Simultaneous Estimation of Menthol, Camphor and Methyl Salicylate in Anti-Arthritic Spray Using Gas Chromatography

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Research Article

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ABSTRACT

A simple, rapid, accurate, precise and robust Gas Chromatographic (GC) method has been developed for the simultaneous estimation of Menthol, Camphor and Methyl Salicylate from an anti-arthritic spray. The separation of these three constituents was performed on DB-23 column (60 m × 250 µm × 0.25 µm). The Nitrogen was used as carrier gas at a flow rate of 2.5 mL/min. The inlet temperature and detector temperature were set as 250°C and 280°C respectively. The initial oven temperatures was 125°C for 5 min, then increase to 150°C at the ramp of 3°C/min and finally reach to 200°C at the ramp of 10°C/min. The ALS (Automatic Liquid Sampler) system was used for sample injection. The injection volume was 2 µl. The run time of chromatogram was 20.333 min. The proposed GC method provides a good resolution of Menthol, Camphor and Methyl Salicylate. The method was validated as per ICH guidelines. The linearity of Menthol, Camphor and Methyl Salicylate were found to be in the range of 10-50 µg/mL, 20-60 µg/mL and 100-300 µg/mL respectively. The recovery was calculated by using external standard method and the mean average recovery for Menthol, Camphor and Methyl Salicylate were found to be 100.55%, 99.93% and 99.95%. The tested validation parameters were found to be within acceptable limits. The method was successfully applied for quantification of these three ingredients in anti-arthritic spray formulation.

INTRODUCTION

Menthol, Camphor and Methyl Salicylate are the active ingredients of many topical formulations used for the treatment of rheumatic diseases. These are common ingredients used in certain topical preparation due to their analgesic and anti-inflammatory activity [1] (Figure 1).

Since time immemorial, various herbs and their derived compounds have been used in treatment of inflammation. Inflammation is a normal, protective response to tissue injury caused by physical trauma, noxious chemicals or microbiologic agents, which is a part of the host defense. Sometimes, inflammation seems to produce events that are quite serious and become chronic like occurrence of rheumatoid arthritis and hay fever which may be life threatening. Rubor (redness), calor (heat), tumor (swelling) and dolor (pain) are the main signs of inflammation [2].

S. P. Sethy has found from his review that GC with capillary column can be suitable for simultaneous identification and detection of active substance as well as purity evaluation in the presence of matrix constituents. Thus such an injection method increase the stability of the injected sample, thus also decreasing the number of peaks in the chromatogram Therefore an attempt has been made to establish conditions for identification and determination active constituents in the presence of a base constituents, while considering the effect on the results obtained from an gel containing menthol and methyl salicylate by using capillary gas chromatography [3].

Because of the volatility of these constituents, it is not surprising that these compounds are generally determined with GC. Few reports can be found about the determination of these constituents in pharmaceutical products [4].

A recent trip to a community pharmacy revealed 35 different ingredients. Of those products, 16 contained only menthol in amount ranging from 1.27% to 16%. One spray product listed menthol as the only active ingredients but no content was given. Two products contained menthol and camphor only, but neither listed content for camphor and only one product listed menthol at 2.5%. Fourteen products contained menthol and 30% methyl salicylate. One of the store brand product contained menthol and methyl salicylate as active ingredients in unlisted amounts. Three products contained all the ingredients, with the most concentrated product containing 30% methyl salicylate, 10% menthol and 4% camphor. These products are manufactured and sold in a variety of forms including: creams, gels, balms, rub-on-sticks, patches, pain reliever mousses etc [5].

Assay methods for the quantitative determination of combinations of any two of these drugs have been reported. These methods were based on Gas-Liquid Chromatography (GLC) since these drugs are volatile substances. However, only a few methods were published which allowed the assay of mixtures containing all three drugs [6].

Since many pharmaceutical preparations contain camphor and menthol, it is desirable to use a method which is both fast and accurate for the simultaneous determination of camphor and menthol [7].

In this paper, we have reported a gas chromatographic method for simultaneous estimation of these three constituents from anti-arthritic spray which contains Menthol, Camphor and Methyl Salicylate 3.0%, 4.0% and 20% respectively. The literature review reveals that till now, no any published method is reported for estimation of these three constituents from anti-arthritic spray which is research product yet to be commercialized. To confirm suitability of developed method for routine analysis, method was validated as per the ICH guidelines [8].This method can be successfully applied for routine quality control analysis.

Figure 1. Structures of active ingredients.
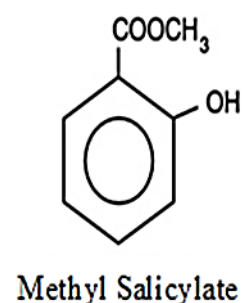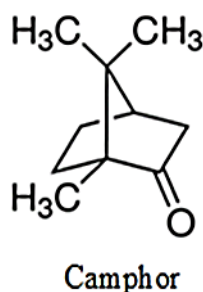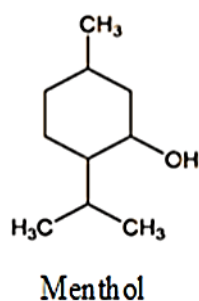
MATERIALS AND METHODS

The method was developed on GC of Agilent Technologies equipped with FID (Flame Ionization Detector) in 2017-18 at ICPA healthcare products ltd. Ankleshwar by using following parameters.

Chromatographic parameters

- **Column**: DB-23 (60 m × 250 µm × 0.25 µm)
- **Oven temperature**: 125°C(5 min), increase to 150°C (ramp 3°C/min), reach to 200°C (ramp 10°C/min)
- **Time of run**: 20.333 min
- **Inlet temperature**: 250°C
- **Detector temperature**: 280°C
- **Mode**: Split
- **Split ratio**: 20:1
- **Injection volume**: 2 µl
- **Carrier gas**: Nitrogen
- **Flow rate**: 2.5 mL/min
- **H2 flow**: 30 mL/min
- **Air flow**: 300 mL/min
- **N2 makeup flow**: 25 mL/min

Reagents and materials

The following reagents and materials were used: Menthol of Hindustan Mint and Agro Products Pct. Ltd., Camphor of Camphor and Allied Product, Methyl salicylate of John Aromas and Methanol of Merck (all ingredients available at ICPA healthcare product ltd. Ankleshwar, Gujarat, India).

Preparation of standard mixture

Accurately weighed quantity of 1 gm Menthol, 1 gm Camphor and 4 gm Methyl Salicylate were transferred into separate 100 mL volumetric flasks; dissolved each in 50 mL of methanol and sonicated for 15 minutes. Final volume was made upto 100 mL with methanol of each flask to prepare standard stock solutions having 10000 μg/mL Menthol, 10000 μg/mL Camphor and 40000 μg/mL Methyl Salicylate concentrations. From those stock solutions, 6 mL of Menthol, 8 mL of Camphor and 10 mL of Methyl Salicylate were withdrawn from respective flasks and transferred into one common 100 mL volumetric flask and final volume was made up with methanol.

Preparation of test solution

The anti-arthritic spray sample was shaken well and 2 mL was withdrawn and transferred to 100 mL volumetric flask; dissolved in 50 mL of methanol and sonicated for 20 minutes. Final volume was made up to 100 mL with methanol.

Chromatographic procedure

Using ALS (Automatic Liquid Sampler) equipped with 10 μl syringe, 2 μl of standard solution and 2 μl of test solution were injected into gas chromatographic system under the operating conditions described above and chromatograms were recorded (Figures 2 and 3).

Figure 2. Standard mixture.

Back Signal Results Name	Retention Time	Area	Area Percent	Resolution (USP)
DILUENT	2.635	1395478344	98.25	0.00000
MENTHOL	6.879	3830185	0.27	56.46910
CAMPHOR	7.387	4975939	0.35	5.14268
WINTER GREEN OIL	9.410	16004743	1.13	19.52121
Totals		1420289211	100.00	

Figure 3. Test sample.

Name	Retention Time	Area	Area Percent	Resolution (USP)
DILUENT	2.637	1395848793	97.21	0.00000
	2.849	22847	0.01	1.35539
	4.706	6375309	1.31	14.01070
	6.129	127212	0.03	10.73472
MENTHOL	6.888	3832174	0.16	56.45313
CAMPHOR	7.380	4903372	0.22	5.14266
WINTER GREEN OIL	9.408	15934890	1.02	19.53362
	11.274	287780	0.04	8.95498
Totals		1427332377	100.00	

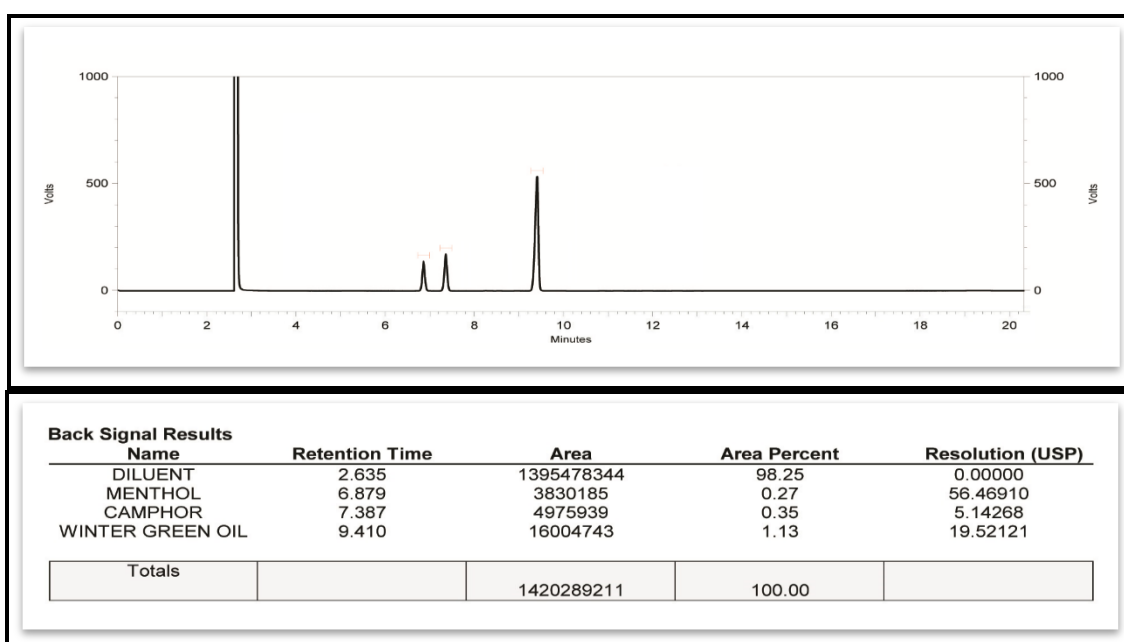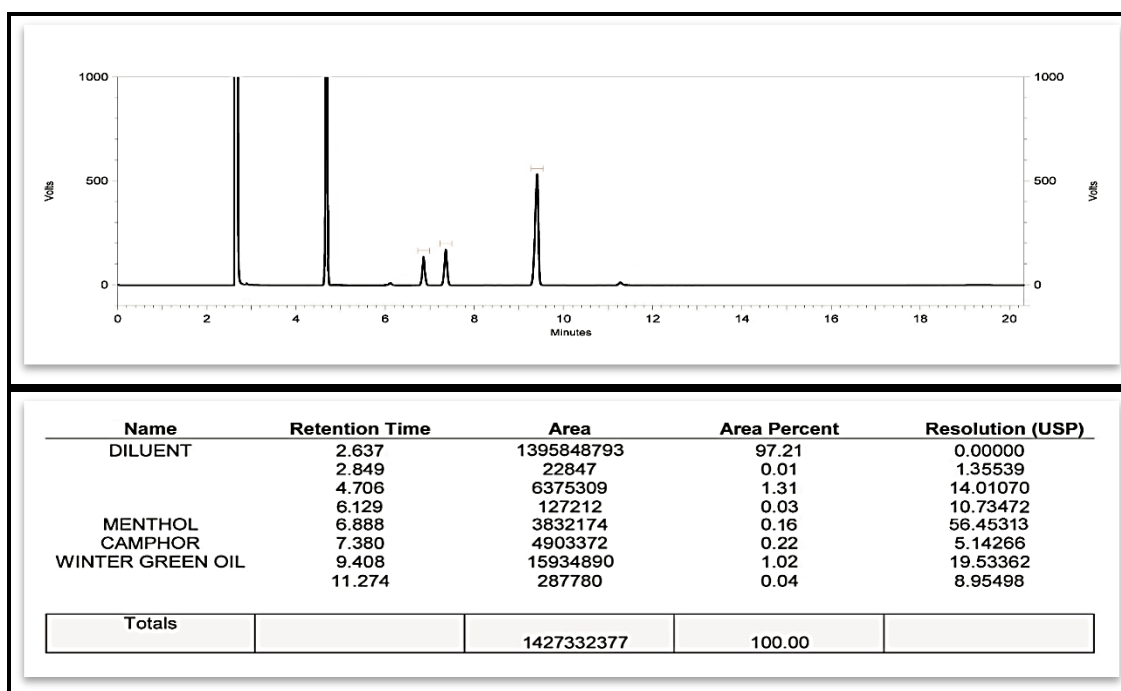## Assay

The individual solutions of Menthol (600 ppm), Camphor (800 ppm) and Methyl Salicylate (4000 ppm) were injected to the GC system with respect to developed chromatographic method and then sample solution was injected (Table 1). The area of the standard solutions as well as sample was determined and assay was performed by using following equation:

$$\% \, Assay = \frac{Test \, area}{Std \, area} \, X \, \frac{Std \, conc}{Test \, conc} \, X \, \frac{purity \, of \, std}{Label \, Claim} \, X \, 100$$

Method validation

Specificity: Specificity is a procedure to detect quantitatively the analytes of interest in presence of components that may be present in the sample matrix. Specificity was tested against standard compounds and in the formulation against potential interferences of excipients.

Linearity and range: The linearity is evaluated through a linear regression analysis. Linearity levels for Menthol, Camphor and Methyl Salicylate at range of 10-50 µg/mL, 20-60 µg/mL and 100-300 µg/mL respectively (as per their concentrations in formulation) were determined in terms of correlation coefficient (Figures 4-6) (Tables 2-4).

Accuracy: The analytical method should be able to recover actual amount of the analyte from the formulation. Accuracy was determined by calculating the %recovery by External Standard method in which three Placebo samples were taken and known amount of Menthol, Camphor and Methyl Salicylate were added in them at concentrations of 80%, 100% and 120% respectively. These solutions were injected in

GC system and %Recovery was calculated. These were repeated 3 times and mean recovery was calculated (Tables 5-7).

Precision: Precision was considered at different levels.

Repeatability: Repeatability expresses the precision under same operating conditions over short interval of time. Take 0.1 mL of test solution in 100 mL volumetric flask and diluted up to mark with Methanol to get concentrations of 30 μg/mL Menthol, 40 μg/mL Camphor and 200 μg/mL Methyl Salicylate and analyze the procedure for 10 times and get the result (Table 8).

Intermediate precision: Intermediate precision was determined by analyzing the test solution (0.1 mL test sample in 100 mL Methanol) 10 times by different analysts and by same analyst in different days (day 1 and day 8) and %RSD was calculated (Tables 9 and 10).

Intraday precision: Intraday precision was determined by analyzing the test solution (0.1 mL test sample in 100 mL Methanol) 10 times in the same day and %RSD was calculated (Tables 11 and 12).

Robustness: The robustness of the method was established by making deliberate minor variations in the method parameters (Tables 13-15).
Change following parameters one by one and observe their effect on assay.
i. Change in mobile phase flow rate by ± 0.2 mL/min
ii. Change in initial oven temperature by ± 5 °C

System suitability: System suitability testing is an integral part of many analytical procedures. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. Here, Resolution between all three peaks, Theoretical plates and Peak Asymmetry were calculated by injecting test samples 5 times and % RSD was calculated (Tables 16-18).

RESULTS AND DISCUSSION

Assay

Table 1. Assay of developed formulation.

Constituents	% Assay
Menthol	99.97%
Camphor	98.54%
Methyl Salicylate	99.56%

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Specificity

No interference was detected at the retention time of Menthol, Camphor and Methyl Salicylate in sample solution.

Linearity and range

Figure 4. Linearity of menthol.
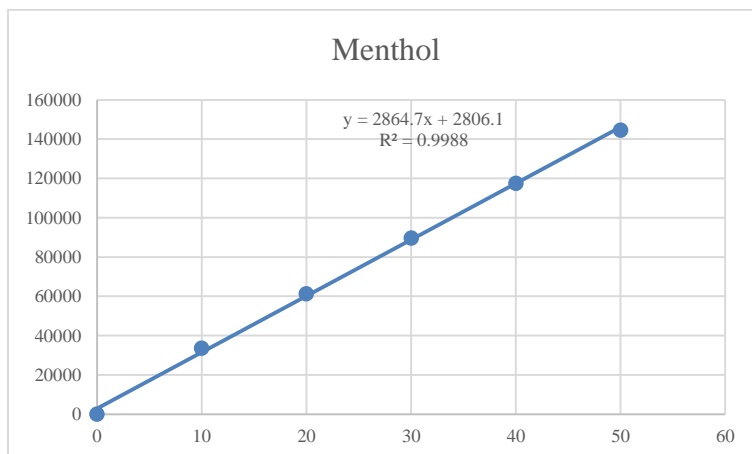
Table 2. Linearity and range of menthol.

Menthol	
Concentration	Peak Area
0	0
10	33548
20	61339
30	89669
40	117476
50	144503

Figure 5. Linearity of camphor.
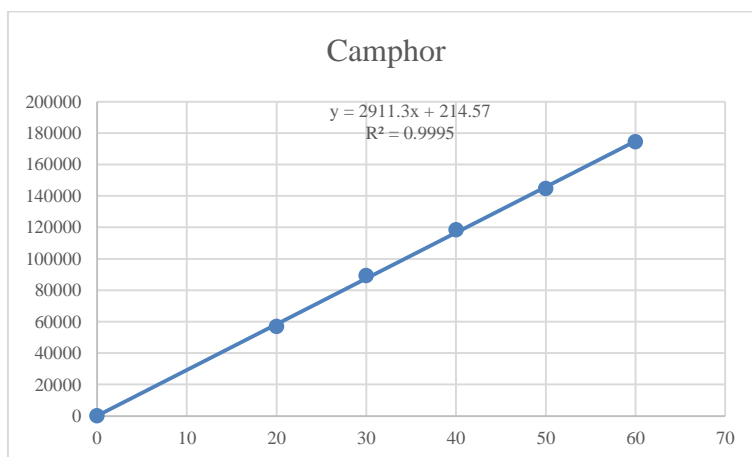
Table 3. Linearity and range of camphor.

Camphor	
Concentration	Peak Area
0	0
20	56922
30	89223
40	118385
50	144700
60	174320

Figure 6. Linearity of methyl salicylate.

Methyl Salicylate

$y = 2008.1x + 397.79$
$R^2 = 0.9998$
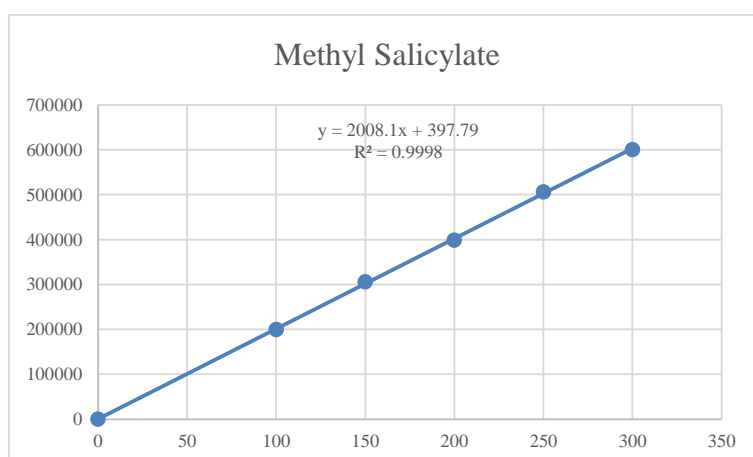
Table 4. Linearity and range of methyl salicylate.

Methyl Salicylate	
Concentration	Peak Area
0	0
100	199567
150	305570
200	398666
250	506184
300	600486

Accuracy

Table 5. Recovery study of menthol.

Menthol						
Sr. No.	% Level	% of menthol added in placebo	% Assay	% RSD	% Recovery	Average % Recovery ± SD
1	80	81.35	80.89	0.4247	101.11	101.07 ± 0.1923
2		80.84	80.65		100.81	
3		81.19	81.02		101.28	
1	100	100.40	100.78	0.4587	100.78	100.09 ± 0.4452
2		100.69	99.43		99.43	
3		100.28	100.07		100.07	
1	120	121.00	120.62	0.3013	100.51	100.50 ± 0.4558
2		120.74	121.26		101.05	
3		121.20	119.92		99.93	
Mean % Average Assay						100.55 ± 0.4007

Table 6. Recovery study of camphor.

			Camphor			
Sr. No.	% Level	% of camphor added in placebo	% Assay	% RSD	% Recovery	Average % Recovery ± SD
1	80	80.58	80.14	0.6126	100.17	99.85 ± 0.6042
2		80.75	79.20		99.00	
3		80.84	80.29		100.37	
1	100	100.32	99.91	0.5126	99.91	99.69 ± 0.4602
2		100.25	100.12		100.12	
3		100.16	99.05		99.05	
1	120	120.24	120.13	0.1788	100.11	100.24 ± 0.1481
2		120.28	120.20		100.16	
3		120.34	120.54		100.45	
Mean % Average Assay						99.93 ± 0.4041

Table 7. Recovery study of methyl salicylate.

			Methyl Salicylate			
Sr. No.	% Level	% of camphor added in placebo	% Assay	%RSD	% Recovery	Average % Recovery ± SD
1	80	80.05	80.16	0.4696	100.20	99.68 ± 0.4849
2		80.08	79.23		99.03	
3		80.07	79.84		99.80	
1	100	100.06	99.92	0.3464	99.92	100.34 ± 0.3358
2		100.07	100.35		100.35	
3		100.08	100.74		100.74	
1	120	120.09	119.67	0.2747	99.73	99.82 ± 0.2838
2		120.10	119.44		99.53	
3		120.07	120.25		100.21	
Mean % Average Assay						99.95 ± 0.3681

Precision

Repeatability

Table 8. Repeatability.

Sr. no.	Menthol		Camphor		Methyl salicylate	
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	6.867	89651	7.362	117294	9.355	397922
2	6.878	88852	7.36	118485	9.362	398568
3	6.861	89769	7.366	118395	9.351	397025
4	6.861	89689	7.372	119285	9.354	398190
5	6.869	89702	7.365	117385	9.361	398126
6	6.864	89569	7.369	118402	9.36	397590
7	6.873	88669	7.362	118345	9.359	399489
8	6.865	89675	7.364	116585	9.36	389012
9	6.871	88969	7.37	118485	9.355	398663
10	6.868	90193	7.365	119385	9.356	389564
Mean	6.8677	89473.8	7.3655	118204.6	9.3573	396414.9
SD	0.00508	455.2893	0.00364	832.8762	0.003407	3619.129
%RSD	0.0739	0.5088	0.0494	0.7046	0.0364	0.9129

Intermediate precision

Day 1 of interday precision and analyst 1 precision are considered to be same as repeatability.

Table 9. Interday precision at day 8.

	DAY–VIII, ANALYST-I					
	Menthol		Camphor		Methyl salicylate	
Sr. no.	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	6.865	89640	7.360	117405	9.354	398129
2	6.877	88765	7.361	118398	9.362	398568
3	6.881	88799	7.366	118612	9.351	397025
4	6.879	89486	7.372	119081	9.355	398190
5	6.864	89512	7.385	117854	9.371	398126
6	6.865	88956	7.369	118366	9.360	397590
7	6.870	89570	7.362	117447	9.349	399489
8	6.866	87999	7.364	118476	9.345	389012
9	6.876	88406	7.370	118333	9.355	398663
10	6.869	89454	7.365	118994	9.356	389564
Mean	6.8712	89058.7	7.3674	118296.6	9.3558	396435.6
SD	0.006129	534.4996	0.00696	544.2794	0.006911	3628.27
%RSD	0.0891	0.6001	0.0944	0.4600	0.0738	0.9152

Table 10. Intermediate precision by analyst 2.

	ANALYST-II					
	Menthol		Camphor		Methyl salicylate	
Sr. no.	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	6.860	90177	7.372	117899	9.364	398875
2	6.864	89635	7.368	117885	9.356	398112
3	6.859	89225	7.370	119045	9.365	389898
4	6.861	90368	7.371	117466	9.360	398853
5	6.861	88996	7.378	119154	9.359	398213
6	6.864	89235	7.369	117980	9.358	397562
7	6.865	89534	7.368	118036	9.365	395633
8	6.860	89662	7.370	119256	9.359	398863
9	6.863	89457	7.361	118622	9.368	386900
10	6.862	90111	7.368	118765	9.368	398876
Mean	6.862	89640.0	7.370	118410.8	9.362	396178.500
SD	0.0019	428.5340	0.0040	599.6747	0.0041	4056.2496
%RSD	0.0280	0.4781	0.0544	0.5064	0.0437	1.0238

Intraday precision

Table 11. Intraday precision analysis 1.

Sr. no.	Menthol		Camphor		Methyl salicylate	
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	6.861	89888	7.369	118645	9.361	398850
2	6.862	88354	7.368	117840	9.359	389523
3	6.861	88569	7.370	118752	9.361	397564
4	6.870	89567	7.366	116662	9.357	398047
5	6.871	89043	7.368	118003	9.362	398856
6	6.869	90111	7.363	118753	9.367	398740
7	6.872	88045	7.370	119874	9.359	398500
8	6.870	90256	7.372	118630	9.355	398014
9	6.861	89114	7.367	118554	9.368	386483
10	6.865	88236	7.368	118976	9.357	389711
Mean	6.866	89118.3	7.368	118468.9	9.361	395428.800
SD	0.0044	767.8271	0.0023	795.8635	0.0040	4577.5865
%RSD	0.0641	0.8616	0.0318	0.6718	0.0428	1.1576

Table 12. Intraday precision analysis 2.

Sr. no.	Menthol		Camphor		Methyl salicylate	
	RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area
1	6.868	89874	7.364	117459	9.359	397985
2	6.862	88463	7.369	117946	9.358	398457
3	6.860	89156	7.366	119350	9.360	398024
4	6.859	88687	7.368	118868	9.362	397843
5	6.865	90041	7.370	118705	9.361	397825
6	6.864	89337	7.365	117985	9.357	398051
7	6.868	89024	7.364	117563	9.368	389921
8	6.866	88647	7.368	118912	9.365	397853
9	6.862	90178	7.366	118175	9.358	386631
10	6.861	89630	7.372	119045	9.362	395530
Mean	6.864	89303.7	7.367	118400.8	9.361	395812.000
SD	0.0030	579.5888	0.0025	625.1182	0.0033	3912.0454
%RSD	0.0443	0.6490	0.0342	0.5280	0.0348	0.9884

Robustness

Robustness of menthol

Table 13. Robustness of menthol.

Condition	% RSD of MEN in Std mixture	% RSD of MEN in test	% Average Assay	%Difference in Assay
(1) Change in flow rate ± 0.2 mL/min				
2.5 mL/min (Normal)	0.3357	0.6964	100.08	-
-0.2(2.3 mL/min)	0.5095	0.3778	100.14	0.06
+0.2(2.7 mL/min)	0.1962	0.2887	100.59	0.51
(2) Change in initial oven temperature 5 °C				
125 °C (Normal)	0.3357	0.6964	100.08	-
-5(120 °C)	0.1533	0.3829	99.98	0.1
+5(130 °C)	0.4570	0.1546	100.19	0.11

Robustness of camphor

Table 14. Robustness of camphor.

Condition	% RSD of CAM in Std mixture	% RSD of CAM in test	% Average Assay	% Difference in Assay
(1) Change in flow rate ± 0.2 mL/min				
2.5 mL/min (Normal)	0.2608	0.2620	100.78	-
-0.2(2.3 mL/min)	0.2609	0.3405	99.99	0.79
+0.2(2.7 mL/min)	0.5106	0.5638	100.14	0.64
(2) Change in initial oven temperature 5 °C				
125 °C (Normal)	0.2608	0.2620	100.78	-
-5(120 °C)	0.8100	1.2301	100.00	0.78
+5(130 °C)	0.8271	0.7622	100.70	0.08

Robustness of methyl salicylate

Table 15. Robustness of methyl salicylate.

Condition	% RSD of MeS in Std mixture	% RSD of MeS in test	% Average Assay	% Difference in Assay
(1) Change in flow rate ± 0.2 mL/min				
2.5 mL/min (Normal)	0.7621	0.7526	100.08	-
-0.2(2.3 mL/min)	1.1732	1.1506	100.44	0.36
+0.2(2.7 mL/min)	0.6571	0.3179	101.04	0.96
(2) Change in initial oven temperature 5 °C				
125 °C(Normal)	0.7621	0.7526	100.08	-
-5(120 °C)	0.6869	0.8176	99.46	0.62
+5(130 °C)	1.0876	0.3588	99.72	0.36

System suitability parameters

Table 16. System suitability parameters for menthol.

Sr. no.	Menthol		
	Resolution	Theoretical plates	Asymmetry
1	0	68054	1.10288
2	0	65986	1.09914
3	0	67843	1.09874
4	0	69331	1.10341
5	0	68877	1.09965
Mean	0	68018.200	1.101
SD	0	1150.8949	0.0020
%RSD	0	1.6920	0.1792
Limit	>2	>2000	<2

Table 17. System suitability parameters for camphor.

Sr. no.	Camphor		
	Resolution	Theoretical plates	Asymmetry
1	4.78	87571	1.00019
2	4.79	89631	0.99865
3	4.77	88014	1.00547
4	4.79	85122	1.01372
5	4.78	87103	0.99971
Mean	4.782	87488.2	1.003548
SD	0.0075	1457.5455	0.0056
%RSD	0.1565	1.6660	0.5587
Limit	>2	>2000	<2

Table 18. System suitability parameters for methyl salicylate.

Sr. no.	Methyl Salicylate		
	Resolution	Theoretical plates	Asymmetry
1	20.11	147311	0.87564
2	20.09	150744	0.90411
3	20.10	146682	0.89772
4	20.11	148134	0.89980
5	20.10	152365	0.91003
Mean	20.102	149047.2	0.89746
SD	0.0075	2160.0659	0.0117
%RSD	0.0372	1.4492	1.3031
Limit	>2	>2000	<2

CONCLUSION

A simple, accurate, rapid and economic Gas Chromatographic method has been developed and validated for simultaneous estimation of Menthol, Camphor and Methyl Salicylate in developed anti-arthritic spray formulation. The method assured satisfactory linearity, accuracy and precision. The method is robust and % recovery in spray formulation is good. Analysis of sample containing Menthol, Camphor and Methyl Salicylate showed no interference from the other excipients and additives. The proposed method can be easily and conveniently adopted for routine quality control analysis.

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