retention time of the marker and percentage present in the each Essential oil in assay

Essential oil	Marker compound	Retention time of the pure marker peak in standard solution (Minutes)	Retention time of marker peak in individual oil (minutes)	Assay (% of marker present in oil)
Coriander	Linalool	11.82	11.84	69%
Peppermint	Menthol	13.53	13.53	67%
Fennel	Anethol	16.11	16.08	75%
Caraway	D-Carvone	15.54	15.52	55%
Ajowan	Thymol	17.15	17.15	53%
Cardamom	Cineol	17.66	17.66	55%

Table 6: The table lists the system suitability parameter of the standard of blend of marker compounds in validation

Essential oil	Plate count	Tailing	Resolution
Linalool	32382	1.3	NA
Menthol	23006	0.99	17.3
D-Carvone	21768	1.0	21.1
Anethol	19109	1.1	**4.8**
Thymol	17090	1.2	7.4
Cineol	13003	1.3	**4.4**

But in 2nd pareto chart (Fig. 3), positive dual interaction as well as individual independent effect of three variable was found on resolution between Thymol and Cineol.

The system suitability parameter majorly effect by flow rate, oven temperature followed by dual interaction between thermostat temperature with oven temperature, flow rate with thermostat temperature and flow rate with oven temperature. There was no effect due to individual thermostat temperature as well as mixed interaction of all three variables was found.

Table 7.1: The table lists the inter day precision

Precision	Sample	Coriander	Caraway	Fennel	Ajown	Cardamom
		Linalool	D-carvone	Anethol	Thymol	Cineol
Inter day	Sample -1	66.5%	52.2%	75.1%	51.7%	53.3%
	Sample -2	65.4%	54.7%	76.5%	52.6%	53.9%
	Sample -3	67.3%	53.8%	74.7%	53.1%	52.1%
	Sample -4	67.1%	53.2%	73.1%	53.3%	51.6%
	Sample -5	64.9%	53.9%	76.4%	52.2%	52.4%
	Sample -6	65.4%	52.5%	76.8%	53.7%	51.4%
Average		66.1%	53.4%	53.4%	52.8%	52.5%
%RSD		1.5	1.8	1.9	1.4	1.9

Table 7.2: The table lists intraday precision physical properties of the oils

Precision	Sample	Coriander	Caraway	Fennel	Ajown	Cardamom
		Linalool	D-carvone	Anethol	Thymol	Cineol
Inter day	Sample -1	66.0%	54.2%	77.8%	52.1%	52.9%
	Sample -2	67.1%	53.4%	76.1%	52.1%	52.4%
	Sample -3	66.9%	54.2%	75.3%	53.7%	54.1%
	Sample -4	66.1%	53.7%	75.2%	52.9%	53.1%
	Sample -5	65.2%	52.6%	76.3%	51.4%	52.3%
	Sample -6	64.7%	52.9%	75.8%	52.8%	53.8%
Average		66.0%	53.5%	76.1%	52.5%	53.1%
%RSD		1.3	1.1	1.2	1.4	1.4
Difference		**0.1**	**0.1**	**0.7**	**0.3**	**0.6**

Table 8: The table lists result of bench top stability of standard and sample

Parameter	Time	Coriander	Caraway	Fennel	Ajown	Cardamom
		Linalool	D-Carvone	Anethol	Thymol	Cineol
Similarity factor of standard solution	24 hrs	1.01	0.99	1.01	0.99	0.99
	48 hrs	0.99	0.99	1.01	1.01	1.0
	72 hrs	1.01	1.01	0.99	1.01	1.01
% assay of sample	Initial	67.8%	54.6%	75.3%	52.3%	53.1%
	24 hrs	66.1%	54.2%	73.1%	54.1%	54.3%
	48 hrs	66.8%	53.3%	74.5%	52.9%	53.5%
	72 hrs	66.4%	53.7%	74.8%	52.1%	54.2%
%RSD		**1.1**	**1.1**	**1.3**	**1.7**	**1.1**

Table 9: The table lists result of linearity response of marker compound, LOD, LOQ

Concentration (ppm)	Area Mean Response				
	Linalool	D-carvone	Anethol	Thymol	Cineol
250	1300513	1851214	1608554	2027489	826444
375	1901023	2838901	2446810	2980629	1249871
500	2617050	3867831	3267831	4041707	1656432
600	3081132	4599560	3864040	4853670	1999449
750	3843220	5716347	4836027	6015142	2464321
Regression coefficient	0.9992	0.9992	0.9997	0.9997	0.9996
LOD/LOQ (ppm) by S/N ratio	3/35	2/25	2.2/25	1.5/18	4/50

Table 10: Result accuracy for the developed method

Concentration	Sample	Coriander	Caraway	Fennel	Ajown	Cardamom
		Linalool	D-Carvone	Anethol	Thymol	Cineol
50%	Sample -1	99.2%	98.1%	99.2%	100.4%	98.5%
	Sample -2	99.6%	99.4%	98.8%	101.7%	99.2%
	Sample -3	98.3%	99.6%	99.6%	100.1%	98.7%
	Sample -4	98.4%	100.3%	99.2%	99.4%	98.2%
	Sample -5	101.1%	100.8%	100.4%	99.6%	99.1%
	Sample -6	100.5%	101.4%	100.8%	100.3%	101.2%
Average		99.5%	99.9%	99.7%	100.3%	99.2%
%RSD		**1.1**	**1.1**	**1.2**	**0.8**	**0.8**
100%	Sample -1	99.3%	101.4%	99.5%	98.9%	100.9%
	Sample -2	100.9%	100.4%	98.1%	99.1%	100.2%
	Sample -3	101.1%	99.1%	99.9%	99.7%	99.3%
Average		100.4%	100.3%	99.2%	99.2%	100.1%
%RSD		**1.0**	**1.1**	**1.0**	**0.4**	**0.8**
150%	Sample -1	100.8%	99.3%	99.4%	98.6%	99.6%
	Sample -2	99.2%	101.2%	99.2%	100.3%	98.2%
	Sample -3	101.1%	99.5%	100.5%	99.2%	99.4%
	Sample -4	99.3%	99.1%	100.1%	99.9%	100.5%
	Sample -5	98.9%	98.4%	101.3%	100.8%	101.8%
	Sample -6	98.4%	100.6%	101.7%	100.1%	99.2%
Average		99.6%	99.7%	100.4%	99.8%	99.8
%RSD		**1.1**	**1.0**	**1.0**	**0.8**	**1.2**

To evaluate the desire range of chromatographic condition of these variables, counter desirability plot were established as shown in Fig. 4, Fig. 5 and Fig. 6. These provided the assurance of desire acceptable result obtained in the range of the chromatographic condition of three variables in the desirable space. The red to yellow region in Design space graph indicates the responses are in acceptable range and the green to blue region shows the responses are below the desired level.

Fig. 4 shows that at constant oven temperature 85°C with changing the flow and thermostat time predicted desirability was reach to 1.0 in red region. If we plot a scale Y-axis vs X-axis from the point of 1.0, it means at constant 85°C Oven temperature, the flow rate 1.2 with thermostat time 30 minutes is the optimum

chromatographic condition where maximum resolution between D-carvone- Anethol has been found.

It was found that, the region of desirability value 0.6 is the maximum desire space where the response was found in the desire level. On consideration of this space, the thermostat time 28 minutes to 32 minutes with respect to flow rate 1.0 ml/minutes to 1.3 ml/minutes is the desirable range of the chromatographic condition of these two variables.

Similarly counter plot Fig. 5 demonstrate, the oven temperature can vary from 80°C to 90°C with flow rate 1.0 ml/minuets to 1.3 ml/minutes where the result will be in tolerable range.

From the Fig. 6 it has confirmed that the thermostat range from 28 minutes to 32 minutes with oven temperature 80°C to 90°C is the desire range of the true result when flow was constant.

From the above design plot it facilitated to calculate the chromatographic risk factor of the variable. It has also confirmed the design space inbetween multiple interaction of the varible in the acceptble responses. From the above design, it has proved that the selected chromatographic condition on each variable was lying in middle of the design space and has wide boundary and space. Hence the initial method was finalized and performed method validation.

Analytical method Validation

Method validation is the evidence of degree of tolerability of results of the precise method. The method validation was performed on selected above GC-headspace method by consideration of number of characteristics as System suitability, precision, specificity, linearity, LOD, LOQ establishment, accuracy and range.

System suitability

System suitability parameter was verified from the standard injection which was prepared by taken accurately weighed 5mg of each marker compound in 10 ml volumetric flask and volume made up with methanol used as diluent.

Precision

The precision assess of degree of reproducibility of analytical result. It is expressed by the result of relative standard deviation of six replicate preparation of sample. The method precision was had performed by six replicate preparation of blend of oil in a same concentration and injected in the chromatographic condition and calculated against the standard. The experiment was performed in PerkinElmer gas chromatography with headspace auto sampler.

Linearity

The linearity of an analytical test method defines the ability of response are proportional to the amount of analyte present in the sample. It is measure by correlation of regression line under a specify range.

To demonstrate the linearity of the method, a series of blend of standard solutions in triplicate was prepared in the range of 50% to 150% of the target assay concentration and inject into the GC system. The regression curve was plot by Area (y-axis) vs Concentration (X-axis) and correlation coefficient (r^2) was calculated.

LOD and LOQ

Limit of detection and limit of quantification is the lowest amount of analyte can be detect and quantify in a stated chromatographic condition. Number of preliminary run was taken by prepared of individual lower concentration solution of each marker compound and S/N ration was checked from empower software where LOD was consider not less than 3 and LOQ was considered not less than 10. LOQ Precision was performed by prepared six replicate preparation of blend of standard marker in LOQ concentration level and run in the chromatographic system. The % relative standard deviation of the area of each marker compound was calculated.

Accuracy and range

Accuracy of an analytical method defines the degree of true value in a specified range. It was performed by the method of standard addition at three different levels, by multiple level recovery studies. Three levels of solution were made which correspond to 50, 100 and 150% of the nominal analytical concentration 0.5 mg/ml. Each lower level of 50% and higher level of 150% level was made in replicate six preparations and 100% level in triplicate preparations. These solutions were then analyzed for recovery studies and consistent values replicated preparation on each level were evaluated by calculating relative standard deviation. The range was considered from the % relative standard deviation of six replicate preparations of 50% and 150% level.

Ruggedness

The ruggedness indicates the degree of reproducibility of test results of the same sample analyze under variety of conditions such as different instruments, different temperatures, different days, etc. Precision test once again performed in different make Agilent gas chromatography with headspace auto sampler system. Bench top solution stability also established for 72 hours. The sample and standard was used in precision was kept on bench top and refrigerator and analyzed on day-1 and day-2.

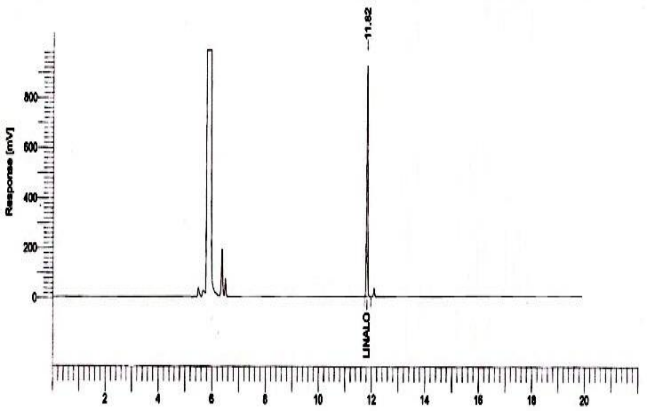

Fig. 7a: Typical chromatogram of marker compound of linalool in standard preparation

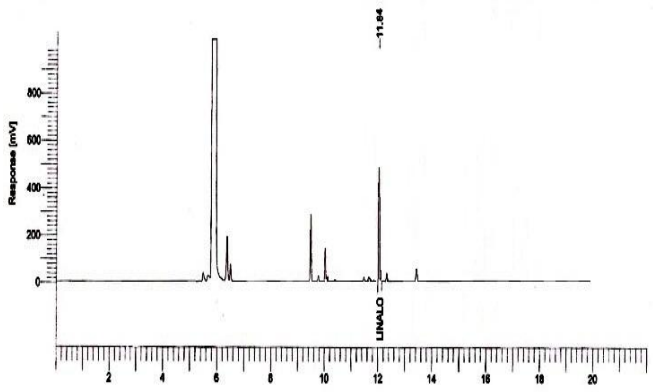

Fig. 7b: Typical chromatogram of coriander oil in assay preparation

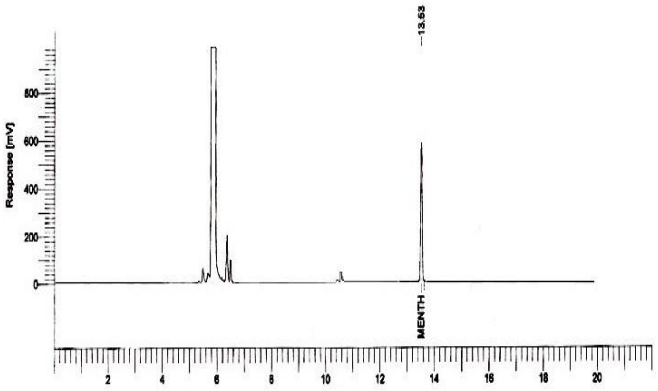

Fig. 8a: Typical chromatogram of menthol in assay standard preparation

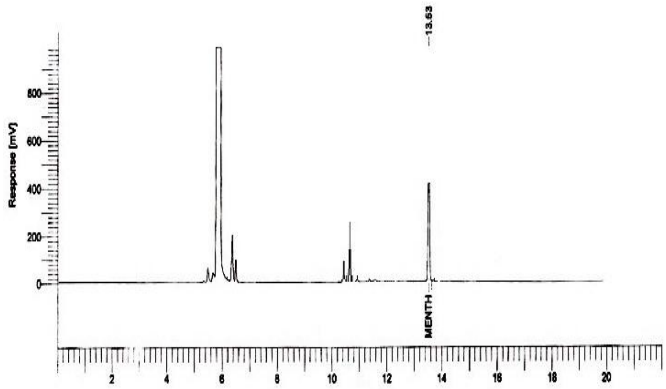

Fig. 8b: Typical chromatogram of peppermint oil in assay preparation

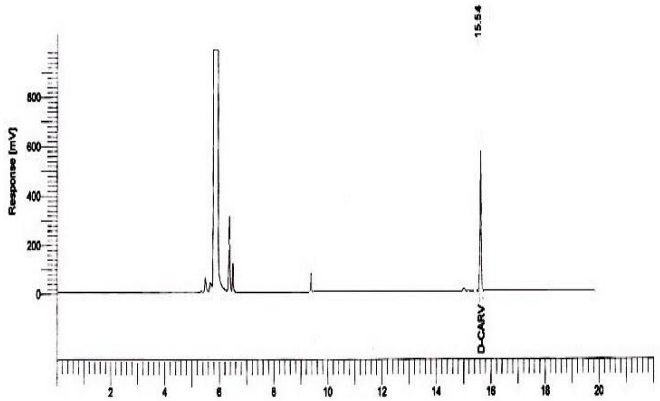

Fig. 9a: Typical chromatogram of marker compound of D-carvone in standard preparation

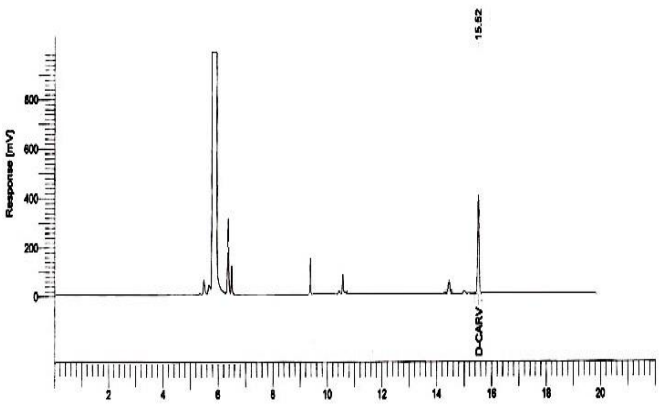

Fig. 9b: Typical chromatogram of caraway in assay preparation

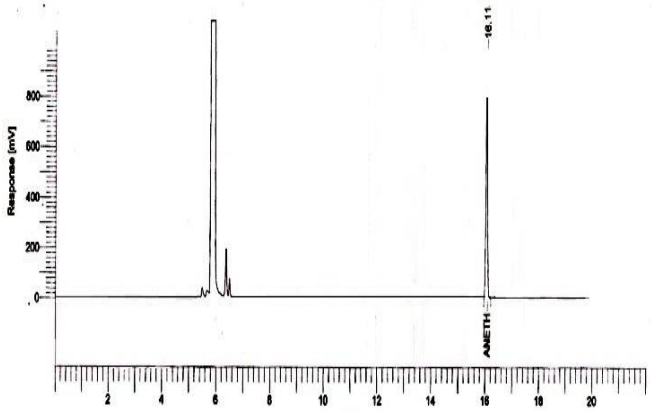

Fig. 10a: Typical chromatogram of marker compound of anethol in standard preparation

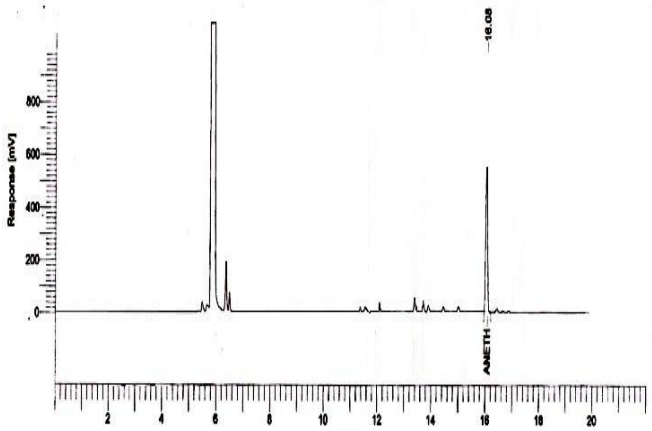

Fig. 10b: Typical chromatogram of fennel oil assay preparation

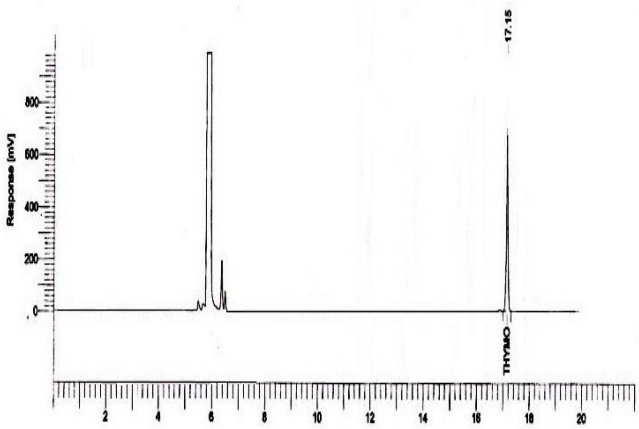

Fig. 11a: Typical chromatogram of marker compound of thymol in standard preparation

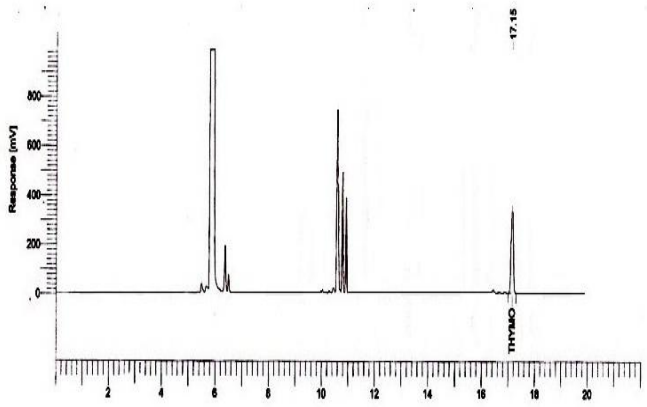

Fig. 11b: Typical chromatogram of ajowan oil in assay preparation

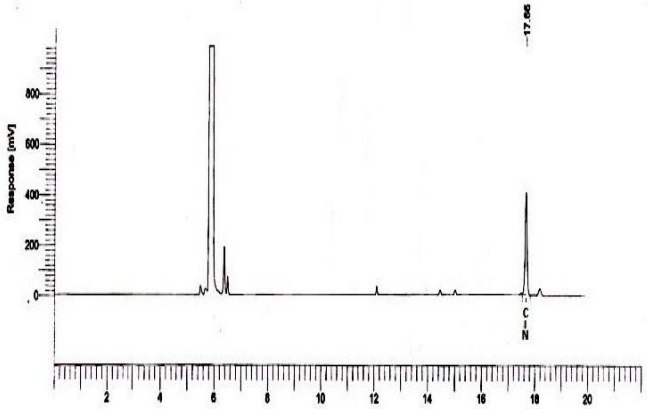

Fig. 12a: Typical chromatogram of marker compound of cineol in assay preparation

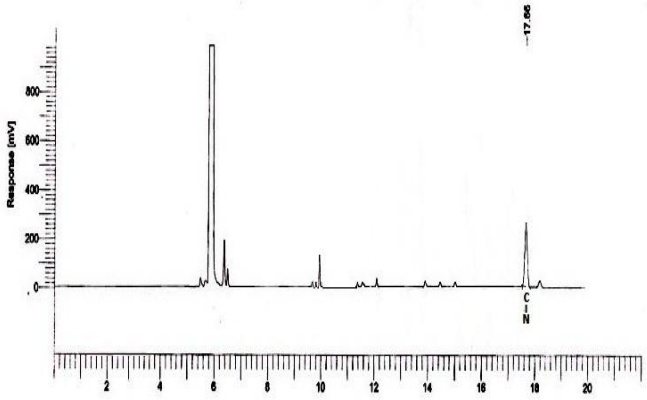

Fig. 12b: Typical chromatogram of cardamom oil in assay preparation

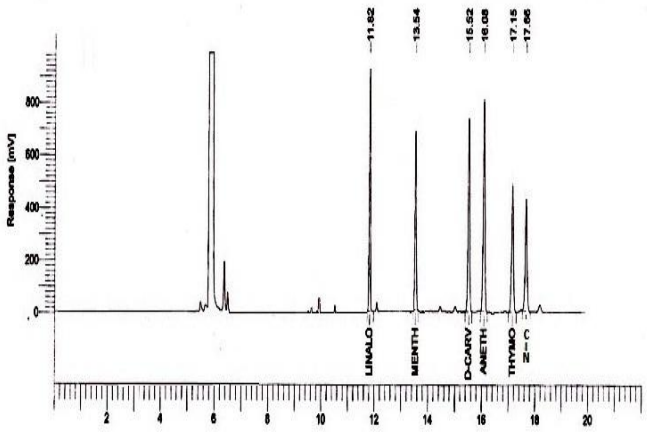

Fig. 13a: Typical chromatogram of system suitability solution of standard

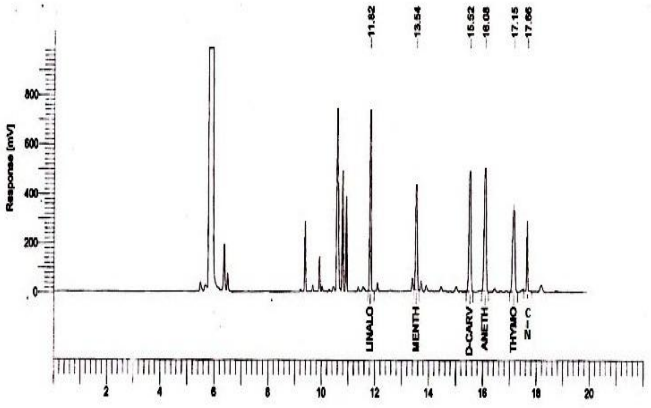

Fig. 13b: Typical chromatogram of blend sample solution in precision

RESULT AND DISCUSSION

Physical characterizations of the oils that odor, color, refractive index and optical rotation had done and match with their specification available in the data bank & literature are tabulated in Table 4.

Mass spectrum of marker compound

The obtained Linalool mass m/z 154 [M+H], m/z 153 [M+H-H_2], m/z 137 [M+H-H_2O] , D-ne mass m/z 151 [M+H], m/z 149 [M-H], m/z 137 [M+H] , m/z 123 [M+H], Anethol mass m/z 148 [M+H], m/z 135 [M+H], m/z 121 [M+H], Thymol mass m/z 151 [M+H], m/z 137 [M+H] , m/z 94 [M+H], Cineole mass m/z 155 [M+H], m/z 137 [M+H-H_2O] $^+$, m/z 111 [M+H-H_2], has identified by comparison of the obtained mass spectra of the peaks with those standards both reported in the literature and available in the data system library.

Identification and determination of purity of individual oil

The identification has made based on finger print comparison between the retention time of each marker peak present in individual oils with their respective marker compound. Linalool is a marker compound of Coriander oil. In the figure 7a Linalool peak was at about 11.84 minutes which is match with the Figure 7b of Coriander oil sample chromatogram. Like this in Figure 8a, 9a, 10a, 11a and 12a of menthol RT about 13.54 minutes, D-carvone at about 15.52, anethol 16.15 minutes, thymol 17.15 and Cineol 17.68 has match with figure 8b, 9b, 10b, 11b and 12b of peppermint, caraway, fennel, ajowan and cardamom oil sample chromatogram. Purity of the oil has determined from assay of individual oil based on the percentage of marker compound present in the respective oil are tabulated Table 5.

System suitability and Precision, Repeatability with intraday precision and Bench top stability

System suitability parameter was verified from the standard injection was tabulated in Table 5 and the chromatogram shown in Figure 13a.

The result of precision, ruggedness as intraday precision and bench top stability of the sample and standard was calculated were tabulated in Table 6, 7.1, 7.2 and 8. From the % relative standard deviation of

assay of six replicate preparation of sample was found less than 2. Ruggedness of intraday precision, bench top stability results shows that the method is precise at any condition and the solution of sample and standard are sable up to 2 day.

Linearity, Limit of detection and quantification

The regression curve was plot by Area (y-axis) vs Concentration (X-axis) has shown in figure 14 and the r^2 value was calculated and found 0.999 in all marker compounds are tabulated in Table 9. LOD & LOQ result was verified based on the signal to noise ratio (S/N).

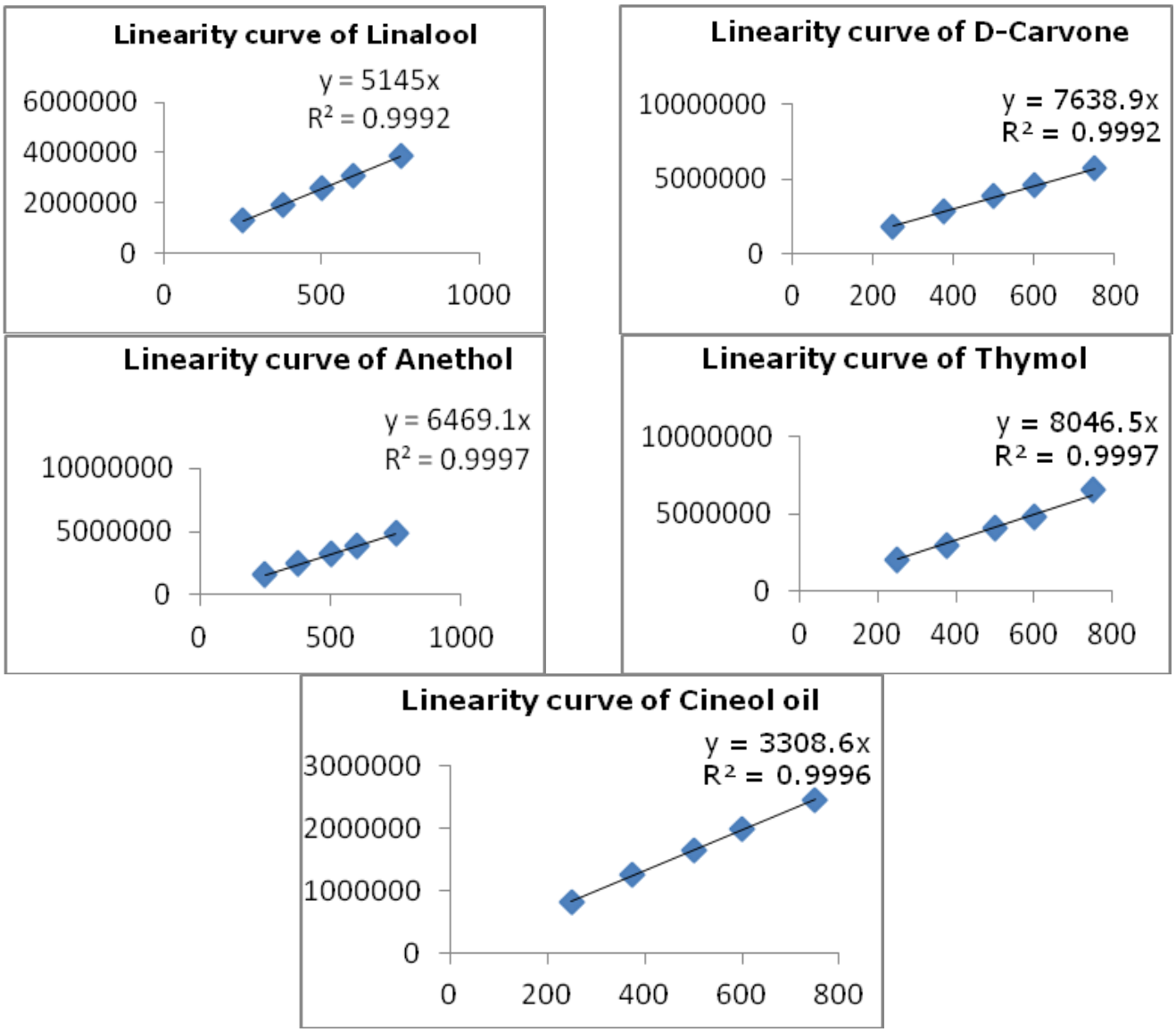

Fig. 14: Linear curve of the marker compound

Accuracy and range

The recovery and relative standard deviation for each of the analytes were calculated and noted down are tabulated in Table 10. From the recovery study it is evident that the method is highly accurate and can give true results from the range of 50% level to 150% level.

In the present study, an accurate and reliable analytical method for the identification and estimation of purity of active compounds of essential oil (Cineol, linalool, Anethol, D-Carvone, thymol) was established by GC-HS method coupled with FID. Good linearity, repeatability, intra-day and inter-day precision, accuracy and reliability were presented in the method validation procedure.

The proposed method makes the possibility to characterize of the compound from the multi component mixer compound with their marker compound in a shorter run time. Headspace is a modern technique that applied in the method to reduce the time of analysis as well as make accurate results. QBD base experiment design gives confidence on the method and it is a tool to prove the design space of critical parameter. Hence it is easy to determine the purity of the compound which gives a tool of analysis of herbal industry.

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