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Extraction, purification and analysis of thymol and carvacrole from thyme plant by Modern Chromatographic techniques

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بسم الله الرحمن الرحيم
﴿لا الشمس ينبغي لها أن تدرك القمر ولا الليل سابق
النهار وكل في فلك يسبحون﴾
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صدق الله العلي العظيم

الإهداء

الى من جرع الكأس فارغاً ليسقيني قطرة حب

الى من كلت انامله ليقدم لنا لحظة سعادة

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اهدي ثمرة جهدي المتواضع مع خالص تقديري

Indexing

ABSTRACT

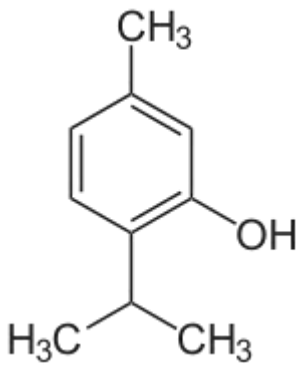
The procedures for obtaining thyme (Thymus vulgaris L.) extracts, steam distillation (prescribed by Yugoslav pharmacopoeia), extraction with periodical exchange of extract (Soxhlet extraction) using methylene chloride, and supercritical fluid extraction (SFE) by carbon dioxide (p = ١٠٠ bar, T = ٤٠°C, d = ٠,٦٣٠٢ g/mL) were used.

For the qualitative analysis of obtained extracts, thin-layer hromatography (TLC) was used. The content of pharmacologically active phenolic compounds, thymol and carvacrol, was determined by developed HPLC (High Performance Liquid Chromatography) method. The comparison of qualitative and quantitative composition of obtained extracts was done.

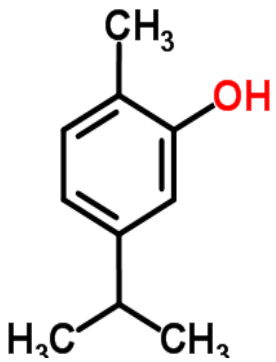
Keywords: Carvacrol, thymol, Thymus vulgaris, SFE and HPLC.

INTRODUCTION

Thyme (Thymus vulgaris L.) is an excellent antiseptic and tonic, and today is used as a respiratory (antitussive) remedy, as well as being important for variety of other ailments. The strong antiseptic and antifungal activities of thyme, i.e. thyme essential oil, are mainly due to the presence of phenolic compounds, thymol and carvacrol.



Thymol Structure



Carvacrol Structure

The thyme essential oil yield is ٠,٣-٦,٣٪ (١-٥). The Yugoslav pharmacopoeia prescribes the minimal yield of ١,٥٪ (٦). The content of thymol in thyme essential oil (up to ٦٠٪) is much higher compared to carvacrol content (to ٦٪) (٧،٨). thymol shows ٣٠ times higher antiseptic effect and ٤ times lower toxic effect than phenol (٩). The thyme phenolic compounds can form oxygen free radicals (١٠).

The classical procedures for separation of active substances from the plant material by steam distillation and extraction with organic

solvents have serious drawbacks. The distillation procedure allows only the separation of volatile compounds (essential oils), which, to a greater or lesser extent, are transformed under the influence of the elevated temperature. On the other hand, extraction with organic solvents can hardly render an extract free of traces of the organic solvent, which are undesirable for either organoleptic and/or health reasons. Besides, organic solvents are insufficiently selective, so that, in addition to the active substances, they also dissolve some concomitant compounds.

For these reasons supercritical fluid extraction (SFE) with supercritical carbon dioxide ($CO_{٢}$) has recently gained in importance as an alternative to the classical procedure. Extraction procedures involving supercritical $CO_{٢}$ belong to "clean technologies", with no secondary products polluting the environment. $CO_{٢}$ is most widely used in SFE because it is simple to use, inexpensive, non-flammable, nontoxic, chemically stable, shows great affinity to volatile (lipophilic) compounds, and can be easily and completely removed from any extract. Bychanging pressure and/or temperature above the critical point of Cf), (Tc=٣١,٣°C; pc=٧٢,٨ bar; dc=٠,٤٦٧ g mL-١) , a pronounced change in the density and dielectric constant, i.e. solvent power of supercritical $CO_{٢}$ can be achieved. SFE of essential oil from wild thyme (Thymus serpyllumiwas investigated (II). HPLC method for

determination of thymol and carvacrol (٧،١٢،١٣-١٥), i.e. GC analysis of thyme essential oil compounds (٨،١٦), were used by many authors. In this study the comparison of qualitative and quantitative composition of thyme extracts obtained by steam distillation, Soxhlet extraction using methylene chloride, and supercritical fluid extraction (SFE) by $CO_{٢}$ was done.

EXPERIMENTAL

Chemicals

Standard samples of thymol and carvacrol (Carl Roth KG, Karlsruhe, Germany) were used. Commercial carbon dioxide was used as the extracting agent. All other chemicals were analytical reagent grade.

Chromatographic Procedures

TLC: Glass plates (٢٠ x ٢٠ em) coated in our laboratory with silica gel G ٦٠ for TLC (Fluka AG, Switzerland), thickness ٠,٢٥ rnm, were used. A methylene chloride solutions of thyme essential oil, extract obtained by Soxhlet extraction using methylene chloride and extract obtained by SFE using $CO_{٢}$ as well as standard of thymol, were put on the start (see Figure I). The mobile phase was benzol: ethylacetate

(٩٣:٧; V/V) (٥). Detection was by spraying the plate with I% vanillin solution and heating (briefly at ١٠٥°C).

HPLC: The HPLC instrument was a Waters ٦٠٠E Multi solvent Delivery System with Waters Multi wavelenght Detector(Millipore Corporation, Waters Chromatography Division, Milford, MA, USA) and HP ٣٣٩٦ Series Integrator (Hewlett-Packard Gmbl-l, Waldbronn, Germany). A column NovaPak CI٨ (Waters) (٣,٩ mm LD. x ١٥ ern: ٤ urn) and Precolumn WatersGuard-Pakl't/Resolve" (I ٠ urn) were used. The mobile phase was acetonitrile-water (٥٠:٥٠: V/V) (isocratic elution) with the flow rate ٠١'٠,٨mL min'. After filtration (٠,٤٥ urn Millipore filter - Millipore, Bedford, MA USA), I٠ ~L of each sample were used. Detection was carried out at ٢٧٦ nm. The recorder chart speed was ٠,٤ cm min:'. The quantitative determination was carried out using the external standard method.

SFE: The supercritical fluid extraction (SFE) with carbon dioxide was carried out using a laboratory- scale high pressure extraction plant - HPEP (NOVA-Swiss, Effretikon, Switzerland) described previously (١٧). The main parts and characteristics of the plant were: the diaphragm-type compressor (up to ١٠٠٠ bar), extractor with the internal volume ٠١'٢٠٠mL(Pmax=٧٠٠ bar), separator with the internal volume ٢٠٠ mL(Pmn,=٢٥٠ bar), maximum$CO_٢$ flow rate of about ٥,٧ kg h:'. The

sample mass of thyme (mean particle size ٠,٣٥ mm) in extractor: ٥٠ g; pressure: ١٠٠ bar; temperature: ٤٠°C:CO٢, flow rate: ٩٧,٧٢٥ L h"; extraction time: ٢,٥ hours. Separator conditions were: pressure ١٥ bar and temperature ٢٥°C.

Essential oil density was measured on a Calculating Digital Density Meter, Tip PAAR DMA ٤٦, Gratz, Austria.

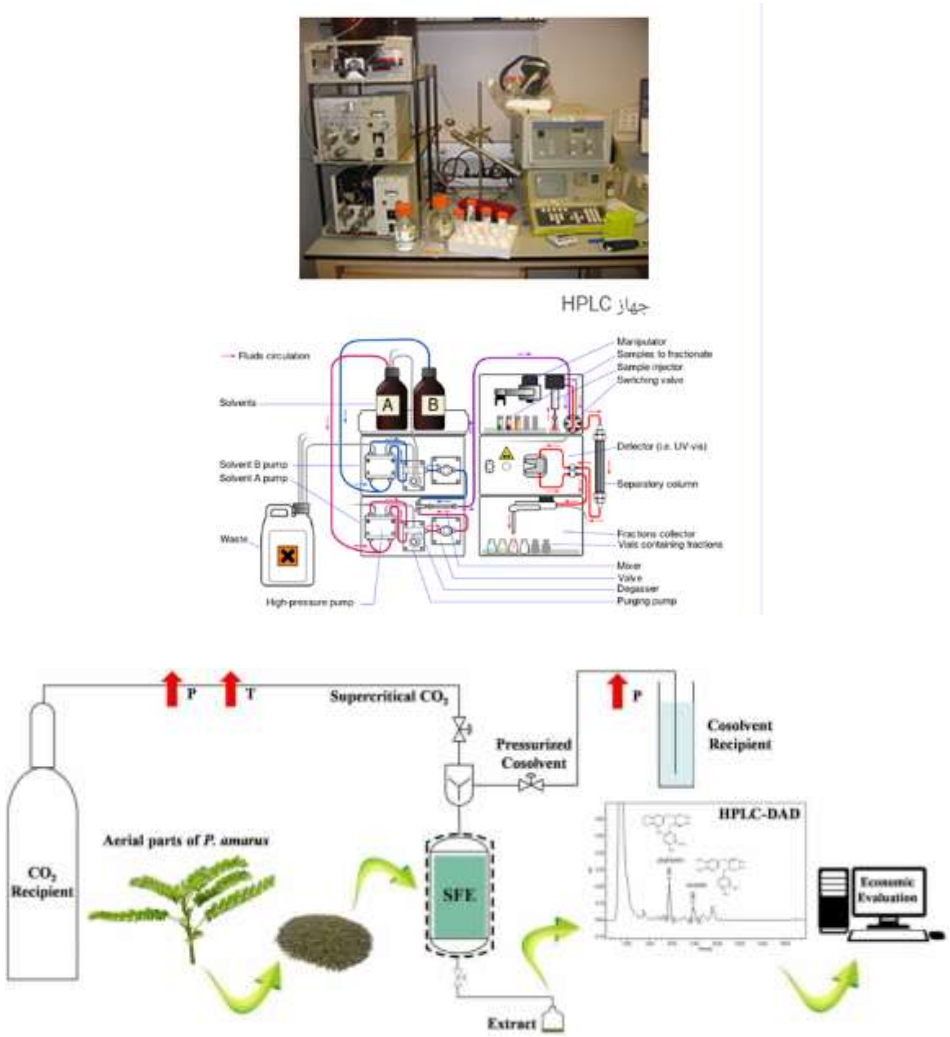


Figure ١: Schematic diagram of SFE apparatus

The system must contain a pump for the CO_2, a pressure cell to contain the sample, a means of maintaining pressure in the system and a collecting vessel. The liquid is pumped to a heating zone, where it is heated to supercritical conditions. It then passes into the extraction vessel, where it rapidly diffuses into the solid matrix and dissolves the material to be extracted. The dissolved material is swept from the extraction cell into a separator at lower pressure, and the extracted material settles out. The CO_2 can then be cooled, re-compressed and recycled, or discharged to atmosphere.

Carbon dioxide (CO_2) is usually pumped as a liquid, usually below ٥ °C (٤١ °F) and a pressure of about ٥٠ bar. The solvent is pumped as a liquid as it is then almost incompressible; if it were pumped as a supercritical fluid, much of the pump stroke would be "used up" in compressing the fluid, rather than pumping it. For small scale extractions (up to a few grams / minute), reciprocating CO_2 pumps or syringe pumps are often used. For larger scale extractions, diaphragm pumps are most common. The pump heads will usually require cooling, and the CO_2 will also be cooled

Simple model of SFE:

There are two essential steps to SFE, transport (by diffusion or otherwise) from with the solid particles to the surface, and dissolution in the supercritical fluid. Other factors, such as diffusion into the particle by the

SF and reversible release such as desorption from an active site are sometimes significant, but not dealt with in detail here. Figure ٢ shows the stages during extraction from a spherical particle where at the start of the extraction the level of extractant is equal across the whole sphere (Fig. ٢a). As extraction commences, material is initially extracted from the edge of the sphere, and the concentration in the center is unchanged (Fig ٢b). As the extraction progresses, the concentration in the center of the sphere drops as the extractant diffuses towards the edge of the sphere (Figure ٢c)

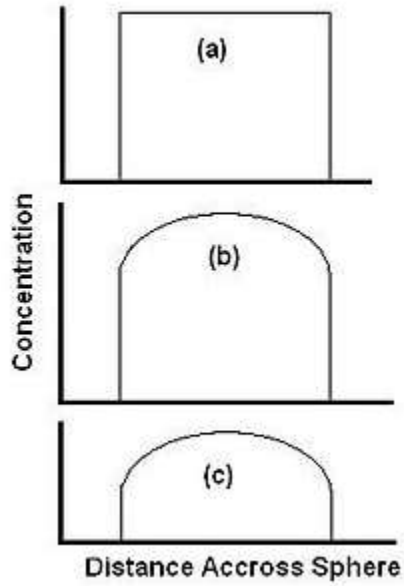


Figure ٢. Concentration profiles during a typical SFE extraction

The relative rates of diffusion and dissolution are illustrated by two extreme cases in Figure ٣. Figure ٣a shows a case where dissolution is fast relative to diffusion. The material is carried away from the edge faster than it can diffuse from the center, so the concentration at the edge drops to zero. The material is carried away as fast as it arrives at the surface, and the extraction is completely diffusion limited. Here the rate of extraction can be increased by increasing diffusion rate, for example raising the temperature, but not by increasing the flow rate of the solvent. Figure ٣b shows a case where solubility is low relative to

diffusion. The extractant is able to diffuse to the edge faster than it can be carried away by the solvent, and the concentration profile is flat. In this case, the extraction rate can be increased by increasing

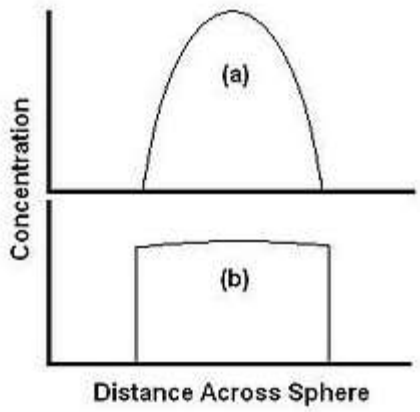


Figure ٣. Concentration profiles for (a) diffusion limited and (b) solubility limited extraction

the rate of dissolution, for example by increasing flow rate of the solvent.

The extraction curve of % recovery against time can be used to elucidate the type of extraction occurring. Figure ٤(a) shows a typical diffusion controlled curve. The extraction is initially rapid, until the concentration at the surface drops to zero, and the rate then becomes much slower. The % extracted eventually approaches ١٠٠٪. Figure ٤(b) shows a curve for a solubility limited extraction. The extraction rate is almost constant, and only flattens off towards the end of the extraction. Figure ٤(c) shows a curve where there are significant matrix effects, where there is some sort of reversible interaction with the matrix, such as desorption from an active site. The recovery flattens off, and if the ١٠٠٪ value is not known, then it is hard to tell that extraction is less than complete.

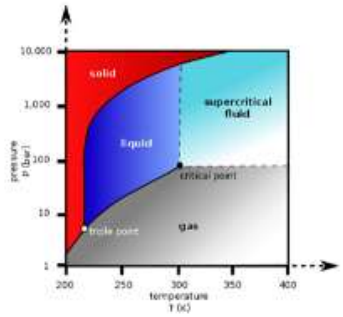


Figure ٤. Extraction Profile for Different Types of Extraction

RESULTS AND DISCUSSION

The dependence of the mass of carvacrol (mK : mg), i.e., of the mass of thymol (rn.; mg), in the investigates sample, on the integrated peak area (P), can be expressed by the following equations (١٨):

$$m_K = (P + ٣١١٩١,٥)/٦٨١٣٣٧٠$$

$$r = ٠,٩٩٨٨$$

and

$$m_K = (P + ٣١١٩١,٥)/٦٨١٣٣٧٠$$

$$r = ٠,٩٩٨٨$$

$$m_T = (P-٦٨١١٤٩)/٧٨٧١٧٢٠$$

$$r = ٠,٩٩٨٥$$

where r is the coefficient of regression. The yield of thyme essential oil (Table I) in plant material obtained by steam distillation (١,٤٥٪) is a little lower than that prescribed by Yugoslav pharmacopoeia. The

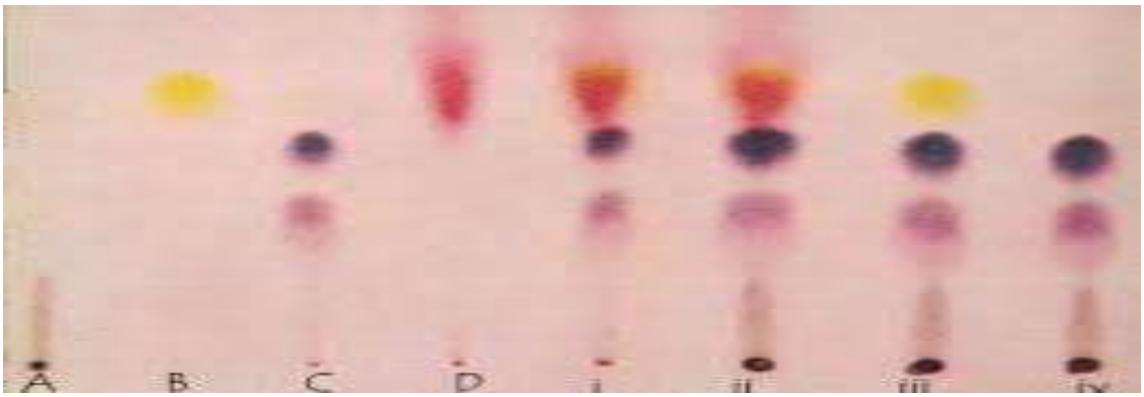
qualitative composition of extracts obtained by investigated extraction procedures, determined by TLC (Figure I), shows that they are very similar (hR_f, values of detected thyme compounds are given in Table ٢).

Table ١. Yields of thyme extraction.

Procedure No.	Extraction procedure	Extraction yield (%)	Thymol content (%; w/w)	Carvacrol conten (%; w/w)
1	Steam distillation	1.45*	52.19	6.72
2	Soxhlet extraction	5.52**	12.37	1.60
3	SFE	1.85**	37.22	4.76

* - Yield in $cm^{٣}$/١٠٠ g of thyme (essential oil density of ٠،٩١ $g/cm^{٣}$).

** - Yield in g/١٠٠ g of thyme.



T ١ ٢ ٣ T

Figure ١. TLC-chromatogram of investigated samples

T – Thymol.

I - Essential oil obtained by steam distillation.

٢ - Methylene chloride extract.

٣ - Extract obtained by supercritical fluid extraction(SFE).

Table ٢. hR_f values of thyme compounds

Compound	hR_f (%)	Color
Terpene alcohols (borneol, geraniol, linalool)	28.1÷45.6	Grey
Thymol+carvacrol	68.7	Red-orange
Terpene esters (bornyl and linalyl-acetate)	81.3	Blue
Terpene hydrocarbons	93.7	Violet

The contents of carvacrol and thymol in the essential oil (Procedure No.١) were determined by HPLC(Figure ٢) using Eqs (I) and (٢). Thymol content (٥٢,١٩٪)in that sample is much higher than content of carvacrol (٦,٧٢٪).

HPLC conditions:

Column: NovaPak C١٨ (Waters; ٣,٩ mm LD. x ١٥ ern; ٤ urn)

Precolumn: Waters Guard-Pak (١٠ urn)

Mobile phase: Acetonitrile - water (٥٠:٥٠; VIV)

Flow rate: ٠,٨ mL/min

Pressure: ١٢٥ bar

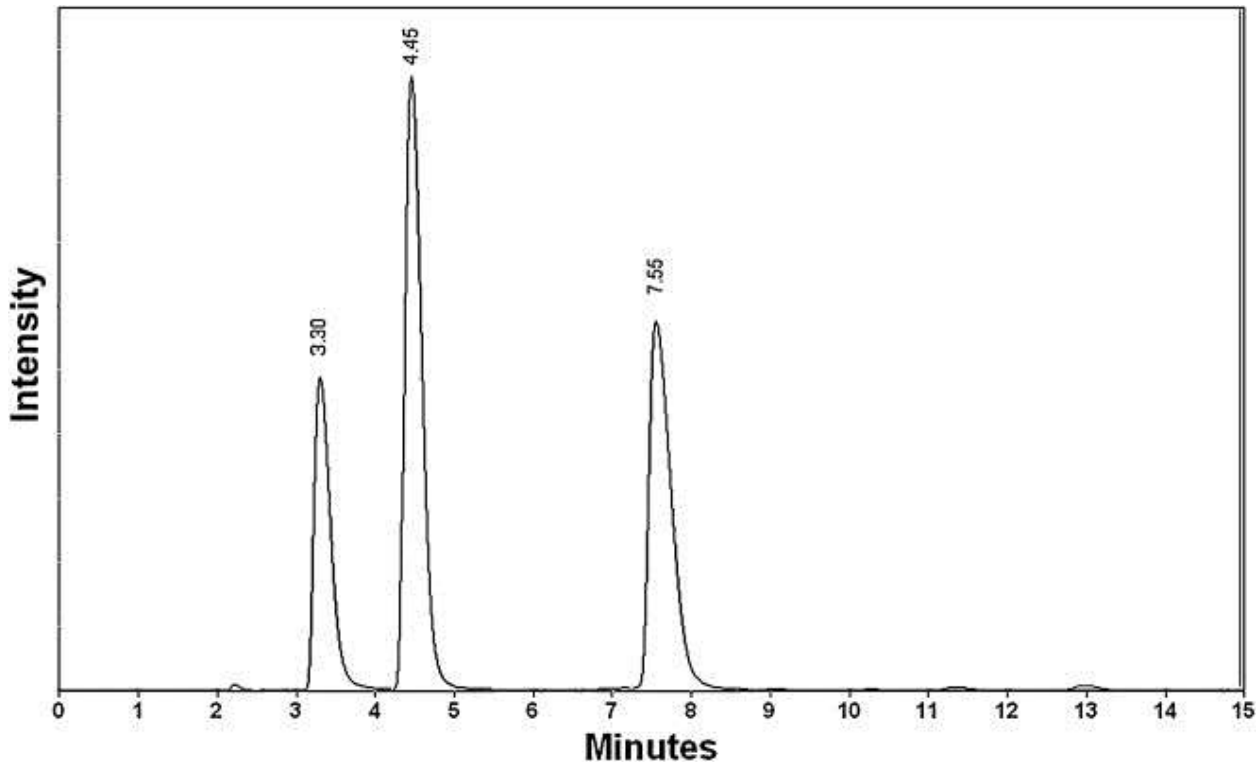
Detection: UV at ٢٧٦ nm / ١.٠ AUFS

Chart speed: ٠,٤ cm/min

Concentration of solution: ٢,٤٧ mg/mL

Injected volume: ١٠ µL

Figure ٢. HPLC-chromatogram of thyme essential oil Carvacrol (t_R= ٣. ٣ min) and Thymol (t R =٤.٤٥ min).



CONCLUTION

In the total extract of thyme obtained by methylene chloride (Soxhlet extraction; Procedure No.٢), yield of ٥,٥٢٪, content of thymol, as well

as of carvacrol, is about ٤,٢ times lower than that in thyme essential oil. On the other hand, contents of investigated phenols in the extract obtained by $CO_٢$-SFE (Procedure No.٣), yield of I.٨٥٪. are only about ٢٩٪ lower than that in thyme essential oil.

To obtain a thyme extract whose qualitative and quantitative characteristics are more similar to those of the essential oil obtained using official procedure by steam distillation, it is necessary to use a supercritical fluid extraction by carbon dioxide at determined extraction conditions.

THE REFERENCES

١. Chevallier, A. in: The Encyclopedia of Medicinal Plants, Dorling Kindersley Limited, London (١٩٩٦). p. ١٤٢.

٢. Kisgeci, ١., Adamovic D. i M. Soldatovic: Sistematski prikaz privredno znacajnih lekovitih, arornaticnih i zacinskih biljaka i njihov izbor za gajcnjc u Vojvodini, Bilten za hmelj, sirak i Ickovito bilje, Vol. ١٧،٤٩-٥٠ (١٩٨٥),٤٤-٧١.

٣. Pekic, B. in: Hemija i tehnologija farmaceutskih proizvoda (alkaloidi i etarska ulja), Tehnoloski fakultet Univerziteta u Novom Sadu (١٩٨٣), p. ٢٢٣.

٤. The Merck Index, Tenth Edition, Merck & Co., Inc., Rahway,N. J., U.S.A. (١٩٨٣). p. ٩٧٨.

٥. Wagner, H., Bladt S. and E.M. Zgainski, in: Plant Drug Analysis (Translated by A. Scott). Springer-Verlag, Berlin HeidelbergNew YorkTokyo(١٩٨٤), p. ١٣.

٦. Pharmacopoeia.Iugoslavica, Editio quarta (Ph. Jug. IV), Savezni zavod za zdravstvenu zastitu, Beograd (١٩٨٤).

٧. Pekic, B., Lepojevic Z. i Z. Zekovic, L. Pekic: Ispitivanjc sadrzaja etarskog ulja i fenolnih jedinjenja u nekim biljnim vrstama roda Thymus i Satureja, Proceedings of Faculty of Technology (Novi Sad) ٢٢ (١٩٩١), ٨٧-٩٢.

٨. Fehr, D. und G. Stenzhorn: Untersuchungen zur Lagerstabilitat von Pfefferminz-blattern, Rosmarinblattern und Thymian, Pharmazeutischc Zeitung ١٢٤،٤٦ (١٩٧٩),٢٣٤٢-٢٣٤٩.

٩. Lukic, P. in: Farrnakognozija, Farmaceutski fakultet Univerziteta u Beogradu (١٩٨٩).

١٠. Deighton,N., Glidewell S.M., S.G. Deans, B.A.Goodman: Identification by EPR Spectroscopy of Carvacrol and Thymol as the Major Sources of Free-Radicals in the Oxidation of Plant Essential Oils, .Iournal of the Science of Food and Agriculture ٦٣, ٢ (١٩٩٣),٢٢١-٢٢٥.

II. Aleksovski, S., Sovova H., Poposka F. and B. Curapova: Supercritical fluid extraction of essential oils from peppermint and wild thyme and its comparison with hydrodistillation, Xlii CHISA '٩٨, Summaries ٤ (١٩٩٨), ١١٨.

١٢. VanDen Broucke, e.٠. and lA. Lemli:Chemical Investigationof the Essential Oil of Origanum compactum, Planta medica ٣٨ (١٩٨٠), ٢٦٤-٢٦٦.

١٣. Solinas, v., Oessa e. and L. Falchi Delitala: High-performance Liquid Chromatographic Analysis of Carvacrol and Thymol in the Essential Oil of Thymus capitatus,.I. Chromatogr. ٢١٩ (١٩٨١),٣٣٢-٣٣٧.

١٤. VanDen Broucke, C.O. and lA. Lemli: Pharmacologicaland Chemical Investigation of Thyme Liquid Extracts, Plantamedica٤١ (١٩٨١),١٢٩-١٣٥.

١٥. Pekic, P., Lepojevic Z. i Z. Zekovic: Odredivanje timola i karvakrola u etarskom ulju timijana (Thymus vulgaris L.) primenom HPLC, Proceeding for Natural Sciences (Novi Sad) ٨٠ (١٩٩١),١٧-٢٢. ٦٢١

١٦. Jackson, S.A.L. and R.K.M. Hay: Characteristics of Varieties of Thyme (Thymus vulgaris ١,.) for Use in the UK: Oil Content, Composition and Related Characters, Journal of Horticultural Science٦٩, ٢ (١٩٩٤),٢٧٥-٢٨١.

١٧. Pekic, B., Zekovic Z., Petrovic L. and A. Tolic: Behavior of (-)-a-Bisabolol and (-)-a Bisabololoxides A and B in Camomile Flower Extraction with Supercritical Carbon Dioxide, Separation Science andTechnology ٣٠, ١٨(١٩٩٥),٣٥٦٧-٣٥٧٦.

١٨. Zekovic, Z., Lepojevic Z. and Dj. Vujic: Supercritical Extraction of Thyme (Thymus vulgaris L.), Chromatographia ٥١،٣/٤ (٢٠٠٠), ١٧٥-١٧٩.