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Subcritical water extraction of essential oils from *Thymbra spicata*

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Abstract

Essential oils from the leaves of *Thymbra spicata* L. were extracted using subcritical water. The extraction efficiencies of different temperatures (100, 125, 150 and 175 °C), pressures (20, 60, and 90 bar) and flow rates (1, 2, and 3 ml min^{-1}) were investigated. The components of essential oils of *Thymbra spicata* were removed from the aqueous extract by C18 solid phase extraction. The identification of components was carried out using two-dimensional gas chromatography (GC)- Time of flight/Mass Spectrometry (TOF/MS). The essential oil yields were found to be 2.5, 2.7, 3.7, 3.5% for 100, 125, 150 and 175 °C, respectively. Subcritical water extraction of essential oils from *Thymbra spicata* exhibited the highest extraction efficiency at 150 °C, 2 ml min^{-1} and 60 bar for 30 min. The minimum time for a complete extraction of the leaves of *Thymbra spicata* was 20 min at 150 °C and 2 ml min^{-1}.

Keywords: Subcritical water extraction; Solid phase extraction; *Thymbra spicata*; Essential oil; Two dimensional GC-TOF/MS

1. Introduction

Muller-Riebau, Berger, and Yegen (1995) reported that carvacrol- and thymol- containing plants such as *Thymus, Origanum, Thymbra, Cordothymus* and *Satureajaare* are used as condiments or as herbal teas, taken especially to cure various medical disorders. *Thymbra spicata* (Labiatae) grows wild in some eastern Mediterranean countries and the dried leaves are used as a spice and as a herbal tea. The essential oils of *Thymbra spicata* are found to inhibit mycelial growth of the fungi *Fusarium moniliforme, Rhizoctonia solani, Sclerotinia sclerotiorum, and Phytophthora capsici*. The conventional methods used for the preparation of essential oils and spice oleoresins are steam distillation and solvent extraction, respectively. Recently, more efficient extraction methods, such as supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE), have been used for the isolation of organic compounds from various plants (Kubatova, Lagadec, Miller, & Hawthorne, 2001; Moldao-Martins, Palavra, Beirao da Costa, & Bernarda-Gil, 2000; Okihashi, Obana, & Shinjiro, 1998; Schafer, 1998; Simandi, Oszagyan, Lemberkavics, Kery, Kaszacs, Thyrion, & Matyas, 1998). More recently, a continuous subcritical water extraction (SWE) technique has been used for the extraction of solid samples in a number of studies (Ayala & Castro, 2001; Fernandez-Perez, Jimenez-Carmona, & Luque de Castro, 2001; Kubatova, Miller, & Hawthorne, 2001).

Subcritical or superheated water extraction is a technique based on the use of water as an extractant, at temperatures between 100 and 374 °C and at a pressure high enough to maintain the liquid state. Previous workers (Ayala & Luque de Castro, 2001; Gamiz-Gracia & Luque de Castro, 2000; Rovio, Hartonen, Holm, Hiltunnen, & Riekkola, 1999) have reported that subcritical water for the extraction of essential oils is a powerful alternative, because it enables a rapid extraction, and the use of low working temperatures. This avoids the loss and degradation of volatile and thermo labile compounds. Additional positive aspects of the use of SWE are its simplicity, low cost, and favourable environmental impact.

The aim of this study was to determine the optimum conditions (temperature, time of extraction, pressure and water flow rate) for the continuous SWE of essential oils of *Thymbra spicata* and to

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investigate the effect of temperature on the composition of extracted essential oils.

2. Materials and methods

2.1. Materials

Thymbra spicata L., was collected in June 2002 in Nizip, Gaziantep (South-Eastern Turkey). The leaves were separated from the branches and the air-dried leaves were stored in a polyethylene bag. Undecane (as an internal standard) and NaCl (as a demulsifier) were provided by Aldrich (Gillingham, Dorset, UK). Hexane, methanol and water were of HPLC grade, supplied by Fisher Scientific (Loughborough, UK). ODS-6 (C18) solid phase extraction (SPE) cartridges (500 mg/unit) were purchased from Whatman (Clifton, New Jersey, USA).

2.2. Subcritical water extraction

SWE was performed in laboratory built apparatus (Fig. 1). The water was purged with nitrogen to remove dissolved oxygen prior to the extraction. Deoxygenated water was used in an HPLC pump programmed for a constant flow of 1–3 ml/min^{-1}. A Carlo Erba series 4200 GC oven heated the extraction system. A 3 m long pre-heated coil (0.76 mm i.d. × 1.6 mm o.d.) was used to equilibrate the water to the desired temperature. A 10.4 ml extraction cell (Keystone Scientific, Bellefonte, PA, USA), equipped with 0.5 μm frit at the inlet and outlet, was connected to a 1 m cooling loop (in ice water) outside of the oven. A pressure control valve was placed between the cooling loop and the collection vial.

SWE was carried out using 1.5 g of *Thymbra spicata* leaves, an extraction cell which contained a stainless steel filter and glass wool at both ends, a 1–3 ml min^{-1} flow rate, a temperature of 100–175 °C, a pressure of 20–90 bar and 30 min of extraction time. For the kinetic experiment, the collection vial was replaced every 5 min.

2.3. Solid phase extraction

An end-capped ODS-6 (C-18) SPE cartridge was used to re-extract the analytes with hexane. The sample was loaded onto a 0.5 g ODS-6 SPE cartridge, previously washed with 5 ml of 50/50 (v/v) water-methanol and dried completely by means of a vacuum. Essential oils were eluted with 4 ml of hexane. The collected eluent was concentrated under a nitrogen stream to about 0.5 ml of volume. An appropriate amount of undecane was added into the concentrate as an internal standard. The mixture was directly injected into the two-dimensional GC-TOF (Time of Flight)/MS and GC-FID.

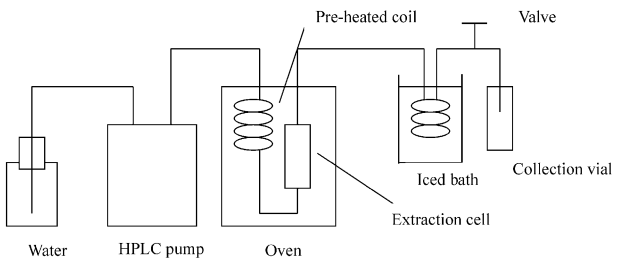

Fig. 1. Laboratory built SWE apparatus.

2.4. Chromatographic analysis

Qualification and quantification were carried out using GC-TOF/MS and GC-FID, respectively. The two dimensional GC-TOF/MS system consisted of an HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with two electronic pressure controlled split/splitless injectors and two columns with independent retention properties. One microlitre of sample volume was injected using the splitless method. The first column was a non-polar BP1 (25 m × 0.32 mm i.d., 1 μm film thickness) and the second column a BP20 (3 m × 0.1 mm i.d., 0.1 μm film thickness). Both columns were purchased from SGE (Ringwood, Australia). Helium was used as a carrier gas. The initial temperature of the GC oven was 60 °C for 3 min and the subsequent temperature programme was a heating rate of 4 °C min^{-1} until 240 °C was reached.

The two columns were coupled in an orthogonal manner using a fast switching (less than 80 ms actuation time) 10 port two position valve with 10 μl sampling loop. Eluting analytes from the first column passed through the sampling loop, which was actuated to produce a pulsed injection to the second column every 4 s. The flow rates through the first and second columns were controlled independently at 4 ml min^{-1}and 2 ml min^{-1} respectively. Peak identification was made using a Pegasus III reflectron time-of-flight mass spectrometer (Leco, Las Vegas, USA) with electron impact ionisation. The mass spectrometer used a push plate frequency of 5 kHz, with transient spectra averaging to give unit resolved mass spectra between 45 and 350 amu at a rate of 50 spectra/second. The linear response data from the 2D separation and detection system was transposed at the valve injection frequency (4s) to give a two dimensional chromatogram, scaled by detector response intensity. A typical 2D separation/total ion chromatogram is shown in Fig. 2.

Quantification of extracts was performed using gas chromatography with a flame ionisation detector (GC-FID) on a Hewlett-Packard 5890 series GC. One microlitre of extract was injected, cold, into a BPX5 fused silica capillary column (25 m × 0.32 mm i.d., 0.5 μm film thickness). Nitrogen was used as a carrier gas. The initial temperature of the GC oven was 60 °C for 3 min; then the temperature was increased at a rate of 4 °C min^{-1} until 240 °C was reached. Undecane was used as

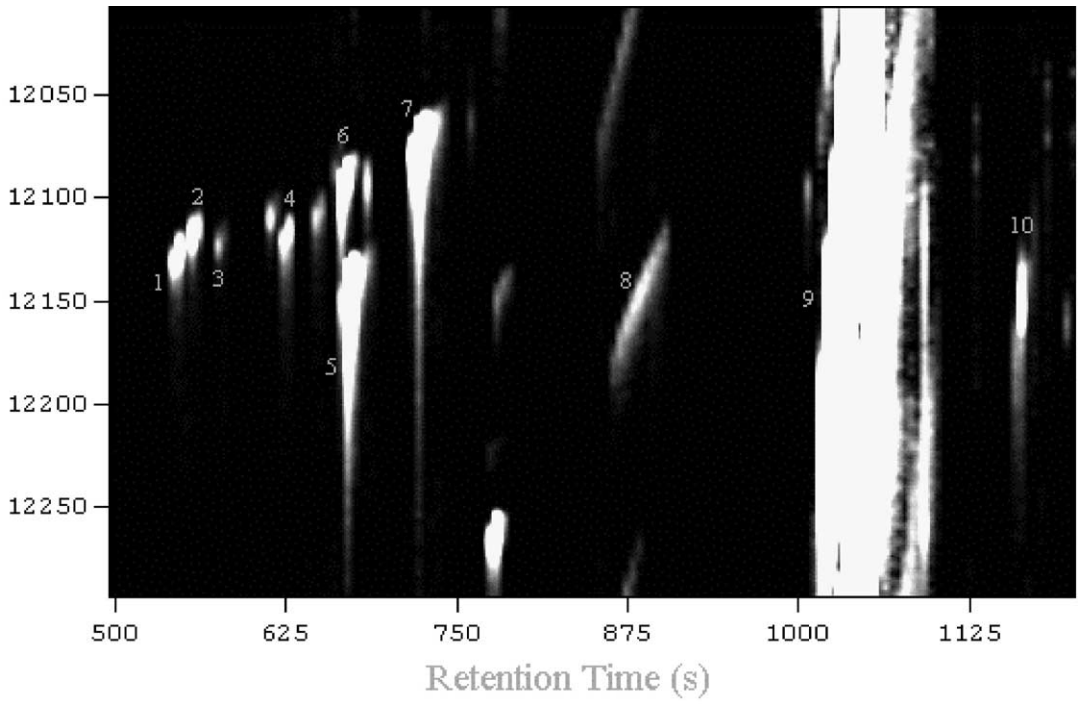

Fig. 2. 2D separation of *Thymbra spicata* extract at 150 °C (1: α-thujene, 2: (−)-α-pinene, 3: camphene, 4: (+)-α-pinene, 5: *p*-cymene, 6: *γ*-terpinene, 7: E-3-caren-2-ol, 8: terpinen-4-ol, 9: 1-carvone, thymol and carvacrol, 10: caryophyllene).

an internal standard for quantification. Percentage of total peak area was used for control purposes.

3. Results and discussion

Prior to the optimisation studies, the critical stage was the removal of the compounds from the aqueous extract. A liquid–liquid extraction technique was applied to remove the compounds from the aqueous extract, using hexane as the extractant. Various volumes of extractant and a process of stepwise extraction were investigated in order to achieve a successful removal of compounds by breaking the emulsion. In addition to the extractant volume change and stepwise extraction trials, NaCl was added to facilitate the emulsion breakdown. However, it was found that even though NaCl helped in the breaking the emulsion, the complete removal of compounds was not successful with this technique and an alternative route was sought.

The separation of organic compounds from aqueous systems by solid phase extraction has been applied in many cases (Thurman & Mills, 1998; Moret & Conte, 2002). Rovio et al. (1999) also have reported that the essential oils of clove were successfully removed from aqueous extract by solid phase extraction of C18 materials. Therefore, it was decided to use C18 material SPE for the removal of compounds from the aqueous phase. The efficiency of the C18 material was tested with a steam distilled sample of the essential oil of *Thymbra spicata* with a known composition. It was found that there was almost no change in the composition of the sample before and after the application of SPE of the C18 material.

The optimal subcritical water extraction conditions were decided upon by using various pressures, water flow rates and temperatures. The experimental variables were optimised to obtain the maximum yield of essential oil in a defined time. The time for the subcritical water extraction was chosen to be 30 min, to make sure all the essential oil had been extracted (Ayala & Luque de Castro, 2001). The kinetic studies were then carried out under these chosen conditions of the subcritical water extraction. The results are the means of the three experiments and the relative standard deviation was in a range of 2–7%.

The effect of pressure, at an arbitrarily selected constant flow rate of 2.0 ml min^{-1} and a temperature of 125 °C, was studied by using a needle type pressure control valve. The pressures studied were 20, 60 and 90 bar. It was observed that there were no significant difference in the amounts of extracted oils. A pressure of 60 (±5) bar was selected for further experiments because of its lower percentage error and for being an intermediate value.

The yields of essential oils for a 30-min extraction at a flow rate of 2 ml min^{-1} and 60 bar, and at four different temperatures (100, 125, 150 and 175 °C) are given in Table 1. The yield increased with temperature up to 150 °C. A further increase to 175 °C resulted in a small

Table 1
The effect of temperature on the essential oil contents (% w/w; on the basis of 100 g of dried leaves), and their constituents (% w/w; on the basis of 100 g of essential oil) extracted from the leaves of *Thymbra spicata* collected in Nizip[a]

Thymbra spicata, Nizip	100 °C	125 °C	150 °C	175 °C
Essential oil content	2.0	2.5	3.4	3.2
α-Thujene	0.19	0.18	0.22	0.24
(−)-α-Pinene	0.37	0.43	0.44	0.42
Camphene	nd	0.19	0.25	0.36
(+)-α-Pinene	0.50	0.69	0.62	0.62
Sabinen	nd	nd	nd	0.84
α-Terpinene	nd	nd	nd	0.22
p-Cymene	0.64	2.52	2.93	2.69
Limonene	nd	nd	nd	0.18
o-Cymene	nd	nd	nd	0.49
γ-Terpinene	1.90	0.38	0.58	0.37
E-3-caren-2-ol	3.08	6.72	7.71	7.16
Terpinen-4-ol	0.41	0.37	0.39	0.39
1-carvone	0.26	0.22	0.21	0.20
Thymol	3.67	1.92	1.24	0.97
Carvacrol	86.2	83.5	82.5	79.5
Caryophyllene	0.29	0.48	0.86	1.94
Spathulenol	nd	nd	nd	0.28
Caryophyllene oxide	nd	nd	nd	0.68
Unknown	2.52	2.41	2.10	2.46

% w/w = percent (weight/weight); nd: not detected.
[a] Each temperature was held for 30 min.

decrease in the yield. Therefore, 150 °C was chosen for the further kinetic studies. Jimenez-Carmona, Ubera, and Luque de Castro (1999), for marjoram extraction, and Gamiz-Gracia and Luque de Castro (2000), for fennel extraction by SWE, also found that the yield reached its maximum at 150 °C over a temperature range of 50–175 °C. In addition, Rovio et al. (1999), for clove extraction, and Basile, Jimenez-Carmona, and Clifford (1998), for rosemary extraction, selected 150 °C as an optimum water extraction temperature because of processing difficulties in further stages at higher temperatures.

It has also been seen that the change in temperature may also result in a change of components and compositions of the extracted essential oils. In general, more components are extracted when the temperature is elevated, brought about by their increasing solubility. Miller and Hawthorne (2000) reported that the solubilities of d-limonene, carvone, eguenol, 1,8-cineole and nerol increased with increasing temperature from 25 to 200 °C. The change in the composition is mainly because of the additional components at elevated temperatures.

Conventional one-dimensional gas chromatography generally does not provide sufficient separation for complex mixtures. Since essential oils contain numerous components, it is possible that some components can obscure the analytes of interest (Dalluge, Rijn, Beens, Vreuls, & Brinkman, 2002). Fig. 2 shows the two dimensional gas chromatogram of *Thymbra spicata* extract at 150 °C. As the essential oil contains only 12 compounds, it is easy to see all of them here. The horizontal axis, a one dimensional chromatogram, shows separation by boiling point in a non-polar column. The vertical axis shows separation by polarity using a polar column and the inclusion of this makes this overall a two dimensional chromatogram. In this study, it was observed that other than the appearance of new components in the essential oil of *Thymbra spicata*, the identification of enantiomers of α-pinene was also possible.

The extraction flow rate was studied in the range 1.0–3.0 ml min^{-1} at a constant temperature of 150 °C and a pressure of 60 (±5) bar. It was found that there was little effect of flow rate on the yield of essential oil. However, as can be seen in Fig. 3, the essential oil recovery changes with changing flow rate as a function of time. Extraction was mostly finished in 20 min for 2–3 ml min^{-1} flow rates, while 97% of oil was extracted with 1 ml min^{-1} in the same time. The difference in the rate of extraction is more apparent up to 10 min. So, it can be concluded that at flow rates less than 2 ml min^{-1}, the rate of extraction is slower and extraction needs more time to be completed. Gamiz-Gracia and Luque de Castro (1999) found that a flow rate of 2 ml min^{-1} was optimum for the extraction of individual components of essential oil from fennel. Fernandez-Perez, Jimenez-Carmona, and Luque de Castro (2000) and Clifford, Basile, and Al-Saidi (1999) also preferred to use a 2 ml min^{-1} flow rate in their studies.

The kinetics of SWE under the optimum working conditions was studied. The influence of the extraction time on the extraction kinetics is shown in Fig. 4. The experiments were carried out for 30 min to obtain a value for the total amount of 15–16 components. The rate of the extraction for five compounds can be quantitatively inferred from the plot. The extraction was mostly completed in 15 min. In the first 5 min 68–86% of the five main compounds were extracted with carvacrol and thymol extraction was completed in 20 min. The other components in the sample (not shown in Fig. 4) were completely extracted in the first 5 min. The kinetic study performed here clearly demonstrated SWE to be a faster technique than conventional essential oil production techniques. This is in good agreement with, for example, the extraction of essential oil of thymus or origanum species within 1 h using supercritical CO_2 or steam distillation (Maldao-Martins et al., 2000) compared with three hours using hydrodistillation (Ayala & Leque de Castro, 2001) and 4–24 hours using Soxhlet extraction (Simandi et al., 1998). Muller-Riebau, Berger, and Yegen, (1995) used steam distillation for the preparation of essential oil from *Thymbra Spicata*. This essential oil contained only 69% of the phenolic compounds. However, in this study, oil containing over 87% of the phenolic compounds was produced using

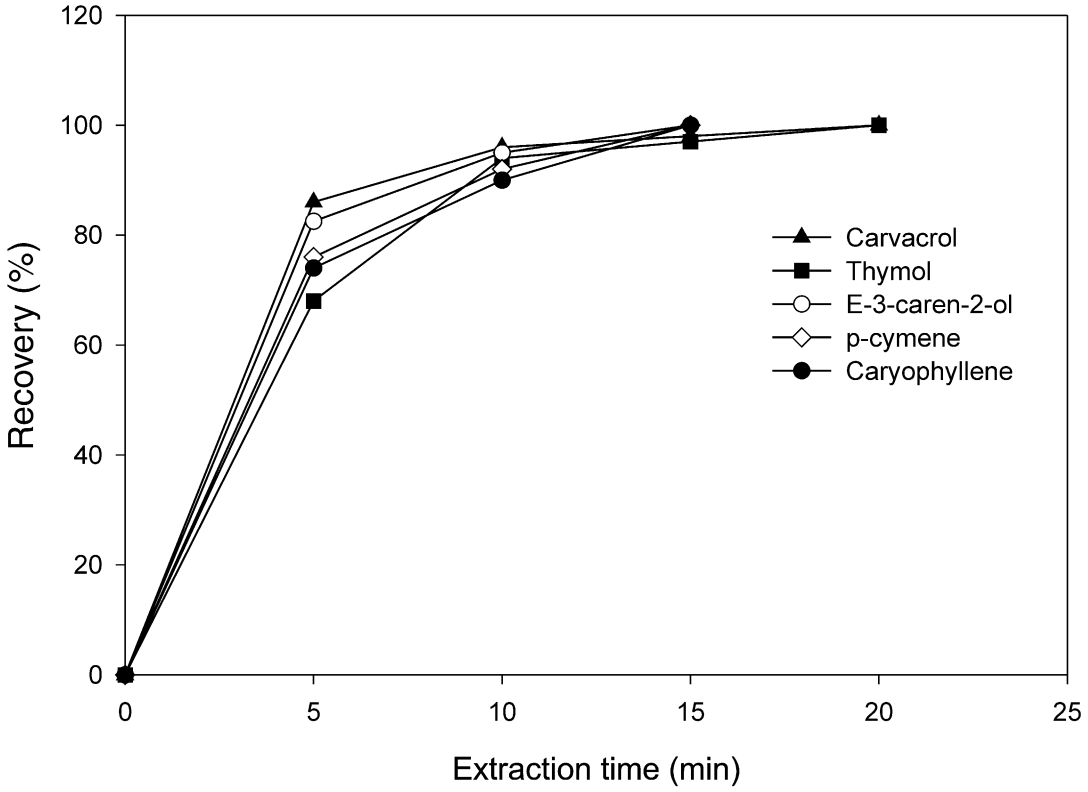

Fig. 3. Effect of extraction time on the extraction efficiency of some compounds of essential oils of *Thymbra spicata* using subcritical water extraction at a temperature of 150 °C and a flow rate of 2 ml min^{-1}.

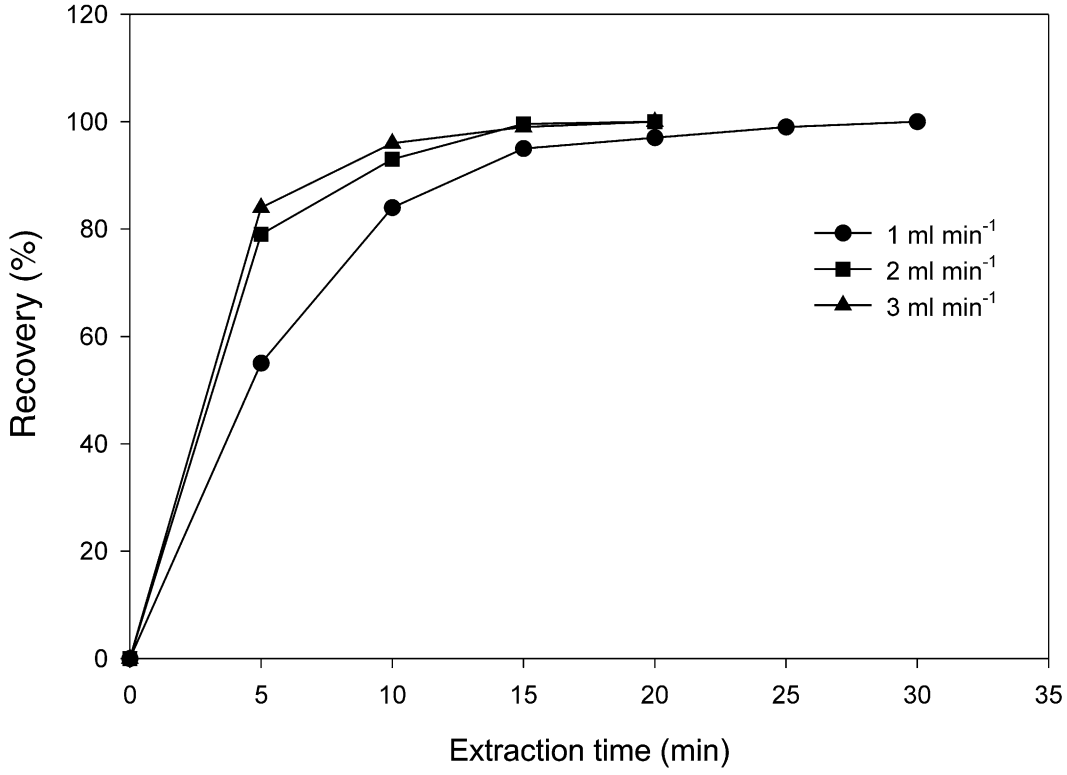

Fig. 4. Effect of flow rate on the extraction efficiency of essential oils of *Thymbra spicata* using subcritical water extraction at a temperature of 150 °C.

SWE. As a consequence of the optimum conditions, the extraction by the SWE system is quite rapid for many of the compounds. The aroma of the essential oil produced by this method was found to be more concentrated and powerful than that of the herb although this was not compared to oils produced by other methods.

4. Conclusions

A method for the extraction of *Thymbra spicata* essential oil using subcritical water was combined with two-dimensional GC-TOF/MS and GC-FID for the identification and quantification of the oil components. The water flow rate and pressure were not as effective as temperature in controlling the overall essential oil yield. The extract composition obtained in this study was similar to the essential oil obtained from Thymus species. However, the carvacrol and thymol contents were seen to be higher ($\sim$90%) which is advantageous since they are known as antioxidants when used in foods. Other compounds, such as E-3-caren-2-ol and enantiomers of α-pinene, were identified in the extract by the two dimensional GC-TOF-MS analytical technique. Overall we demonstrate that, for *Thymbra spicata*, SWE is a notably faster extraction method than conventional essential oil production techniques, in addition to being of low cost and environmentally friendly.

References

Ayala, R. S., & Luque de Castro, M. D. (2001). Continuous subcritical water extraction as a useful tool for isolation of edible essential oils. *Food Chemistry*, *75*, 109–113.

Basile, A., Jimenez-Carmona, M. M., & Clifford, A. A. (1998). Extraction of rosemary by superheated water. *Journal of Agriculture and Food Chemistry*, *46*, 5205–5209.

Clifford, A. A., Basile, A., & Al-Saidi, S. H. R. (1999). A comparison of the extraction of clove buds with supercritical carbon dioxide and superheated water. *Fresenius Journal of Analytical Chemistry*, *364*, 635–637.

Dalluge, J., Rijn, M. V., Beens, J., Vreuls, R. J. J., Brinkman, U. A. T. (2002). Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection applied to the determination of pesticides in food extracts. *Journal of Chromatography A* [in press].

Fernandez-Perez, V., Jimenez Carmona, M. M., & Luque de Castro, M. D. (2001). Continuous liquid–liquid extraction using modified subcritical water for the demetalisation of used industrial oils. *Analytica Chimica Acta*, *433*, 47–52.

Gamiz-Gracia, L., & Luque de Castro, M. D. (2000). Continuous subcritical water extraction of medicinal plant essential oil: comparison with conventional techniques. *Talanta*, *51*, 1179–1185.

Jimenez-Carmona, R. S., Ubera, J. L., & Luque de Castro, M. D. (1999). Comparison of continuous subcritical water extraction and hydrodistillation of marjoram essential oil. *Journal of Chromatography A*, *855*, 625–632.

Kubatova, A., Lagadec, A. J. M., Miller, D. J., & Hawthorne, S. B. (2001). Selective extraction of oxygenates from savory and peppermint using subcritical water. *Flavour and Fragrance Journal*, *16*, 64–73.

Kubatova, A., Miller, D. J., & Hawthorne, S. B. (2001). Comparison of subcritical water and organic solvents for extracting kava lactones from kava root. *Journal of Chromatography A*, *923*, 187–194.

Miller, D. J., & Hawthorne, S. B. (2000). Solubility of liquid organic flavour and fragrance compounds in subcritical (hot/liquid) water from 298 K to 473 K. *Journal of Chemical Engineering Data*, *45*, 315–318.

Moldao-Martins, M., Palavra, A., Beirao da Costa, M. D., & Bernardo-Gil, M. G. (2000). Supercritical CO_2 extraction of *Thymus zygis* L. subsp. *Sylvestris* aroma. *Journal of Supercritical Fluids*, *18*, 25–34.

Moret, S., & Conte, L. Y. S. (2002). A rapid method for polycyclic aromatic hydrocarbon determination in vegetable oils. *Journal of Separation Science*, *25*, 96–100.

Muller-Riebau, F. J., Berger, B. M., & Yegen, O. (1995). Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey. *Journal of Agricultural Food Chemistry*, *43*, 2262–2266.

Okihasni, M., Obana, H., & Hari, S. (1998). Determination of N-methyl carbamate pesticeides in foods using an accelerated solvent extraction with a mini-column cleanup. *Analyst*, *123*, 711–714.

Rovio, S., Hartonen, K., Holm, Y., Hiltunen, R., & Riekkola, M. L. (1999). Extraction of clove using pressurized hot water. *Flavour and Fragrance Journal*, *14*, 399–404.

Schafer, K. (1998). Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material. *Anaytica Chimica Acta*, *358*, 69–77.

Simandi, B., Oszagyan, M., Lemberkovics, E., Kery, A., Kaszacs, J., Thyrion, F., & Matyas, T. (1998). Supercritical carbon dioxide extraction and fractionation of oregano oleoresin. *Food Research International*, *31*, 723–728.

Thurman, E. M., & Mills, M. S. (1998). *Solid phase extraction: principles and practice*. New York: John Wiley & Sons.