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Quality Control Criteria of Volatile Ingredients in Certain Mouthwash Products in the Egyptian Market

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Abstract

Quality control criteria of active volatile ingredients in the commercial mouthwash products are not sufficient and not probably specify the quantity of active herbal ingredients. Therefore, it is important to apply the most recent advanced techniques including chromatographic methods as GC, HPLC and HPTLC for analysis of these active volatile ingredients. Comparison of different methods was carried out depending on their validation parameters to choose the best method for analysis. The total amount of volatile ingredients in all the selected mouthwashes was in the effective limit. HPTLC was the simplest method for identification of volatile ingredients. Moreover, HPLC technique was recommended instead of GC for determination of volatile ingredients that have chromophore in the commercial mouthwash products as resulted from their validation parameters, as well as GC analysis needs a lot of extraction solvent that lead to loss of active volatiles.

Key words: Quality control, mouthwash, volatile ingredients, HPLC, HPTLC, GC

Introduction

Dental caries and periodontitis are common oral diseases worldwide caused by cariogenic plaque bacteria and anaerobic microbiota as *Streptococcus mutans* and *Porphyromonas gingivalis*, respectively [1].

These diseases can be prevented and treated by regular oral hygiene practices as brushing with toothpastes or Miswak (tooth stick), daily rinsing with a mouthwash is important in removing the dental plaque, as it can reach areas not easily accessed by a toothbrush and removing bad breath [2].

Cariogenic plaque bacteria have developed antimicrobial resistance [3]. Therefore, demand for new source of antimicrobial agent of botanical origin has led to incorporation of antibacterial herbal ingredients as volatile oils against these cariogenic bacteria [4].

Most of the composition of commercial mouthwashes is synthetic compounds and the incorporated volatile ingredients are added only as flavoring agents. The pharmaceutical companies labeled the active synthetic ingredients in a mouthwash, as for example, chlorohexidine (1.25 mg/mL), however the active volatile ingredients present in the same mouthwash are not labeled and not analyzed by the companies or quality control organization. GC and HPLC techniques are widely used for analysis of natural products constituents [5], [6], [7] and [8]. This study aims at both comparing the most common analytical methods (HPLC, GC & HPTLC) in addition to quantitative determination of total volatile ingredients in the selected mouthwash products.

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Experimental

Chemicals, standards and products:

- Sigma Aldrich (USA) supplied the required authentic materials thymol, eucalyptol, benzyl isothiocyanate (BITC), methyl salicylate, menthol and chemicals (acetonitrile, dichloromethane *p*-anisaldehyde, sulphuric acid and methanol) of HPLC grade.
- Four mouthwash products (P I-IV) were selected from the Egyptian market for evaluation and quantitative determination of the volatile ingredients. Their composition was; **P I**: menthol, thymol, methyl salicylate, eucalyptol, caffeine and *Camellia sinensis* leaf extract, **P II:** menthol and thymol, **P III:** menthol and thymol, **P IV:** menthol, thymol, eucalyptol, methyl salicylate and *Salvadora persica* bark extract.

Material for chromatographic study:

- HPTLC precoated silica plates 60 F_{254} (20 × 20 cm), Merck, Germany.
- Analytical HPLC columns, C_{18} and C_8, 5µm Thermo (4.6 × 250 mm), USA.
- HPLC column C_{18} reversed-phase column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 3.5 µm, Agilent,USA).
- Analytical GC columns, HP-5 capillary column Thermo (60 m, 0.53 mm ID, 1.5 µm film thickness), USA.
- Spraying reagent: *p*-anisaldehyde sulphuric acid reagent.

Apparatus and equipment for chromatography:

- **Glass jars** of different dimensions for solvent development of HPTLC plates.
- **Microliter syringe**: Hamilton 25µl (Switzerland), for sample application on TLC plates and injection into HPLC injector.
- **Ultraviolt lamp** (λmax = 254 and 365 nm, Spectroline, USA), for detection of spots on TLC plates.
- **Gas liquid chromatography (GC)** for analysis of volatile and fatty compounds. Hewlett Packard GC, (Agilent, USA), with flame ionization detector (FID).
- **Densitometer for phytochemical analysis**: Cammag TLC scanner (Muttenz, Switzerland) operating with CATS 3 software.
- **High Performance Liquid Chromatography (HPLC)** Dionex Ultimate 3000, (Thermo scientific, USA) with a quaternary pump, autosampler and photodiode array detector (PDA).

Chromatographic Techniques

a) HPTLC Identification of volatile ingredients in (P I-IV).

Solvent system for thin layer chromatography (TLC): n-hexane-ethyl acetate: (9:1 v/v) for benzyl isothiocyanate and volatile compounds [11].

Preparation of test solutions: ten mL of (P I-IV) were separately extracted with (3×10 mL) dichloromethane (DCM). The DCM layers were separately collected, filtered, concentrated and transferred to 10 mL volumetric flasks and the volume was adjusted with DCM to 10 mL.

Preparation of authentic solutions: ten mg of each standard (menthol, thymol, eucalyptol, benzyl isothiocyanate (BITC) and methyl salicylate) was dissolved separately, in 7 mL DCM and transferred to 10 mL volumetric flask and the volume was adjusted to 10 mL with DCM.

Procedure: 5µL of each sample was spotted on TLC silica gel plate; the developed plates were examined before and after spraying with *p*-anisaldehyde sulphuric acid reagent.

b) GC analysis of volatile ingredients in (PI-IV) [10].

The samples were injected on HP-5 capillary column. The initial temperature was set at 60°C and programed to 280°C at a rate 20°C/min. The injector and detector temperatures were kept at 250°C. The flow rate of nitrogen carrier gas was 1 mL/min.

Sample preparation: fifty mL of each product was transferred to a separating funnel and shaken with 100 mL of DCM. The mixtures were left overnight. The DCM layers were separately concentrated to 25 mL.

Stock standard preparation: thymol, eucalyptol, methyl salicylate and menthol standard solutions (1 mg/mL) were prepared in DCM.

Working Standard preparation: different concentrations of thymol, eucalyptol, methyl salicylate and menthol standards were prepared by serial dilution with DCM to produce the following concentrations (0.062, 0.125, 0.25 and 0.5, 0.75 mg/mL).

c) HPLC conditions for determination of:

i) Thymol in (P I-IV)

HPLC analysis was performed using a C8 reversed-phase (Hypersil BDS C-8 column) (4.6×250

mm, 5µm) at room temperature. The PDA detector was set at wavelength 274 nm. The injection volume was 20 µL. Isocratic elution was performed using the mobile phase water: methanol (40: 60 v/v) at a flow rate 1 mL/min. Standard calibration curve of thymol was constructed. The concentration of thymol in tested products was measured using the regression equation [10].

*Sample preparation***:** three mL of each product (P I-IV) was transferred to volumetric flask 5 mL. The volume was adjusted to mark with methanol; the solutions were mixed well, filtered through membrane filter 0.45µ and finally arranged in the autosampler tray in a suitable vial

*Stock standard preparation***:** thymol standard solution (1mg/mL) was prepared in methanol.

Working Standard preparation: different concentrations of thymol standard were prepared by serial dilution with methanol to produce the following concentrations (0.125, 0.25, 0.50 and 0.75 mg/mL).

ii) BITC in P IV.

HPLC method was performed applying isocratic elution of water and ethanol in the ratio (30:70). The PDA detector was set at 254 nm. Reversed phase Hypersil BDS C-18 column (4.6×250 mm, 5µm) was used. Temperature was maintained at room temperature at flow rate of 1 mL/min. Twenty microliters of each concentration of standard and test samples were injected into HPLC. The retention time and the peak area were used to identify and calculate the amount of BITC [12].

Preparation of BITC standard: BITC standard was dissolved in methanol (10 mg/mL) then serial dilution was prepared to obtain the following concentrations (0.05, 0.1, 0.25, 0.5 mg/mL).

Extraction of BICT from PIV: twenty five mL of P IV was extracted using DCM (50 mL× 3). The DCM extract was collected, concentrated to 5ml and transferred to 10ml volumetric flask. The volume was adjusted with the mobile phase (water: ethanol) (30:70), filtered through 0.45µ membrane filter and injected into HPLC.

Determination of alcohol content in selected mouthwash products (P I-IV) [10].

Distillation of 25mL of each product was done after addition of 100-150 mL of distilled water and a few pieces of pumice .Ninety mL of distillate was collected in a 100 mL volumetric flask and dilution to 100 mL with distilled water was made. Determination of the relative density at 20 ± 0.1 °C using a densitometer was carried out.

Results and Discussion

A. Qualitative analysis of volatile ingredients incorporated in selected products in the Egyptian market.

1. Physical properties of mouthwash products (P I-IV)

Physical properties of selected mouthwash products (P I-IV) in the Egyptian market are recorded in table (1)

2. HPTLC Identification of volatile ingredients in products (PI - VI).

Results revealed that: P I and P IV showed quenching at (R_f 0.4, 0.7 0.85) related to eucalyptol, thymol and methyl salicylate, respectively, at 254 nm. Quenching at (R_f 0.6 in S_1) belonged to BITC in P IV only. After spraying with *p*-anisaldehyde sulphuric acid reagent, PI and PIV showed blue brown spot at (R_f 0.4) related to eucalyptol, as well as a blue spot at (R_f 0.28) referred to menthol and dark pink spot at (R_f 0.7) related to thymol. P II and P III exhibited quenching at (R_f 0.7) related to thymol at 254 nm. After spraying with *p*-anisaldehyde sulphuric acid reagent, both products showed blue spot at (R_f 0.28) referred to menthol and dark pink spot at (R_f 0.7) referred to thymol. P IV showed quenching at (R_f 0.6) attributed to BITC. Thymol and menthol was identified in products (PI - IV), BITC was identified in P IV; methyl salicylate and eucalyptol were identified in P I and P IV (table 2).

B. Quantitative estimation of volatile ingredients in the selected oral care products

1. Estimation of volatile ingredients in (P I-VI) by GC

The GC method showed sharp and high resolved peaks of thymol, menthol, eucalyptol and methyl salicylate for both standard and all tested mouthwash products (P I-IV) (Figures 1-3). Validation of GC method was achieved by the external standard method and calibration curves were constructed showing linearity (r^2 = 0.9937, 0.9972, 0.9933 and 0.9989) for thymol, menthol, eucalyptol and methyl salicylate, respectively (Figures 4 and 5). The concentration of volatile oil ingredients to be effective in removal or inhibition of oral microorganisms in the oral cavity is (0.007 - 2.0% w/v) or (0.07-20 mg/ml) [13]. The concentration of menthol in (P I-IV) was calculated from regression equation to be 0.464, 0.24, 0.43, 0.46 mg/mL,

respectively (table 6). The concentration of thymol in (P I-VI) was calculated from regression equation to give 0.6, 0.29, 0.54, 0.59 mg/mL respectively (table 6). Eucalyptol concentration was calculated in P I and P IV to give the 0.53 and 0.414 mg/mL, respectively (table 6). Methyl salicylate concentration was calculated in P I and P IV to give the following results, 0.51, 0.436, mg/mL respectively (table 6). The validation parameters, linear regression equation, linear ranges, determination coefficients (r^2), RSD, LOD and LOQ were recorded in table (3).

2. HPLC determination of thymol in (P I- IV)

The HPLC method showed sharp and high resolved peak of thymol for standard and all tested mouthwash products (P I-IV) (Figures 6 and 7). Validation of HPLC method was achieved by the external standard method. Calibration curve showed linearity (r^2 = 0.9968) (Figure 8a). It was found that thymol concentration in (P I-IV) was 0.608, 0.224, 0.542, 0.572 mg/mL respectively. The validation parameters, linear regression equation, linear ranges, calibration equation, determination coefficients r^2, LOD and LOQ were tabulated in table (4). LOD recorded was equal to 74 µg/mL, lower than 109, the value recorded in GC method, so HPLC method was more sensitive than GC method in determination of thymol in mouthwash products.

3. HPLC determination of benzyl isothiocyanate (BITC) in P IV

HPLC analysis showed sharp and high resolved peak of BITC for standard BITC, however no peak identified at the same retention time of BITC in (P IV) as in (Figures 9a-9b), respectively. Peak spiking (enrichment) was performed by addition of standard BICT on P IV resulting in no increment in peak height which is near to the retention time of BITC in P IV as in (figure 9c).Validation of HPLC method was achieved by the external standard method applying a sequence of serial dilutions of BITC standard and calibration curve was constructed showing linearity (r^2 = 0.99٨١) (Figure 8b). The validation parameters, linear regression equation, linear ranges, calibration equation, determination coefficients (r^2), LOD and LOQ were recorded in table (5). The absence of BICT in the oral P IV may be attributed to degradation of the extracts of *Salvadora persica* incorporated in it.

Conclusion

The total amount of volatile ingredients in all the selected mouthwashes was in the effective limit. HPTLC was the simplest method for identification of volatile ingredients but it can't be used for identification and quantification of BITC in the selected mouthwash product as it is not a specific method of identification of BITC and the result confirmed by HPLC that the selected mouthwash doesn't contain BICT. HPLC technique was superior over GC for determination of volatile ingredients that have chromophore in the commercial mouthwash products as the GC analysis needs a lot of extraction solvent that led to loss of active volatiles. LOD recorded in HPLC analysis of thymol was lower that recorded in GC analysis, so HPLC method was more sensitive than GC method in determination of thymol in mouthwash products.

Conflicts of interest

There are no conflicts to declare.

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Table (1): Physical properties of selected mouthwash products in the Egyptian market

Product	Composition	Colour/ Odour/ Taste	PH		RI		sp. gr.	Ethanol content%	
			Limit	Result	Limit	Result		Limit	Result
P I	menthol, thymol, methyl salicylate, eucalyptol, caffeine and *Camellia sinensis* leaf extract	faint yellow green clear solution with characteristic odour and pleasant taste	3.6 - 6.9	3.8	1.36-1.57	1.37	1.038	0.01-30% **[13]**	0
P II	menthol and thymol	red clear solution		4.5		1.43	1.11		10%
P III	menthol and thymol	faint yellow clear solution with banana taste		5.8		1.53	0.98		12%
P IV	menthol, thymol, eucalyptol, methyl salicylate and *Salvadora persica* bark extract.	dark yellow clear solution with acidic taste		4.0		1.36	1.05		0

P: product, RI: refractive index, sp. gr.: specific gravity.

Table (2): HPTLC Identification of the volatile ingredients in the selected mouthwash products (P I-IV).

Volatile ingredient	Product	R_f	UV (254 nm)	Colour after spraying with *p*-anisaldehyde sulphuric acid reagent
BITC	P IV	0.6	Fluorescence Quenching	----------
Eucalyptol	P I, P IV	0.4	----------	Blue brown
Menthol	P I, P II, P III, P IV,	0.28	----------	Blue
Methyl salicylate	P I, P IV	0.85	Fluorescence Quenching	----------
Thymol	P I, P II, P III, P IV	0.7	Fluorescence Quenching	Dark pink

BITC: benzyl isothiocyanate, R_f: rate of flow

Table (3): GC validation parameters for determination of menthol, thymol, methyl salicylate and eucalyptol in (P I-VI).

Parameter	Menthol	Thymol	Methyl salicylate	Eucalyptol
Linear regression equation	y = 1.3602x + 36.092	y = 2.4981x + 4.0679	y = 1180.6x + 30.34	y =2464.5 x + 35.47
r^2	0.9970	0.9937	0.9989	0.9933
Linear range (µg/mL)	60-1000	60-1000	125-1000	125-1000
Precision (RSD %)				
P I	0.48	1.9	1.44	1.49
P II	1.82	0.57	----	----
P III	0.98	1.5	----	----
P IV	0.81	0.22	1.48	0.69
LOD (µg/mL)	87.5	109	51.6	128
LOQ (µg/mL)	265	330	156	388

LOD: limit of detection, LOQ: limit of quantification, RSD: relative standard deviation

Table (4): HPLC validation parameters for determination of thymol in (P I- IV).

Linear regression equation	r^2	RSD% Precision				Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
		P I	P II	P III	P IV			
y = 0.1668x + 4.5	0.9968	1.3	1.8	1.8	1.5	200-1000	74	224

LOD: limit of detection, LOQ: limit of quantification, RSD: relative standard deviation

.

Table (5): HPLC validation parameters for determination of BICT in P IV.

Linear regression equation	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
y = 917x + 27.5	0.9985	100-400	20.08508	60.86389

LOD: limit of detection, LOQ: limit of quantification

Table (6): Summary of the content of active volatile ingredients in the selected mouthwash products (P I-VI).

Volatile ingredient	Concentration (mg/mL)				
	P I	P II	P III	P IV	Method
Thymol	0.60	0.29	0.54	0.59	GC
	0.601	0.22	0.54	0.57	HPLC
Menthol	0.464	0.24	0.43	0.46	GC
Methyl salicylate	0.51	---	---	0.44	GC
Eucalyptol	0.53	---	---	0.41	GC
BITC	---	---	---	ND	HPLC
Total	2.1	0.53	0.97	1.9	GC

BITC: benzyl isothiocyanate, ND: not detected, P: product, ---, not labeled

*The amount of total volatile ingredients in each product was in the reported effective range (0.07-20 mg/ml).

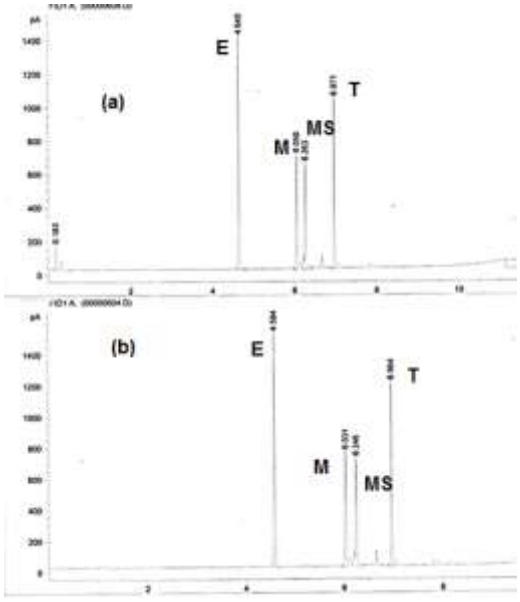

Figure 1: GC Chromatograms of (a): P I, (b): P IV
E: Eucalyptol, **M:** Menthol, **MS:** Methyl salicylate, **T:** Thymol

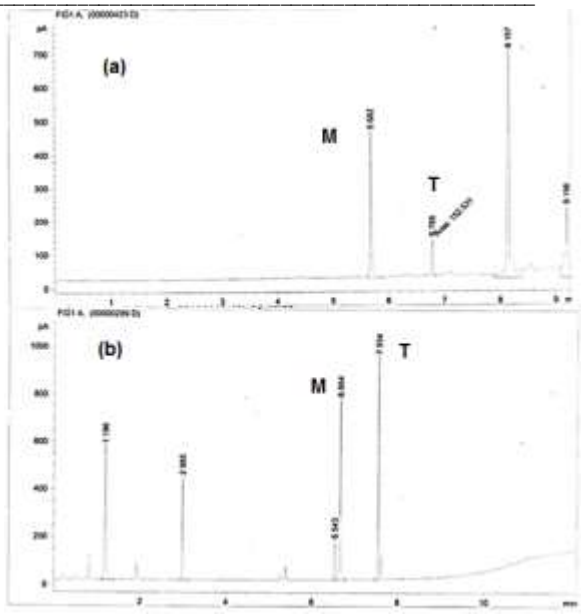

Figure 2: GC Chromatograms of (a): P II, (b): P III
M: Menthol, **T:** Thymol

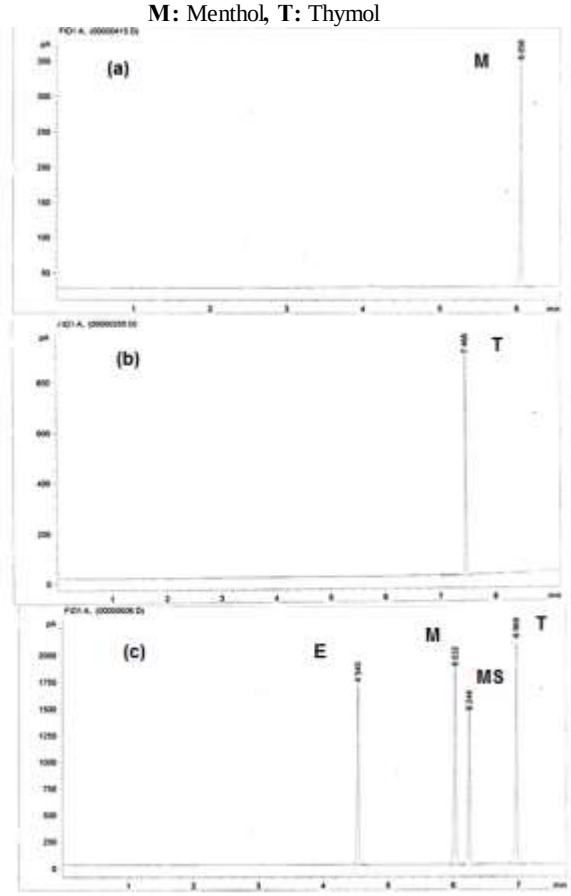

Figure 3: GC Chromatograms of (a): Menthol, (b): Thymol, (c): Mixure (Eucalyptol, Menthol, Methyl salicylate and Thymol) standards
E: Eucalyptol, **M:** Menthol, **MS:** Methyl salicylate, **T:** Thymol

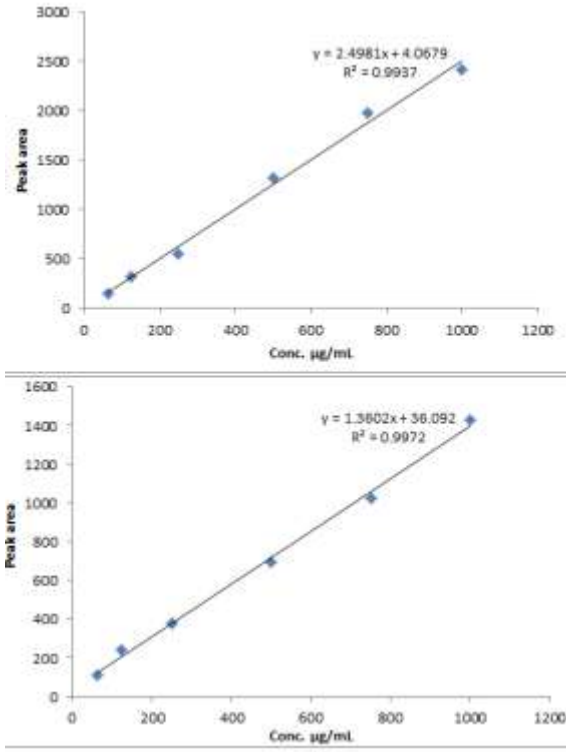

Figure 4: GC calibration curve of (a): Thymol, (b): Menthol

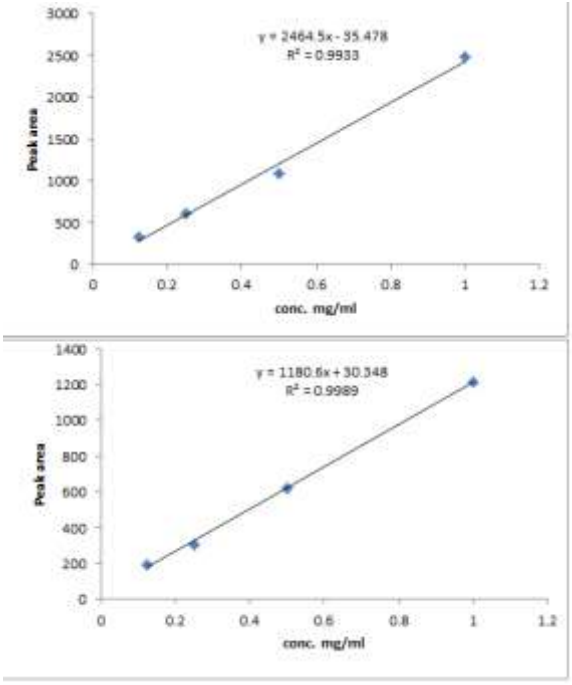

Figure 5: GC calibration curve of (a): Eucalyptol, (b): Methyl salicylate

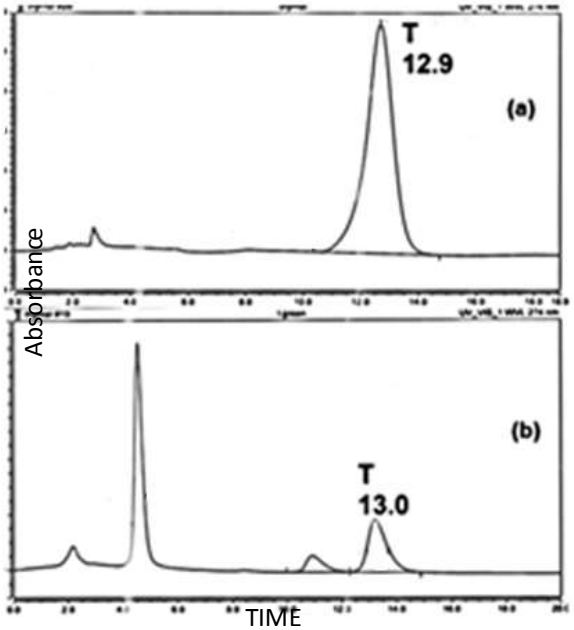

Figure 6: HPLC chro matograms of (a): Thymol, (b): P I , T: Thymol

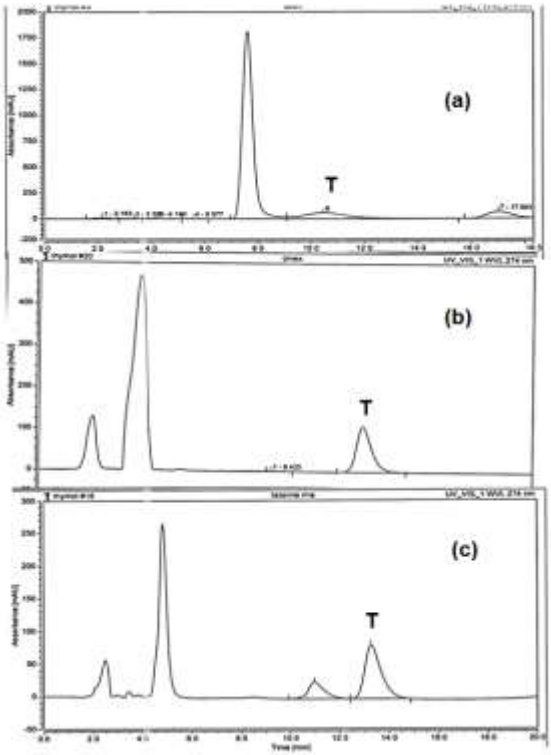

Figure 7: HPLC chromatograms of (a): P II (b): P III (c): P IV
T: Thymol

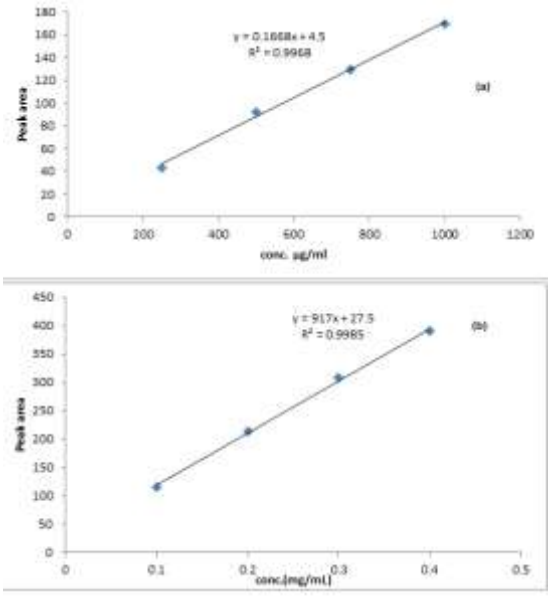

Figure 8: HPLC calibration curve of (a): Thymol, (b): BITC

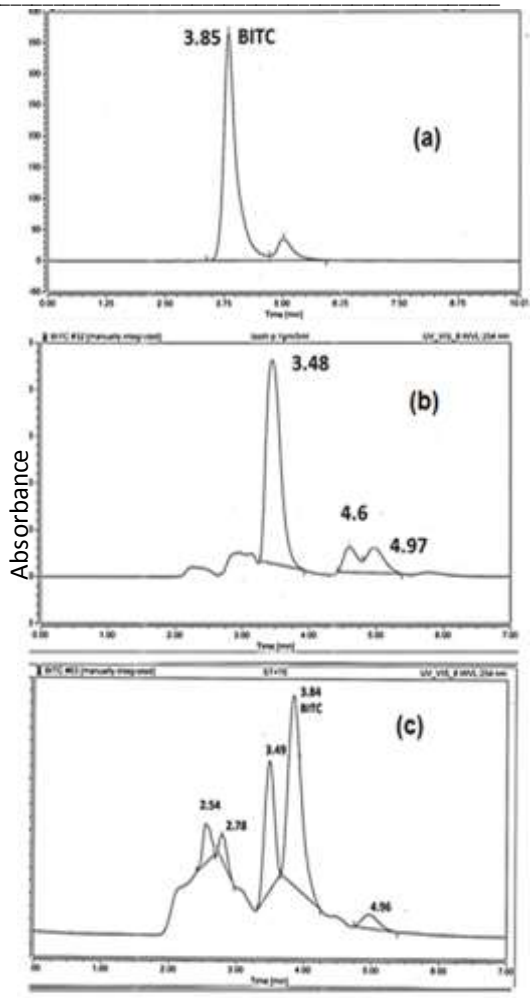

Figure 9: HPLC chromatograms (a): BITC standard, (b): P IV (c): peak enrichment of BITC in P IV