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RESEARCH ARTICLE

Densitometric HPTLC Methods for Qualitative and Quantitative Analysis of Carvone, Dillapiole, Methylparaben and Propylparaben in Dill, Caraway seeds and Pharmaceutical Formulations Utilizing Normal and Reversed-Phase Silica Gel Plates

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SUMMARY

The monoterpene ketone (4S)-(+)-carvone is found naturally in many essential oils such as caraway (Carum carvi) and dill (Anethum graveolens) seeds. Popular marketed baby formulas for relief of colic contain dill or caraway oils or extracts. In attempts to use HPTLC for estimation of carvone in such preparations as well as dill and caraway oils or extracts two methods were developed. Some formulations showed two prominent spots identified as methylparaben and propylparaben spectroscopically after chromatographic purification. Method I was developed and validated with 10 × 20 cm glass-backed plates coated with 0.2 mm layers of silica gel 60 F_{254} using n-hexane-ethyl acetate (9:1 v/v) as developing system. The used system resulted in sharp and symmetrical peak at Rf value of 0.34±0.02 and linearity were found in range 100-800 ng/spot (R2 = 0.9986) for carvone. However, in case of dill seeds extract an overlapping spot was detected and carvone could not be estimated properly. Chromatographic purification and spectroscopic analysis enable the identification of the overlapping spot as dillapiole. Consequently, method II was carried out with 10 × 20 cm glass-backed plates coated with 0.2 mm layers of RP-18 silica gel 60 F254 using methanol-water (8:2 v/v) as mobile phase. Using method II simultaneous determination of carvone, dillapiole, propylparaben and methylparaben was achieved. The R_f values were 0.39 ± 0.04, 0.28 ± 0.04, 0.51 ± 0.02 and 0.59 ± 0.02 and linearity were found in range 100-800 ng/spot (R^2 = 0.9982), 100-1000 ng/spot (R^2 = 0.9984), 50-1000 ng/spot (R^2 = 0.9984) and 50-1000 ng/spot (R^2 = 0.9983) for carvone, dillapiole, methylparaben and propylparaben respectively. The two methods were found to be simple, precise, specific, and can be applied for standardization of different pharmaceutical formulations.

Key Words: *Carvone; Dillapiole; Methylparaben; Propylparaben; HPTLC; ICH guidelines.*

Dereotu ve Kimyon tohumlarındaki Karvon, Dillapiol, Metilparaben ve Propilparabenin Kalitatif ve Kantitatif Analizleri için Densitometrik HPTLC Metodu ve Farmasötik Formülasyonlarda Normal ve Ters Faz Silika Jel Plaklardan Yararlanılması

ÖZET

Monoterpen keton olan (4S)-(+)-karvon kimyon (Carum carvi) ve dereotu (Anethum graveolens) tohumlarında olduğu gibi pek çok uçucu yağda doğal olarak bulunur. Piyasada oldukça popüler olan ve kolik ağrısına karşı rahatlatıcı özellikteki bebek formülasyonları dereotu ve kimyon içeren yağlar veya ekstreleri içermektedir. Dereotu ve kimyon yağları veya ekstrelerin yanı sıra böyle preparatlardaki karvonun değerlendirilmesi için HPTLC çalışmalarında iki metot geliştirilmiştir. Bazı formülasyonlar kromatografik saflaştırma sonrasında spektroskopik olarak propilparaben ve metilparaben olarak tanımlanan iki belirgin noktayı göstermiştir. Metot I geliştirilmiş ve sistem, n-hekzan-etil asetat (9:1 h/h) kullanılarak 0.2 mm kalınlıkta silika jel 60 F254 ile kaplanmış 10 × 20 cm cam destekli plaklar ile valide edilmiştir. Kullanılan sistem, 0.34±0.02 Rf değerinde keskin ve simetrik piklerle sonuçlanmıştır ve karvon için doğrusallık, 100-800 ng/nokta (R2 = 0.9986) aralığında bulunmuştur. Ancak dere otu tohumu ekstresinde üst üste binen bir nokta belirlenmiştir ve karvon düzgün bir şekilde değerlendirilememiştir. Kromatografik saflaştırma ve spektroskopik analizler üst üste binen noktanın dillapiol olarak tanımlanmasına olanak vermiştir. Bu nedenle, Metot II, haraketli faz olarak metanol-su (8:2 h/h) kullanılarak ve 0.2 mm kalınlıkta ters faz-18 silica gel 60 F254 ile kaplanmış 10 × 20 cm cam destekli plaklar ile gerçekleştirilmiştir. Karvon, dillapiol, propilparaben ve metilparabenin eşzamanlı belirlenmesine metot II kullanılarak ulaşılmıştır. Rf değerleri 0.39 ± 0.04, 0.28 ± 0.04, 0.51 ± 0.02 ve 0.59 ± 0.02 dir ve karvon, dillapiol, metilparaben ve propilparaben için doğrusallık sırasıyla 100-800 ng/nokta (R^2 = 0.9982), 100-1000 ng/nokta (R^2 = 0.9984), 50-1000 ng/nokta (R^2 = 0.9984) ve 50-1000 ng/nokta (R^2 = 0.9983) aralığında bulunmuştur. İki metot basit, hassas, özgün ve farklı farmasötik formülasyonların standardizasyonu için uygulanabilir bulunmuştur.

Anahtar kelimeler: *Karvon, Dillapiol, Metilparaben, Propilparaben, HPTLC, ICH Rehberi*

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INTRODUCTION

The monoterpene ketone (4*S*)-(+)-carvone is found naturally in many essential oils, but is most abundant in the oils obtained from caraway (*Carum carvi* L.) and dill (*Anethum graveolens* L.) seeds (1, 2). Caraway seeds are used as a flavouring for many food products and as a source of carvone for cosmetics, toothpaste, chewing gum and pharmaceutical preparations. The seeds have been used in alternative medicine as a laxative, antispasmodic, digestive and as a breath freshener (1). The seeds of *A. graveolens* have been used for relief of digestive problems such as stomachache, indigestion, flatulence, as diuretic and lactagogue (3-5).

It has been reported that chewing its seeds decreases the bad breath (6). Dill essential oil has hypolipidemic activity and could be used as a cardioprotective agent (7). Dill seed oil showed broad antibacterial activity against the Gram-positive bacteria; *Staphylococcus aureus,* MRSA*, Enterococcus* sp. and Gram-negative bacteria; *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* (8). Both fruits and aerial parts extracts significantly decreased indications of pain. The aerial parts showed stronger analgesic effects in the late phase of formalin test. In the hot plate test, fruits and aerial parts extracts showed hyperalgesic properties (9).

(4*S*)-(+)-carvone was effective against *Listeria monocytogenes* (10) and *Candida albicans* (11). It also inhibited viability and proliferation of Hep-2 cells (human epithelial carcinoma cell line) in a dose-dependent manner (12). Several methods were developed for the quantitative analysis of carvone including GC (12-14) unidimensional GC (15) and HPLC (16). The HPTLC methods reported have been used to quantify carvone in plants using normal phase only which are either tedious or expensive methods (17).

To our knowledge, no reports on simultaneous quantitative analysis of carvone, dillapiole, methylparaben and propylparaben in dill, caraway seeds and pharmaceutical formulations utilizing both normal and reversed-phase silica gel plates have been mentioned in the literature. In the present study we have proposed new validated simple HPTLC for the simultaneous determination carvone, dillapiole, methylparaben and propylparaben. The proposed method was validated as per ICH guidelines.

EXPERIMENTAL

Standard and solutions

Standard carvone, dillapiole, methylparaben and propylparaben (Figure 1) were purchased from Sigma Aldrich. Dillapiole was purchased from Shanghai Tauto Biotech Co., Ltd. Pharmaceutical formulations were purchased from local market in Riyadh, Kingdom of Saudi Arabia and Alexandria, Egypt. All the solvents were of HPLC grade and other chemicals used were of analytical reagent (AR) grade.

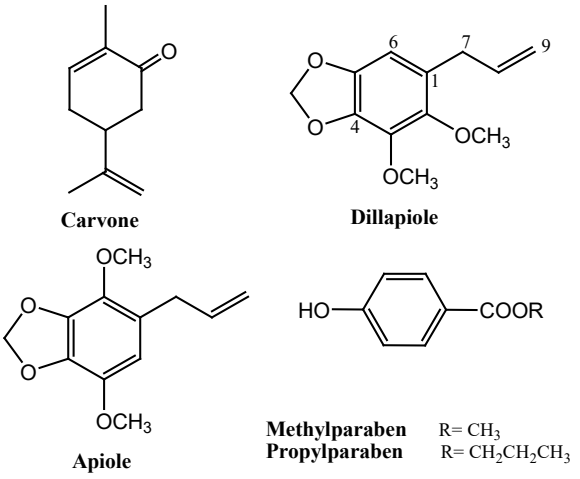

Figure 1. Structures of carvone, dillapiole, apiole, methylparaben and propylparaben

Accurately weighed 10 mg of standard carvone, dillapiole, methylparaben and propylparaben were dissolved in MeOH in a 100 mL volumetric flask to gives concentration of 100 µg/mL used as a reference solution (stock solution).

Sample Preparation for the analysis of carvone, dillapiole, methylparaben and propylparaben in dill, caraway seeds, oils and pharmaceutical formulations

15 g of caraway and dill seeds were separately extract to exhaustion with hot acetone using soxhelt apparatus. The resulted extracts were separately transferred to 50 ml volumetric flasks and completed to volume. Accurately measured 5 ml from each solution was transferred to 25 ml volumetric flask and completed to volume with acetone.

Caraway and dill seed oil were subjected to hydrodistillation. Accurately measure 50 ml from each oil were separately transferred to Clevenger trap apparatus for oils lighter than water, 150 ml of water was added and

distillations were continued for 8 hr. The oil layers and water in the traps were extracted with CH_2Cl_2 and organic layers were concentrated under vacuum, transferred to 25 ml volumetric flasks and completed to volume with CH_2Cl_2.

Three baby water products containing Caraway or Dill essential oils were subjected to liquid-liquid extraction. Accurately measured 50 ml from each product were extracted with CH_2Cl_2 (3x30 ml). The combined organic layer from each product was separately concentrated under reduced pressure and transferred to 25 ml volumetric flask. Volumes were completed to mark with CH_2Cl_2.

Isolation and identification of dillapiole from dill seeds

Dill seeds extract (1 g) obtained from extraction using soxhlet apparatus and acetone as solvent was chromatographed on silica gel column (50 gm x 1cm i.d.) eluted with *n*-hexane. Thirty fractions 25 ml each were collected, screened by TLC and similar fractions were pooled. Fractions 6-8 showed single spot with R_f value similar to carvone. Fractions 9-11 were mixture of carvone and a spot with lower R_f on silica gel TLC using *n*-hexane: ethyl acetate (9:1 v/v) as mobile phase. Fractions 12-15 showed the lower spot with no contamination from carvone. The purity was confirmed on RP-18 plates using methanol: water (8:2 v/v) for development. After complete evaporation of solvents fraction 12- 15 provided 65 mg of pure oily material.

Dillapiole (1-allyl-4, 5-methylenedioxy-2, 3-dimethoxybenzene): $C_{12}H_{14}O_4$, Pale yellow oil; UV λ_{max}: 209, 248, 287. ^{1}HNMR (Bruker UltraShield Plus 500MHz, $CDCl_3$): δ_H 3.33 (2H, d, *J*=6.5, H-7), 3.78 (3H, s, 2-OCH_3), 4.04 (3H, s, 3-OCH_3), 5.05 (1H, s, H-9), 5.07 (1H, d, *J*=6.6, H-9), 5.89 (2H, s, O-CH_2-O), 5.98 (1H, m, H-8), 6.37 (1H, s, H-6). ^{13}CNMR (Bruker UltraShield Plus 125MHz, $CDCl_3$): δ_C 33.92 (C-7), 59.96 (3-OCH_3), 61.29 (2-OCH_3), 101.12 (O-CH_2-O), 102.74 (C-6), 115.57 (C-9), 126.04 (C-1), 135.92 (C-5), 137.41 (C-8),137.41 (C-2), 144.23 (C-3), 144.62 (C-4). EIMS m/z (%): 222 (100, M^+).

Isolation and Identification of Methyparaben and Propylparaben from Pharmaceutical formulation

Residue left after evaporation of the CH_2Cl_2 extract of 100 ml formulation (300 mg) was chromatographed on silica gel column (30 gm x 1cm i.d.) eluting with 5% EtOAc in *n*-hexane. Twenty five fractions 20 ml each were collected screened with TLC and similar fractions were pooled. Fractions 14-18 were subjected to centrifugal preparative TLC (CPTLC) using Chromatotron (Harrison Research Inc. model 7924), 2 mm silica gel P254 disc and 5% EtOAc in *n*-hexane. Two zones were separated and identified as methylparaben and propylparaben by comparison of NMR with the literature (18) then direct comparison with authentic samples.

Chromatographic conditions

In Method I HPTLC densitometric analysis was performed on 10 × 20 cm glass-backed plates coated with 0.2 mm layers of silica gel 60 F_{254} (E-Merck, Germany). In Method II HPTLC densitometric analysis was performed on 10 × 20 cm glass-backed plates coated with 0.2 mm layers of RP-18 silica gel 60 F_{254} (E-Merck, Germany). Samples were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microliter syringe. A constant application rate of 150 nl/s was used. Linear ascending development of the plates to a distance of 80 mm was performed with *n*-hexane: ethyl acetate (9:1 v/v) and methanol: water (8:2 v/v) for Method I and Method II respectively as mobile phase in a Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30 min at 22°C. After development, the plates were scanned at 244 nm for carvone, 209 nm for dillapiole and 260 nm for methylparaben and propylparaben using a Camag TLC scanner IV in absorbance mode, using the deuterium lamp. The slit dimensions were 4.00 × 0.45 mm and the scanning speed was 20 mm/s.

Method validation

The proposed HPTLC densitometric methods were validated according to the guidelines of international conference on harmonization (19). The linearity of carvone was checked between 100-800 ng/spot for method I and linearity were found in range 100-800, 100-1000, 50-1000 and 50-1000 ng/spot for carvone, dillapiole, methylparaben and propylparaben respectively by method II. Graphs were plotted between concentration and peak area for linearity. Linearity data were statistically treated using least square linear regression analysis.

Accuracy

Accuracy was determined by standard addition method. The preanalyzed sample of carvone, dillapiole, methylparaben and propylparaben (300 ng/spot) were spiked with the extra 0, 50, 100 and 150 % of the standard carvone, dillapiole, methylparaben and propylparaben and the solutions were reanalyzed in six replicates by the proposed method or Method I and Method II. The percent recovery and percent relative standard deviation (% RSD) were calculated at each concentration level.

Precision

Precision of the proposed method was determined at two levels i.e. repeatability and intermediate precision. Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of carvone, dillapiole, methylparaben and propylparaben at three different concentration levels of 300, 400 and 500 ng/spot in six replicates for Method I and Method II .

Robustness

Robustness of the proposed HPTLC method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of carvone, dillapiole, methylparaben and propylparaben for method I and method II. Robustness was determined by changing the polarity of the mobile phase.

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

$$LOD = 3.3' SD / S$$

$$LOQ = 10' SD / S$$

Specificity

Specificity of the proposed TLC densitometric was confirmed by analyzing and comparing the R_f values and spectra of the spots for carvone, dillapiole, methylparaben and propylparaben in the samples with that of the standards for method I and method II.

Quantification of Carvone, Dillapiole, Methylparaben and Propylparaben in dill, caraway seeds and pharmaceutical formulations

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard carvone, dillapiole, methylparaben and propylparaben. The area of the peak corresponding to the R_f value of carvone, dillapiole, methylparaben and propylparaben standards were recorded and the amount present were calculated from the regression equation obtained from the calibration plot for Method I and Method II.

RESULTS AND DISCUSSION

The study was designed to develop an HPTLC method for quantification of carvone in its natural sources as well as marketed baby formulations. The use of normal phase silica gel was successful for the estimation of carvone except in case of Dill seeds extract where another spot overlapped with carvone corresponding spot. After identification of the spot as dillapiole method II was developed using RP 18 TLC plates and enable the simultaneous analysis of carvone, dillapiole as well as the two preservatives methyparaben and propylparaben isolated and identified in some formulations.

Identification of Dillapiole, Methylparaben and Probylparaben

The oily material overlapped with carvone in dill extract analyses was isolated by column chromatography on silica gel. ^{13}C NMR of the isolated material showed twelve carbon signals sorted by DEPT experiments into 2 x OCH_3, 3 x CH_2, 2 x CH and 5 quaternary carbons. The chemical shifts of the three methylenes indicated that they include one O-CH-O, one =CH_2 and one benzylic CH_2. These assignments were fully supported by ^1H NMR (experimental). MS data showed an M^+ at m/z 222 consistent with the molecular formula $C_{12}H_{14}O_4$. Such data indicated that the isolated material could be either apiole or its structural isomer dillapiole (20, 21, 22).

Careful study of the ^{13}C NMR of both compounds (20, 21) indicated that the major difference was found in the quaternary carbons and C-6 chemical shifts. The chemical shift of C-1 in apiole is δ_C 110.8 ppm and none of the quaternary carbons chemical shifts exceeded δ_C 140 ppm (20). However, in dillapiole C-1 chemical

shift is δ_C 126.0 ppm while both C-2 and C-3 reading were around δ_C 144 ppm (21). Moreover, C-6 chemical shift was δ_C 108.5 ppm in apiole and δ_C 102.7 ppm in dillapiole (20, 21). Our data (experimental) clearly indicated that the isolated compound is dillapiole. The identity was confirmed after purchase of standard dillapiole and direct TLC comparison.

Two compounds were isolated from pharmaceutical preparations after column chromatography and CPTLC. NMR data for both compounds indicated *para*-hydroxy substituted aromatic nucleus and ester carbonyl. One compound showed signal for OCH_3, while the other for $OCH_2CH_2CH_3$. Comparison with published data indicated that the two compounds are methylparaben and propylparaben (23) used as preservatives in pharmaceutical preparations. Identity was further confirmed by direct comparison with authentic materials.

UV spectra measured for the peaks showed maximum absorbance at approximately 244 nm for carvone by Method I and Method II whereas in case of dillapiole (260 nm) methylparaben and propylparaben (209 nm) by method II. The mobile phase composition was optimized to develop a suitable and accurate TLC densitometric method for analysis of carvone, dillapiole, methylparaben and propylparaben. The mobile phase *n*-hexane: ethyl acetate (9:1, *v/v*) and methanol: water (8:2, *v/v*) were found to have sharp, symmetrical and well resolved peak at R_f value of 0.34±0.02 for carvone by Method I and R_f value of 0.39 ± 0.04, 0.28 ± 0.04, 0.51 ± 0.02 and 0.59 ± 0.02 for carvone, dillapiole, propyl and methylparaben respectively by Method II. The optimized saturation time was found to be 30 min.

The calibration plot of peak area against amount of carvone was linear in the range 100-800 ng/spot by Method I. The linearity of simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 100-800, 100-1000, 50-1000 and 50-1000 ng/spot respectively by Method II. Linear regression data for the plot confirmed the good linear relationship (Table 1). The linear regression equation was Y = 6.556x + 671.2 with correlation coefficient (R^2) of 0.9986 for carvone by Method I whereas simultaneous determination of carvone dillapiole, methylparaben and propylparaben were found to be Y = 6.202x + 627.9, Y = 12.22x + 297.8, Y = 25.79x + 20751 and Y = 17.22x + 1156 with correlation coefficient (R^2) of 0.9982, 0.9984, 0.9984 and 0.9983 respectively by Method II, where Y is response and X is amount of reference standards.

Table 1. Linear regression data for the calibration curve of carvone dillapiole, methyl and propylparaben by Method I and Method II (n=6).

Parameters	Carvone		Dillapiole	Methylparaben	Propylparaben
	(Method I)	(Method II)		(Method II)	
Linearity range (ng/spot)	100-800	100-800	100-1000	50-1000	50-1000
Regression equation	Y = 6.556x + 671.2	Y = 6.202x + 627.9	Y = 12.22x + 297.8	Y = 25.79x + 20751	Y = 17.22x + 1156
Correlation coefficient	0.9986	0.9982	0.9984	0.9984	0.9983
Slope ± SD	6.556 ± 0.03640	6.202 ± 0.03834	12.22 ± 0.06338	25.79 ± 0.1271	17.22 ± 0.08993
Intercept ± SD	671.2 ± 18.38	627.9 ± 19.36	297.8 ± 39.33	2075 ± 75.22	1156 ± 53.22
Standard error of slope	0.014863	0.015655	0.02588	0.0518987	0.036721111
Standard error of intercept	7.505104	7.905267	16.059616	30.714577	21.73132
95% confidence interval of slope	6.483 - 6.629	6.125 to 6.279	12.09 to 12.35	25.53 to 26.04	17.04 to 17.40
95% confidence interval of intercept	634.2 - 708.3	588.9 to 666.9	219.1 to 376.6	1925 to 2225	1049 to 1262
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

The accuracy of the proposed method was calculated by recovery analysis which afforded recovery of 98.28-99.22 for carvone by Method I whereas simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 98.28-99.22, 98.81-99.27, 98.72-99.19 and 98.67-99.16 respectively by Method II after spiking the additional standard drug solution to the previously analyzed test solution. The values of % recovery and % RSD are shown in Table 2. Low values of % RSD was in range of 0.85-1.55 for carvone by method I whereas simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 0.50-1.35, 0.77-1.12, 0.79-1.23 and 0.85-1.37 respectively for method II indicated the good accuracy of the proposed method.

Table 2. Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ± *SD*	% Recovery	% RSD
Carvone (Method I)				
0	300	294.83 ± 4.58	98.28	1.55
50	450	446.50 ± 4.32	99.22	0.97
100	600	594.00 ± 5.02	99.00	0.85
150	750	743.00 ± 7.21	99.07	0.97
Carvone (Method II)				
0	300	284.83 ± 1.35	98.28	1.35
50	450	444.33 ± 4.50	98.74	1.01
100	600	594.50 ± 5.17	99.08	0.87
150	750	744.17 ± 3.71	99.22	0.50
Dillapiole (Method II)				
0	300	286.67 ± 2.50	98.89	0.84
50	450	444.67 ± 3.62	98.81	1.12
100	600	593.83 ± 6.59	98.97	1.11
150	750	744.50 ± 5.75	99.27	0.77
Methylparaben (Method II)				
0	300	296.17 ± 3.66	98.72	1.23
50	450	446.33 ± 3.56	99.19	0.80
100	600	594.00 ± 5.02	99.00	0.85
150	750	743.00 ± 5.87	99.07	0.79
Propylparaben (Method II)				
0	300	296.00 ± 4.05	98.67	1.37
50	450	446.00 ± 4.43	99.11	0.99
100	600	593.67 ± 5.75	98.94	0.97
150	750	743.67 ± 6.35	99.16	0.85

Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in Table 3. RSD was in the range 1.00-1.10 for carvone by Method I whereas simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 0.75-1.07, 0.42-0.82, 0.51-0.73 and 0.34-0.82 respectively by Method II for repeatability, 1.08–1.19 for carvone by Method I whereas simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 1.11-1.31, 0.62-1.01, 0.61-1.02 and 0.55-0.89 for intermediate precision by Method II respectively. These low values indicated that the method is precise.

Table 3. Precision of the proposed method of Method I and Method II.

Carvone (Method I)						
	Repeatability (Intraday precision)			Intermediate precision (Interday)		
Conc. (ng/spot)	Avg Conc. ± SD (n = 6)	Standard error	% RSD	Avg Conc. ± SD (n = 6)	Standard error	% RSD
300	2645.80 ± 28.28	11.55	1.07	2641.80 ± 29.67	12.11	1.12
400	3343.00 ± 33.44	13.66	1.00	3344.20 ± 39.71	16.21	1.19
500	4009.80 ± 44.20	18.05	1.10	4016.20 ± 43.25	17.66	1.08
Carvone (Method II)						
300	2441.20 ± 18.95	7.74	0.78	2449.00 ± 27.14	11.08	1.11
400	3056.60 ± 22.87	9.34	0.75	3026.80 ± 39.55	16.15	1.31
500	3727.60 ± 39.84	16.27	1.07	3728.80 ± 45.68	18.65	1.23
Dillapiole (Method II)						
300	4154.60 ± 34.22	13.97	0.82	4152.80 ± 42.02	17.16	1.01
400	5354.00 ± 31.07	12.69	0.58	5348.60 ± 36.12	14.75	0.68
500	6549.40 ± 27.62	27.62	0.42	6514.00 ± 47.42	19.36	0.62
Methylparaben (Method II)						
300	6431.20 ± 46.97	19.18	0.73	6436.20 ± 65.46	26.73	1.02
400	8289.00 ± 46.94	19.16	0.57	8280.20 ± 56.12	22.91	0.68
500	10084.00 ± 51.69	21.10	0.51	10106.40 ± 61.74	25.21	0.61
Propylparaben (Method II)						
300	10325.00 ± 84.21	34.38	0.82	10312.00 ± 92.29	37.68	0.89
400	12868.80 ± 43.22	17.65	0.34	12838.60 ± 70.26	28.69	0.55
500	14703.00 ± 91.23	37.25	0.62	14691.80 ± 98.06	40.04	0.67

Results of robustness are shown in Table 4. Low values of % RSD (0.99-1.37) for carvone by Method I whereas simultaneous determination of carvone, dillapiole, methyl and propylparaben were found to be 0.94-1.22, 0.73-0.87, 0.44-0.67 and 0.34-0.51 respectively by Method II were obtained after introducing small deliberate changes into the densitometric TLC procedure proved the robustness of the proposed HPTLC method.

Table 4. Robustness of the proposed HPTLC method of Method I and Method II.

Conc. (ng/spot)	Original	Used		Area ± SD (n = 3)	% RSD	Rf
Carvone (Method I)						
Mobile phase composition (hexane: ethyl acetate)						
400	9:1	8.9:1.1	-0.1, +0.1	3438.00 ± 47.23	1.37	0.35
		9:1	0.0	3342.40 ± 33.00	0.99	0.34
		9.1:0.9	+0.1, -0.1	3349.40 ± 40.25	1.20	0.33
Carvone (Method II)						
Mobile phase composition (methanol: water)						
400	8:2	3.9:6.1	-0.1, +0.1	3024.00 ± 36.98	1.22	0.38
		8:2	0.0	3018.40 ± 33.72	1.12	0.39
		4.1:5.9	+0.1, -0.1	3023.80 ± 28.51	0.94	0.40
Dillapiole (Method II)						
400	8:2	7.9:1.1	-0.1, +0.1	5341.20 ± 46.52	0.87	0.27
		8:2	0.0	5328.60 ± 40.47	0.76	0.28
		8.1:1.9	+0.1, -0.1	5336.80 ± 38.80	0.73	0.29
Methylparaben (Method II)						
400	8:2	7.9:1.1	-0.1, +0.1	8264.40 ± 46.64	0.56	0.44
		8:2	0.0	8277.00 ± 55.09	0.67	0.45
		8.1:1.9	+0.1, -0.1	8300.20 ± 36.73	0.44	0.46
Propylparaben (Method II)						
400	8:2	7.9:1.1	-0.1, +0.1	12838.20 ± 62.03	0.48	0.58
		8:2	0.0	12845.20 ± 44.14	0.34	0.59
		8.1:1.9	+0.1, -0.1	12845.20 ± 65.64	0.51	0.57

LOD and LOQ of the proposed method were found to be 7.34 and 21.05 ng/spot for carvone by Method I whereas simultaneous determination of carvone dillapiole, methyl and propylparaben were found to be 7.77 and 20.21 ng/spot, 6.31 and 18.66 ng/spot, 8.11 and 23.56 ng/spot and 8.87 and 23.96 ng/spot respectively by Method II which indicated that the proposed method can be used in wide range for detection and quantification of reference compounds effectively.

The proposed method was found specific by comparing R_f of pharmaceutical formulation and standard as well as the overlaid spectra at peak start, peak apex and peak end position of the spot showing λ_{max} of carvone (244 nm) for method I and Method II and dillapiole (209 nm), methyl and propylparaben (260 nm) for Method II was given in figures 17, 18 and 19 respectively.

Quantification of Carvone, Dillapiole, Methylparaben and Propylparaben in dill, caraway seeds and pharmaceutical formulations

Method I was applied for the estimation of carvone in both caraway and dill seeds extracts. Both extracts contain the cyclic monoterpene ketone carvone. In case of caraway seeds extract (Figures. 2-3) both methods were successful in the analyses. However, in case of dill seeds extract (Figure. 4) the carvone corresponding spot was overlapped with dillapiole. Method II was developed to resolve carvone from dillapiole and estimate them simultaneously (Figure. 5). Several herbalist shops sell oils from natural sources like caraway, dill, anise, fennel and more. We purchase both caraway and dill seeds oils for analyzing carvone contents. Their appearance and odour indicated that they are mainly fixed oils. Both oils were subjected to hydrodistillation (24). The HPTLC analyses using the two methods revealed that dill oil is free from carvone, while caraway oil contains traces of carvone (Table 5).

Table 5. Estimated amounts (%ng/100 gm) of carvone and dillapiole in dill, caraway seeds extracts and oils by Method I and Method II (n=6).

Samples	Carvone		Dillapiole
	Method I	Method II	Method II
Caraway extract	5.67	4.87	-
Dill Extract	-	7.07	5.87
Caraway oil	14.73	13.07	-
Dill oil	-	-	-

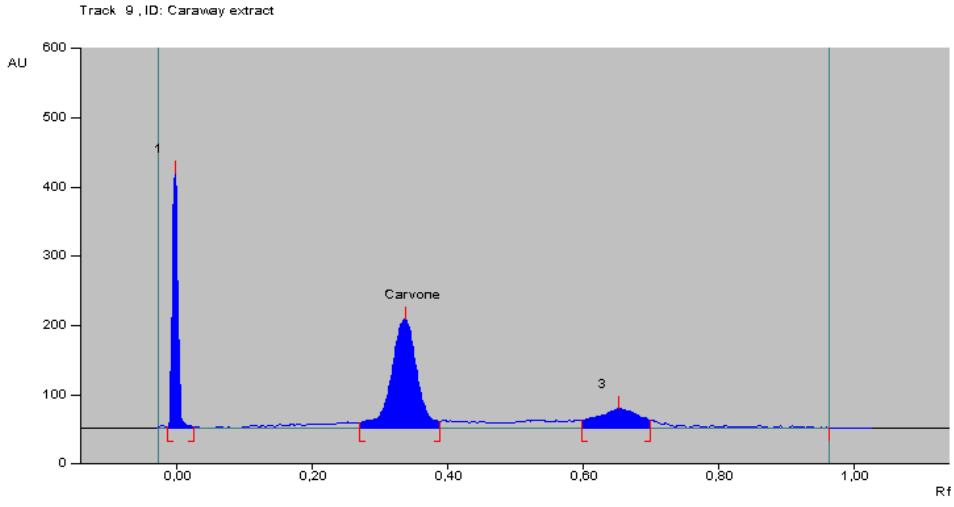

Figure 2. HPTLC densitogram of Caraway extract (Method I).

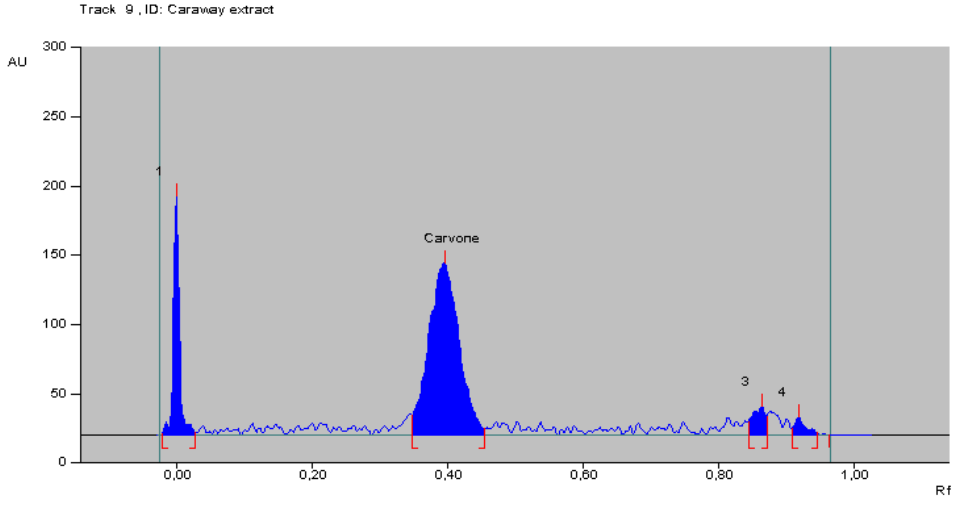

Figure 3. HPTLC densitogram of Caraway extract (Method II).

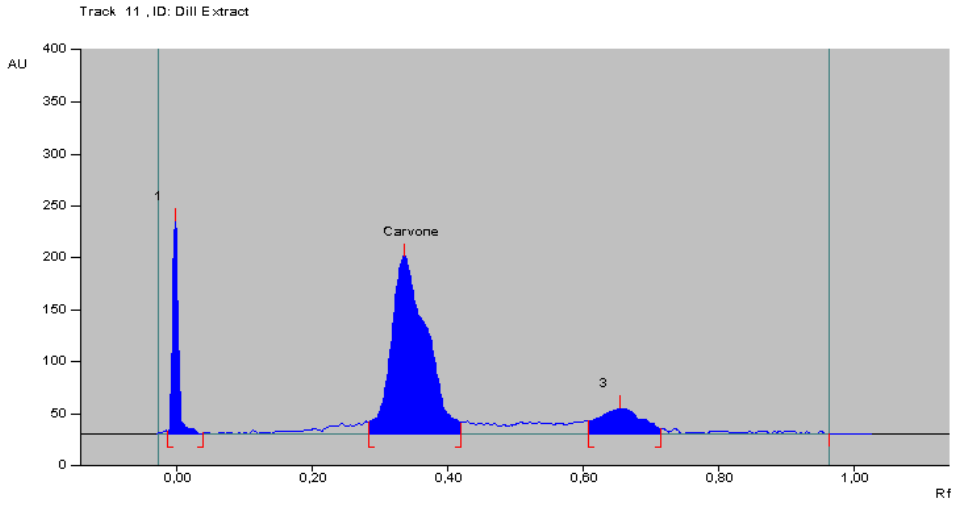

Figure 4. HPTLC densitogram of Dill extract (Method I).

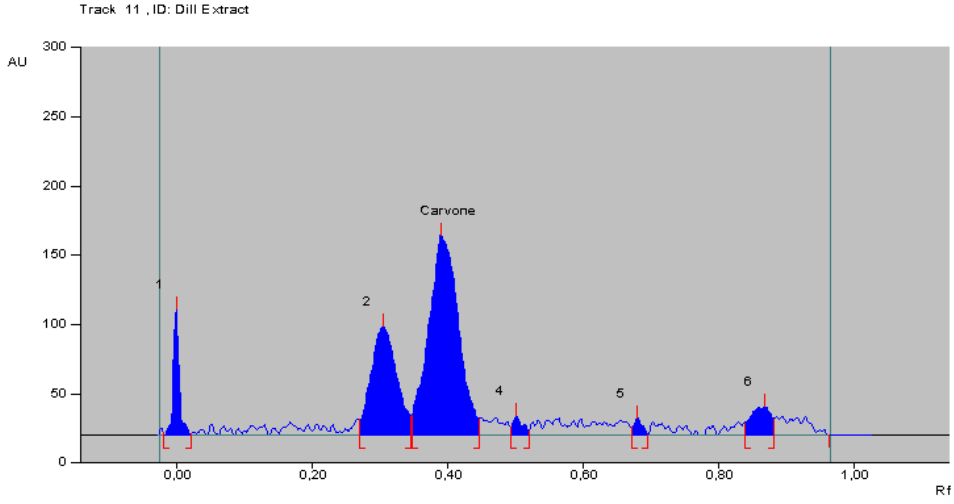

Figure 5. HPTLC densitogram of Dill extract (Method II).

Three marketed formulations were used for the analyses of carvone by the two developed methods (Figures. 6-9). Caraway oil should contain 60-70% carvone, while dill oil should contain 50-60% carvone as well as 15-35% dillapiole (2, 25). Analysis results indicated that one formulation contain more carvone than expected while the other two contain less amounts (Table 6). Moreover, none of the formulations showed the dillapiole corresponding spots. That may indicate the use of synthetic carvone rather than dill seeds essential oil. Two of these formulations showed two well resolved spots in method II with R_f values 0.45 and 0.59 corresponding to methylparaben and propylparaben and their estimation was achieved using Method II (Figures. 10-11). The amount detected for both compounds comply with the safety requirement according to the recommendation of Joint FAO/WHO Expert Committee on Food Additives (JECFA) (26).

Table 6. Estimated amounts (%ng/100 mL) of carvone, dillapiole, methylparaben and propylparaben in pharmaceutical formulations by Method I and Method II (n=6).

Samples	Carvone			Dillapiole			Methylparaben	Propylparaben
	Method I	Method II	Expected	Method I	Method II	Expected	Method II	
A	21.94	20.57	29.70- 35.10	-	-	3.99- 9.32	16.30	22.79
B	16.74	16.89	23.00- 27.06	-	-	6.90- 16.10	11.13	53.85
C	30.85	28.99	22.80- 27.36	-	-	6.84- 15.96	-	-

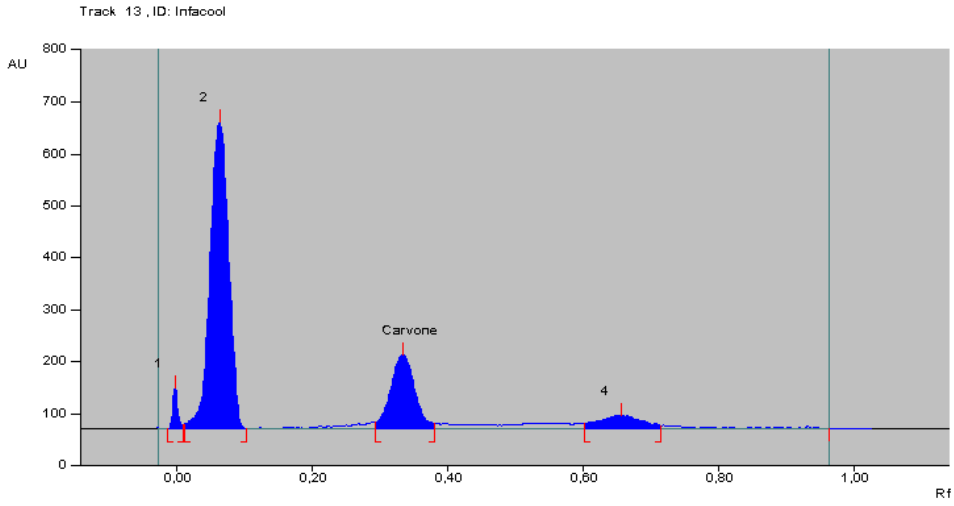

Figure 6. HPTLC densitogram of Formulation A (Method I).

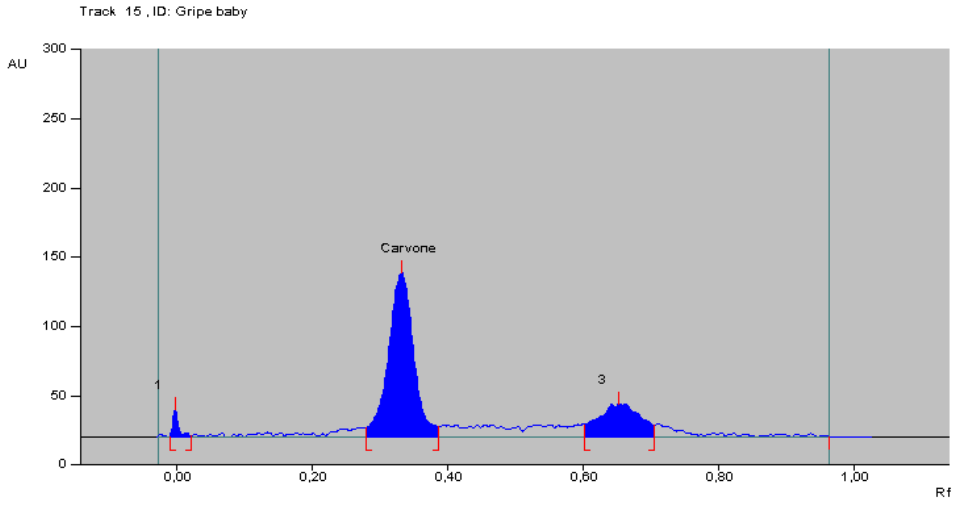

Figure 7. HPTLC densitogram of Formulation B (Method I).

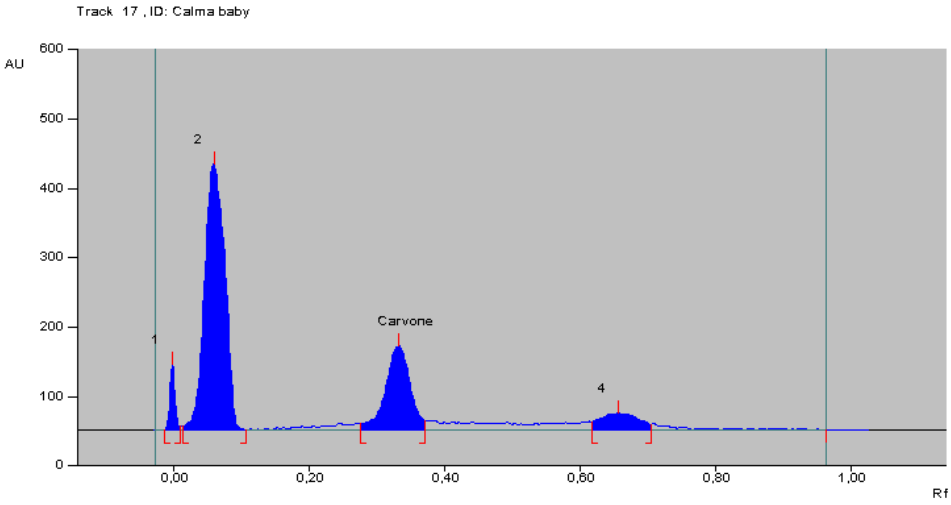

Figure 8. HPTLC densitogram of Formulation C (Method I).

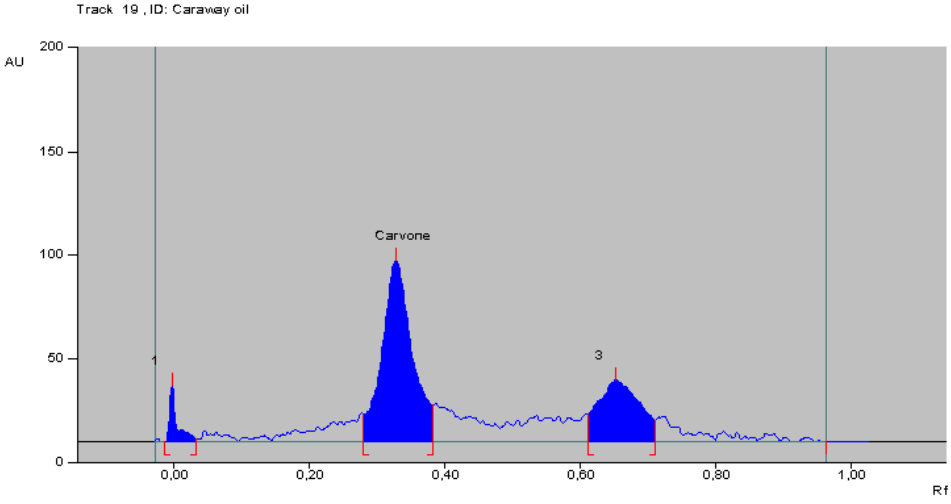

Figure 9. HPTLC densitogram of Caraway oil formulation (Method I).

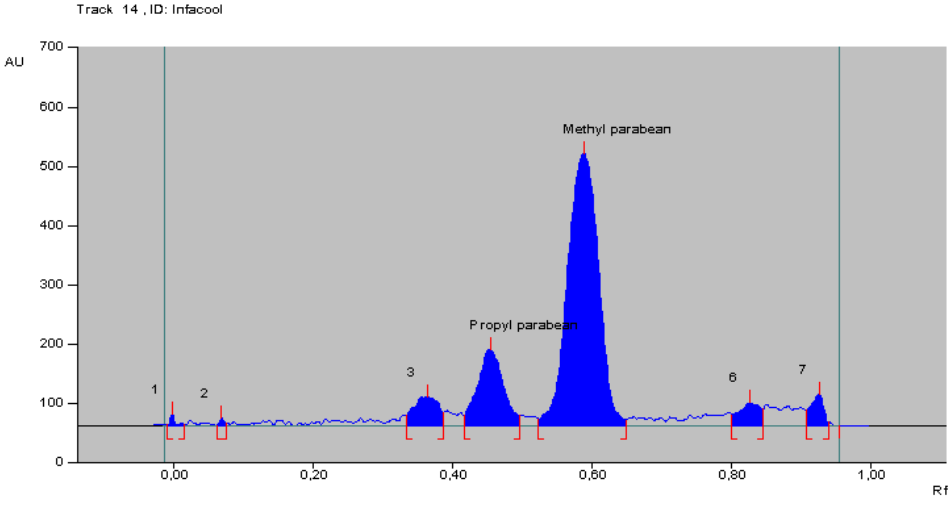

Figure 10. HPTLC densitogram of Formulation A (Method II)

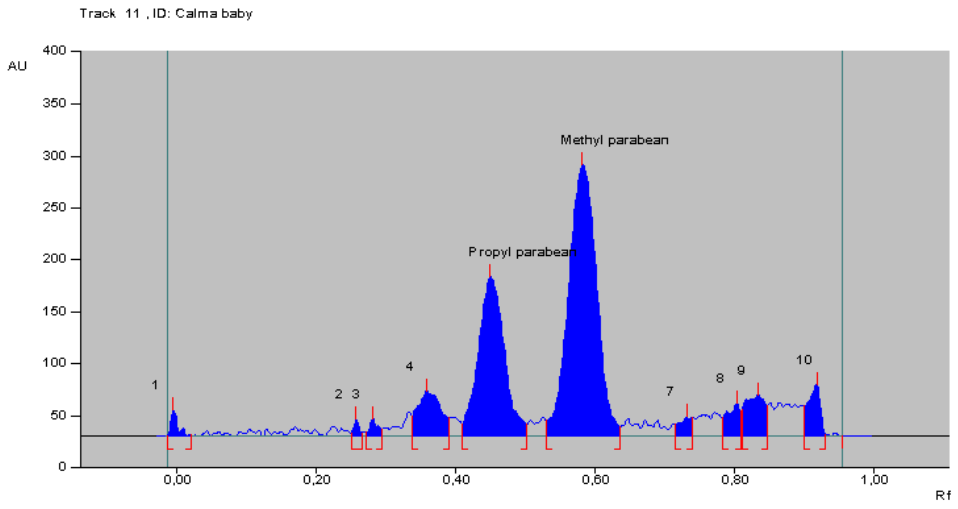

Figure 11. HPTLC densitogram of Formulation C Method II

CONCLUSIONS

The proposed densitometric HPTLC methods are quite simple, accurate, highly precise, sensitive and economic. It is suitable for parallel qualitative and quantitative densitometric analysis of carvone in natural sources and pharmaceutical preparations. Although Method I could be used for the routine analysis of carvone in a relatively short time and at low cost, some other components may interfere with the analyses. Method II using RP18 TLC plates was developed to overcome overlapping of carvone with dillapiole in dill seeds extract. Method II was found useful for the simultaneous analyses of carvone dillapiole, methylparaben and propylparaben. None of the formulations containing dill oil showed spot corresponding to dillapiole. That might be due to the use of synthetic carvone rather than natural dill oil. The marketed natural oils are mainly fixed oils with traces or complete absence of volatile components.

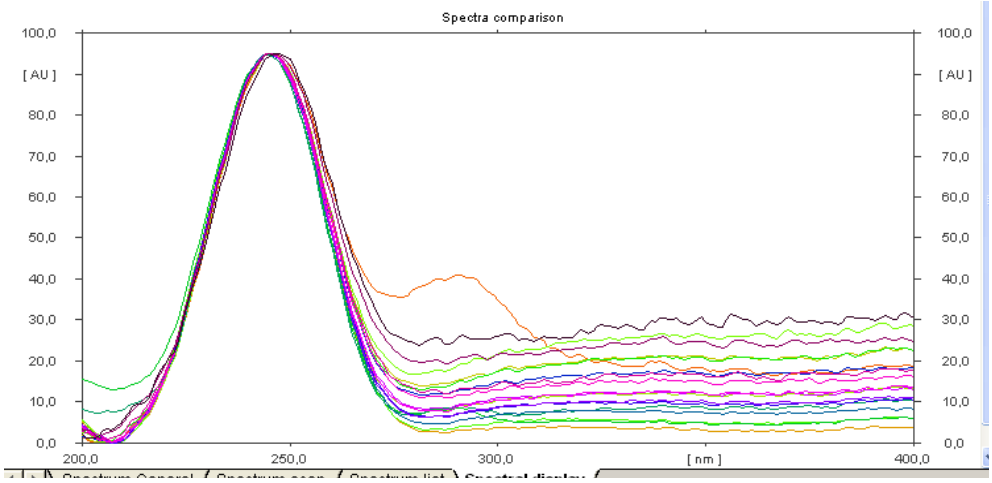

Figure 12. Overlay UV absorption spectra of the standard, caraway seeds extract and pharmaceutical formulations of Carvone.

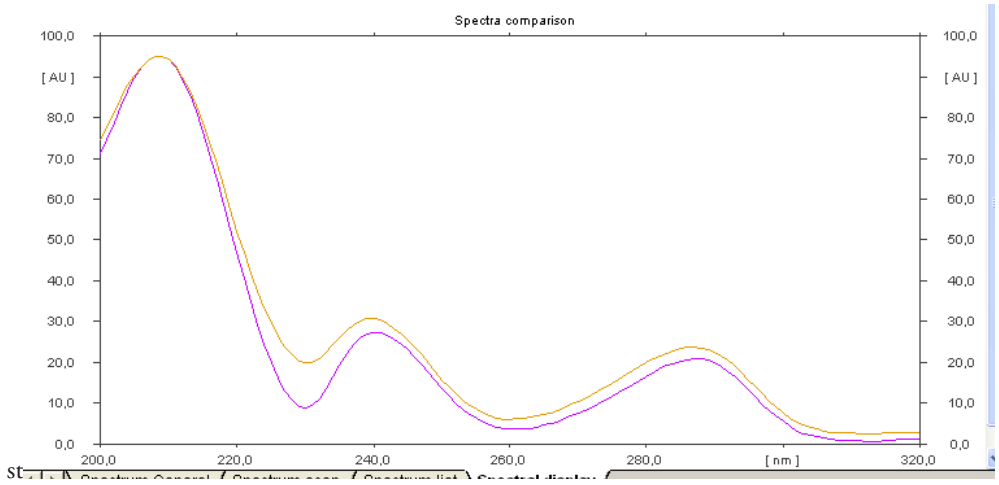

Figure 13. Overlay UV absorption spectra of the standard Dillapiole and Dill seeds extract

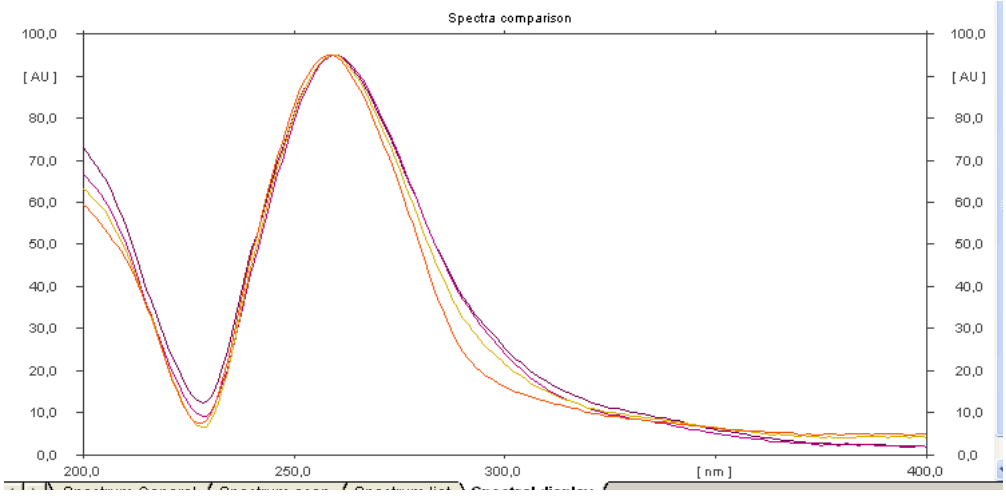

Figure 14. Overlay UV absorption spectra of the standards and pharmaceutical formulations of methyl and propyl paraben

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