Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2018

50

1-6

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: _____

Original Scientific Article

HPLC method for determination the content of thymol and carvacrol in Thyme tincture

Dedić, M.[a*], Bečić, E.[a], Imamović, B.[a], Žiga, N.[b], Medanhodžić-Vuk, S.[c], Šober, M.[a]

[a] *Faculty of Pharmacy University of Sarajevo, Department for Pharmaceutical Analytics, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herczegovina*

[b] *Faculty of Pharmacy University of Sarajevo, Department for Clinical Pharmacy, Zmaja od Bosne 8, 71000 Sarajevo,Bosnia and Herczegovina*

[c]*Pharmamed doo Travnik, Dolac na Lašvi bb Travnik, Bosna i Hercegovina*

Article info
Received: 10/04/2018
Accepted: 06/06/2018

Keywords:
Thyme
Thymol
Carvacrol
Validation
Thyme Tincture

***Corresponding author:**
E-mail: dedicm@gmail.com
Phone: +38761349288

Abstract: Genus *Thymus* contains about 300-400 species, many of which are used in traditional medicine. *Thymus vulgaris (thyme*) is the most commonly used *Thymus*. In official medicine, thyme is used as a general medicine for colds, flu, fever, coughing and bronchitis, such as: an antiseptic, spasmolytic, antifungicide, antitus, tonic, antihelmintic, antioxidant agent, antivirantic agent, carminative, sedative, diaforetic, antibacterial and as refresh remedy. The pharmacological effects of thyme are most closely related to its polyphenolic components, thymol and carvacrol. The most used chromatographic methods for determination of active compounds in herbal preparation is high-performance liquid chromatography. Results obtained by statistical processing are in the reference interval, which is recommended by the ICH guidelines. By analyzing *Thymi tincturae*, it was found that the concentration of thymols was 0.807 mg/g of tincture, while the concentration of carvacrol was 0.082 mg/g tincture. This analysis is very fast, reliable and economical.

The method does not require a complicated sample preparation and as such can be used in the regular control of the content of thymol and carvacrol in the finished product and the semi-product (tincture).

INTRODUCTION

Thymol and carvacrol in Thymus vulgaris L.

Due to the increasing prevalence of resistance to antibiotics, the resorting to treatment with traditional methods is increasing, such as use of herbal remedies with antibacterial effect (Kon and Rai, 2012; Shabnum and Wagay, 2011).

Genus *Thymus* contains about 300-400 species, many of which are used in folk medicine. The most commonly used *Thymus* genus is *Thymus vulgaris* (Hajimehdipoor, Shekarchi, Khanavi, et al., 2010; Zeković, Lepojević, Markov, 2002). *Thymus (Thymus vulgaris L., Lamiaceae)* is an aromatic and medicinal plant used in the production of phytopharmaceutical preparations as a preservative and as an aromatic component (Hajimehdipoor, Shekarchi, Khanavi, et al, 2010; Grigore, Paraschiv, Colceru-Mihul *et al*, 2010).

Most commonly they are prepared as water extracts (infusum and decoct), as well as tinctures, which are used in respiratory infections. Water extracts can also be used externally, locally, for the treatment of rheumatic and skin diseases (Fachini-Queiroz, Kummer, Estevao-Silva, et al., 2012; Zeković, 2000; Zeković, Lepojević, Markov, 2002).

In official medicine, thyme is used as a general remedy for colds, flu, fever, cough and bronchitis such as:

antiseptic, antispasmolytic, antifungicide, antitus, tonic, antihelmintic, spasmolytic, antioxidant agent, antiviral, carminative, sedative, diaforetic, antibacterial agent and refresh remedy. It is often used as a component of toothpaste, or oral tonic and antseptic. It can be a component of perfumes and soaps (Ashnagar, Gharib, Ramazani, 2011; Syamasundar, Srinivasulu, Stephen, et al., 2008).

The pharmacological effects of thyme are most closely related to the polyphenolic components of thymol and carvacrol *(Figure 1.)*

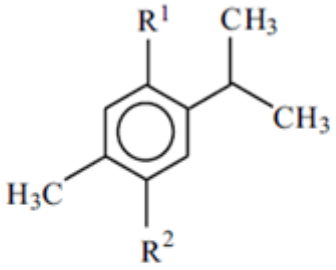

Figure 1*. Chemical structure of thymol and carvacrol (Alekseeva, 2009)*

The content of thymol in essential oil is over 60%, which is much higher than the content of carvacrol which is not more than 6%. Thymol and carvacrol in mixture show 30 times higher antiseptic effect andare 4 times less toxic than phenol. Since thymol and carvacrol are carriers of the activity, most pharmaceutical forms are standardized to their content. Besides thymol and carvacrol, another thirty components in this plant have been identified. According to the order of representation in essential oil, these are: thymol, γ-terpinene, p-cymene, linalool, myrcene, α-pinene, eugenol, carvacrol and α-thujene (Marculescu, Vlase, Hanganu et al., 2007; Syamasundar, Srinivasulu, Stephen, et al., 2008).

Thymian oil and its components exhibit markedly antimicrobial activity (Ezz, Aziz, Hendawy, et al., 2009; Marculescu, Vlase, Hanganu, et al., 2007).
Timol also has an agonistic effect on α_1, αand β-adrenergic receptors. In addition, timol showed analgesic activity through its effect on $\alpha_2$adrenergic nerve cell receptors (Shabnum, Wagay, 2011).

The aim of the research was to develop and optimize the HPLC method for the identification and quantification of thymol and carvacrol and to determine the content of thymol and carvacrol in *Thymi tincture.*

EXPERIMENTAL

Apparatus
The analysis was performed on HPLC apparatus with UV/Vis detector (HPLC system Prominence, type: 3-079, Shimadzu). The stacionary phase was C18, dimensions of 250×4.6 mm, 5μm Microsorb- Varian. Class-VP 7.4 software was used for signal analysis and statistical processing.

Chemicals
Thymol standard (≥ 99,9% purity) - Sigma Aldrich; Carvacrol standard (≥ 98% purity) - Sigma Aldrich; Acetonitrile (HPLC grade) - Sigma Aldrich; Methanol (HPLC grade) - Sigma Aldrich; Sulfuric acid 96% - Lachema; Ethanol absolute- Merck; Purified water for HPLC.

Chromatographic conditions
Stacionary phase: column C18 (4.6 × 250 mm, 5 μm) Microsorb- Varian,
Mobile phase - acetonitrile: water (in 50:50 ratio V:V) - isocratic,
Flow rate: 1 ml/min,
Injection volume: 10 μl,
Column temperature: 25˚C,
Detection: 274 nm.

Preparation of standard solutions of thymol and carvacrol
From the standard substances of thymol and carvacrol, after weighing, and then dissolving in a solvent mixture (acetonitrile: water 80:20 V:V), the basic solutions were prepared: thymol concentration of 3 mg/ml and carvacrol concentration 0.3 mg/ml.

Preparation of Thymitincturae
Thymiherba 2.64 kg
Glycerolum (85%) 1.32 kg
Ethanolum (96%) 4.22
Aqua purificata 7.66 kg

Total: 15.84 kg

The prescribed amount of glycerol was added to the alcoholic mixture and the prepared solvent mixture poured over the drug. Mixture was intesivelystirred left in a dark place to macerate for 5 days with continous mixing several times during a day. The macerates were separated by decanting, then by pressing and tightening and left for 2 days in a cold place protected from light. In the end, the prepared tintcurae was filtered.
This prescription is used for the industrial preparation of intermediate product, from which 5 g was taken for the analysis, due to the concentration of timol and carvacrol in the final product (syrup).

Preparation of the Thymitincturae sample
5 g of *thyme tincture (Thymitinctura, Thymus vulgaris, L., Lamiaceae)* was dissolved in a 50mL in a solvent mixture (acetonitrile: water 80:20 V:V).
Prepared solution was diluted and used for further analysis.

RESULTS AND DISCUSSION

HPLC method was devloped and optimized for identification and quantification of thymol and carvacrol in the thyme tincture (Hajimehdipoor, Shekarchi, Khanavi, et a., 2010). Validation of the analytical method was carried out by examining the following validation parameters: specificity, linearity, accuracy, repeatability, detection limit, quantification limit.

The linearity of the thymol method in a wide range of concentrations of 15-75 μg/ml and for carvacrol in the concentration range of 1.5-7.5 μg/ml was determined. The calibration curves were constructed, the coefficient of correlation was calculated for the thymol R^2 = 0.9981, while the equation of direction was y = 909.46x + 117.27 and for carvacrol R^2 = 0.9981, while the equation of direction was y = 8603.1x + 21.233 (*Figure 2. and Figure 3.*).

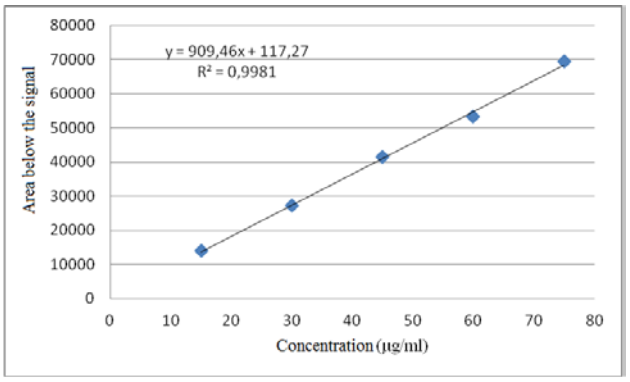

Figure 2. Linearity for thymol

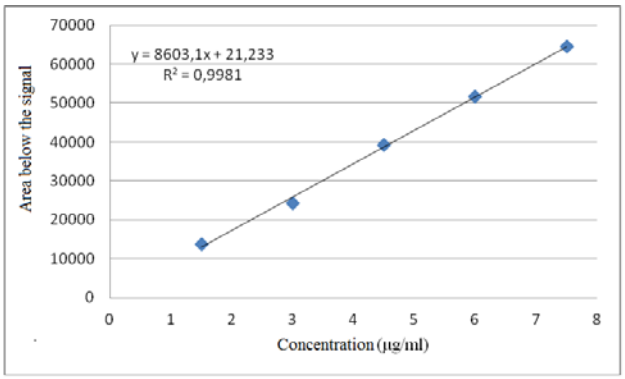

Figure 3. Linearity for Carvacrol

Based on the linearity validation parameter, the values for the detection limits and quantification limit were obtained:

The detection limit for thymol was LD = 0.17 ng/ml, and **the quantification limit** LQ = 0.567 ng/ml.
The detection limit for carvacrol was LD = 0.161 ng/ml, and **the quantification limit** LQ = 0.535 ng/ml.

The accuracy of the test method was determined for thymol concentrations 36; 45; 54 μg/ml and carvacrol 3.6; 4,5; 5.4 μg/ml representing 80, 100, 120% of the base standard concentration. Tables 1 and 2 give the values for areas below the signal, concentration, *recovery (R%)*, standard deviation (SD), relative standard deviations (RSD) and reliability coefficient (tα).

Repeatability of the test method was determined for thymol concentrations 36; 45; 54 μg/ml and carvacrol 3.6; 4,5; 5.4 μg/ml representing 80, 100, 120% of the base standard concentration. Tables 3. and 4. give the values for *recovery (R%)*, standard deviation (SD) and relative standard deviations (RSD%).

Intermediate precision

Intermediate precision for thymol and carvacrol was also assessed, with three analysts separately performing three sample analyzes in two different days.

Sample analysis

The chromatogram of the prepared tincture solution (preparation was previously explained) can be seen in *Figure. 4*, with associated retention times and area below the signal.

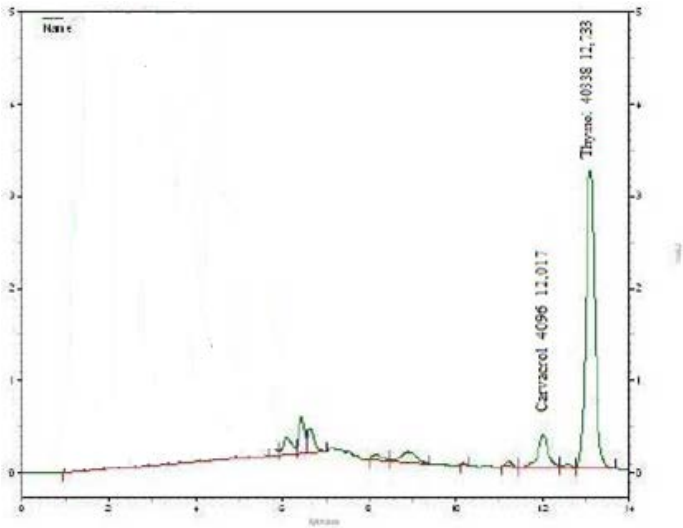

Figure 4. Sample chromatogram

Data analysisi showed that the solution contains 40.34 μg/ml of thymol, and 4.10 μg/ml of carvacrol. Considering the preparation of tincture and the dilution, the content of thymol per gram of tincture is 0.807 mg, and 0.082 mg per gram of tincture of carvacrol.

The obtained ratio of the content between thymol and carvacrol corresponds to available literature and similar studies. The content of the carvacrol are about 1/10 of the thymol content. (Hajimehdipoor, Shekarchi, Khanavi, et al., 2010; Zeković, 2000)

The tincture preparation process must be standardized so that the manufacturer is assured in the exact contents of the active components of thymol and carvacrol, which come from a variety of plant material.

Such preparations form an integral part of a herbal medicine which, in order to be registered on the market in a country, must pass strict quality control in the control laboratories.

This analysis is very fast, reliable and economical.

The method does not require a complicated sample preparation and as such can be used in the regular control of the content of thymol and carvacrol in the finished product and the semi-product (tincture).

Table 1. The validation parameter accuracy for thymol

Concentration (µg/ml)	P_1	P_2	P_3	<P>	<y>	SD	RSD (%)	<R%>	tα
80% (36 µg/ml)	33254	33329	32810	3313.00	36.3	0.03	0.9	100.81	1.32
100% (45 µg/ml)	41572	41822	41730	41708	45.7	0.01	0.3	10.,58	1.28
120% (54 µg/ml)	50139	49956	50077	50057.33	54.9	0.01	0.2	101.63	1.27

Table 2. The validation parameter accuracy for carvacrol

Concentration (µg/ml)	P_1	P_2	P_3	<P>	<y>	SD	RSD (%)	<R%>	tα
80% (3,6 µg/ml)	30231	30161	29777	30056.33	3.49	0.03	0.8	96.98	1.15
100% (4,5 µg/ml)	38646	38877	38759	38760.67	4.5	0.01	0.3	100.07	1.73
120% (5,4 µg/ml)	47160	47276	47508	47314.67	5.5	0.02	0.4	101.8	1.44

Table 3. The validation parameter repeatability for thymol

	Concentration 1			**Concentration 2**			**Concentration 3**		
Number of measurements	**P**	**(µg/ml)**	***R (%)***	**P**	**(µg/ml)**	***R (%)***	**P**	**(µg/ml)**	***R (%)***
1	33254	36.43	101.19	41572	45.56	101.25	50139	54.97	101.80
2	33329	36.51	101.42	41822	45.84	101.86	49956	54.77	101.43
3	32810	35.94	99.83	41730	45.74	101.64	50077	54.90	101.67
4	33512	36.71	101.97	40648	44.55	99.00	50144	54.98	101.81
5	33239	36.41	101.14	41332	45.30	100.66	50157	54.99	101.83
6	33046	36.20	100.55	41565	45.55	101.23	49944	54.76	101.40
<x>	33198.33	36.37	101.02	41444.83	45.42	100.94	50069.5	54.89	101.66
SD		0.27	0.74		0.47	1.04		0.11	0.20
RSD (%)		0.7	0.73		1.0	1.03		0.2	0.19

Table 4. The validation parameter repeatability for carvacrol

	Concentration 1			**Concentration 2**			**Concentration 3**		
Number	**P**	**(µg/ml)**	***R (%)***	**P**	**(µg/ml)**	***R (%)***	**P**	**(µg/ml)**	***R (%)***
1	30231	3.51	97.54	38646	4.49	99.77	47160	5.48	101.47
2	30161	3.50	97.32	38877	4.52	100.37	47276	5.49	101.72
3	29777	3.46	96.08	38759	4.50	100.06	47508	5.52	102.22
4	30066	3.49	97.01	38520	4.47	99.44	47170	5.48	101.49
5	29897	3.47	96.46	38406	4.46	99.15	47392	5.51	101.97
6	29993	3.48	96.77	38535	4.48	99.48	47476	5.52	102.15
<x>	30020,83	3.49	96.86	38623.83	4.49	99.71	47330.33	5.50	101.83
SD		0.02	0.54		0.02	0.45		0.02	0.33
RSD (%)		0.5	0.5		0.4	0.4		0.3	0.3

CONCLUSIONS

The proposed HPLC methods can be used to identify and quantify thymol and carvacrol in the *Thymi tincturae*. Results obtained by statistical processing are in the reference interval, which are recommended by the ICH guidelines.

By analyzing Thymi tincturae, it was found that the concentration of thymol was 0.807 mg/g of the tincture, while the concentration of carvacrol was 0.082 mg/g of the tincture.

This analysis is very fast, reliable and economical.The method does not require a complicated sample preparation and as such can be used in the regular control of the content of thymol and carvacrol in the finished product and the semi-product (tincture).

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Summary/Sažetak

Rod *Thymus* sadrži oko 300-400 vrsta, od kojih se veliki broj koristi u narodnoj medicini. Iz *Thymus* roda se najviše koristi *Thymus vulgaris (timijan).* U službenoj medicini timijan se koristi kao opći lijek za prehladu, gripu, groznicu, kašalj i bronhitis, i to kao: antiseptik, spazmolitik, antifungicid, antitusik, tonik, antihelmintik, antioksidativni agens, antivirotik, sredstvo protiv nadimanja, sedativ, diaforetik, antibakterijsko i osvježavajuće sredstvo. Farmakološki efekti timijana se najviše vezuju za njegove polifenolne komponente timol i karvakrol. Od hromatografskih metoda se najčešće koriste tečna hromatografija visokih performansi. Rezultati dobiveni statističkom obradom se nalaze u referentnom intervalu, koji preporučuju ICH smjernice. Analizom *Thymi tincturae* je dobijeno da je koncentracija timola 0,807 mg/g tinkture, dok je koncentracija karvakrola 0,082 mg/g tinkture.

Ova analiza je vrlo brza, pouzdana i ekonomična. Metoda ne zahtjeva komplikovanu pripremu uzorak i kao takva se može koristiti u redovnoj kontroli sadržaja timola i karvakrola u gotovom proizvodu i poluproizvodu (tinkturi).