Supercritical Extraction of Thyme (Thymus vulgaris L.)

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Summary
Chromatographic methods for the qualitative and quantitative analysis of thyme (Thymus vulgaris L.) extracts (essential oil obtained by steam distillation and extracts obtained by carbon dioxide supercritical fluid extraction and methylene chloride) are described. The composition of extracts obtained at different pressures (from 80 bar to 400 bar) and constant temperature (40 °C) is discussed. The extraction system thyme – supercritical carbon dioxide was modelled by empirical equations defining the dependence of the total extract (TE) solubility and thymol solubility in CO2 on the density of carbon dioxide.

Introduction
The strongly antiseptic and antifungal activities of thyme (Thymus vulgaris L.), i.e., thyme essential oil, is mainly due to the presence of phenolic compounds, primarily of thymol and carvacrol. The yield thyme essential oil is 0.3–6.3 % [1–5]. The Yugoslav pharmacopoeia prescribes a minimum yield of 1.5 % [6]. The thymol content in thyme essential oil (up to 60 %) is much higher than the carvacrol content (to 6 %) [7,8]. Thymol shows a 30 times higher antiseptic effect and 4 times lower toxic effect than phenol [9]. The thyme phenolic compounds can form oxygen free-radicals [10].

The classical procedures for the separation of active substances form 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 accompanying compounds. For these reasons, supercritical fluid extraction (SFE) with supercritical carbon dioxide (CO2) has recently gained in importance as an alternative to the classical procedure. Extraction procedures involving supercritical CO2 belong to “clean technologies”, with no secondary products polluting the environment. CO2 is most widely used in SFE because it is simple to use, inexpensive, non-flammable, nontoxic, chemically stable, shows great affinity for volatile (lipophilic) compounds, and can be easily and completely removed from any extract. By changing pressure and/or temperature above the critical point of CO2 (Tc = 31.3 °C; pc = 72.8 bar; dc = 0.467 g mL-1), a pronounced change in the density and dielectric constant, i.e., solvent power of supercritical CO2, can be achieved.

The SFE of essential oils from wild thyme (Thymus serpyllum) has been investigated [11]. HPLC methods for determination of thymol and carvacrol [7, 12, 13–15], and, GC analysis of thyme essential oil compounds [8, 16], have been used by many authors.

This study describes the chromatographic methods used in our investigations of supercritical fluid extraction of thyme with carbon dioxide.

Experimental
Plant Material
The thyme was produced by Mr. Lazar Oluški (village Sanad near Čoka, Vojvodina, Yugoslavia, 1996).
Chemicals

Standard samples of thymol (Kemika, Zagreb, Croatia) and carvacrol (Carl Roth KG, Karlsruhe, Germany) were used. Commercial carbon dioxide (Tehno-gas, Novi Sad, Yugoslavia) was used as the extracting agent. All other chemicals were analytical reagent grade.

Chromatographic Procedures

HPLC

The HPLC instrument was a Waters 600E Multisolvent Delivery System with Waters Multiwavelength Detector (Millipore Corporation, Waters Chromatography Division, Milford, MA, USA) and HP 3396 Series Integrator (Hewlett-Packard GmbH, Waldbronn, Germany). A column, NovaPak C18 (Waters) (3.9 mm ID × 15 cm; 4 μm) and precolumn, Waters Guard-Pak ResolveTM were used. The mobile phase was acetonitrile-water (50:50; v/v) (isocratic elution) with a flow rate of 0.8 mL min⁻¹. After filtration (0.45 μm Millipore filter- Milipore, Bedford, MA, USA), 10 μL of each sample was used. Detection was carried out at 276 nm. The recorder chart speed was 0.4 cm min⁻¹. The quantitative determination was carried out using the external standard method.

GC-MS

The instrument was GCD HP G 1800 A (Hewlett-Packard, Palo Alto, CA, USA). A column, HP-5 MS (30.0 m × 0.25 mm) was used. Helium flow rate: 0.8 mL min⁻¹. Temperature: injector 250 °C, detector 280 °C, initial 50 °C with a linear increase of 20 °C min⁻¹ to 130 °C (1 min) and 9 °C min⁻¹ to the final temperature of 280 °C (8.33 min). Total analysis time: 30 min. The injected volume of sample solution in n-pentane (40–50 μg mL⁻¹) was 5 μL. Detector: 45–425 D. The compounds were identified using the Wiley data base and the quantitative determination was carried out by the quasi-internal standard method (the tymol content in the sample was determined by HPLC).

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 [17]. The main parts and characteristics (manufacturer specification) of the plant were: the diaphragm-type compressor (up to 1000 bar), extractor with an internal volume of 200 mL (Pmax = 700 bar), separator with an internal volume of 200 mL (Pmax = 250 bar), maximum CO₂ flow rate of about 5.7 kg h⁻¹. The sample mass of thyme (mean particle size 0.35 mm) in extractor: 50 g; pressure: investigated value; temperature: 40 °C; CO₂ flow rate: 97.725 L h⁻¹; extraction time: 2.5 hours. Separator conditions were: pressure 15 bar and temperature 25 °C (see Figure 6).

Results and Discussion

The essential oil density was measured on a Calculating Digital Density Meter, Tip PAAR DMA 46, Gratz, Austria.

UV-spectra were recorded using a 8452A Diode Array Spectrophotometer, Hewlett-Packard GmbH, Waldbronn, Germany.

The content of thyme essential oil (d₂₀⁰C = 0.9118 g mL⁻¹) determined by an official procedure [6] was 1.75 % (V/m).

The UV-spectra of standard samples of thymol and carvacrol are shown in Figures 1 and 2. Figure 3 shows the chromatograms of the standard solution of the investigated phenols and thyme extract obtained after 2.5 hours of SFE-CO₂ (400 bar, 40 °C) under the HPLC conditions.

The dependence of the mass of thymol (mT; μg) (Figure 4), and of carvacrol (mK; μg) (Figure 5), on the integrated peak area (P), can be expressed by the following equations:

\[
m_T = (P - 681149)/7871720 (1)
\]

\[
r = 0.9985
\]

and

\[
m_K = (P + 31191.5)/6813370 (2)
\]

\[
r = 0.9988
\]

where r is the coefficient of regression.

The high values of the regression coefficients obtained confirm the validity of Eqs (1) and (2).

High reproducibility was obtained by the HPLC method developed (CV for thymol 1.29 %; Table I).

The predominant compound of the thyme essential oil is thymol (50.06 %; w/w), while carvacrol is present, practically, in traces (1.15 %; w/w). The results of the GC-MS analysis of the essential oil obtained by steam distillation are shown in Table II.

In order to prevent thermal decomposition of essential oil compounds, a temperature of 40 °C was selected for the SFE-CO₂. The pressure range of 80–400 bar (a pronounced change in the density and dielectric constant of CO₂) for SFE-CO₂ of thyme was investigated (see Figure 6).

The results of the GC-MS analysis of the total extract obtained at the investigated pressures of CO₂ are given in Table II. As an illustration, the CG-chromatogram of thyme TE obtained by CO₂ with high solubility power (400 bar; 40 °C; \(d = 0.9830 \text{ g mL}^{-1}\)) is shown in Figure 7.

The use of CO₂ of low solubility power (80 bar, 40 °C, \(d = 0.1918 \text{ g mL}^{-1}\)) did not yield quantitative extraction of thyme essential oil. The yield of essential oil was 0.475 % and the thymol content in that oil of 69.59 % (w/w) was determined by steam distillation of the thyme after SFE (Table I). The quantitative extrac-