CORVINUS UNIVERSITY OF BUDAPEST
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DOCTORAL THESIS

SUPERCRITICAL FLUID EXTRACTION OF VOLATILE AND NON VOLATILE TERPENIC AS WELL AS PHENOLIC COMPOUNDS, CHARACTERISTIC OF THE *LAMIACEAE* FAMILY

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Scientific background and the aims of the study

In the last few years, the demand on the extracts of natural origin being free from organic solvents has become more and more conspicuous, especially in the food, pharmaceutical and cosmetic industries. Plant extracts and volatile oils produced by traditional extraction methods have been utilized by the pharmaceutical industry for the production of several medicines and other healing preparations, while by the food industry in the form of spices extracts, natural antioxidants or natural colouring agents. Conventional extracts are also applied by the cosmetic, perfume and household chemical industry, especially in natural cosmetics and in perfumes. The actual strict legal requirements declare that plant extracts used in foods, medicines and cosmetics must not contain organic solvent residue.

Supercritical fluid extraction (SFE) has already been applied in several fields of food industry, for example in decaffeination of coffee or in production of hop and other flavouring extracts. Its comprehensive utilization became possible because of the well regulated extraction temperature and pressure, both promoting selectivity.

Beside the traditional extraction methods, SFE provides proper opportunity for extracting several active substances of plants. Solvent power and selectivity in supercritical fluid state can be modified by choosing adequate pressure and/or temperature values as well as by applying entrainers (co-solvents). By using well-selected extraction parameters, only the desired compounds dissolve from plant materials. The extract obtained does not contain residues of solvents or other contaminants. Due to these features, the final product including SFE extract represents a higher quality as well as a higher market value.

The more and more extensive use of SFE can be proven by the increasing number of publications appeared, concerning this research field, in Science Direct database from 1990 till now (33 000<). Similar tendencies of development can be experienced regarding industrial application, too.

Main objectives of my research work were the followings:

- determination of optimal SFE extraction parameters (extraction pressure, temperature, time and modifier %) concerning volatile and non-volatile substances of the drugs produced by species of four selected genera - *Melissa*, *Ocimum*, *Thymus* and *Satureja* – belonging to the *Lamiaceae* family.
- establishment of quality and quantity of extracts obtained.
- Comparison of extract yield and composition obtained by SFE and by conventional methods.

During literature review, in some cases, relevant publications have not been found, consequently we have investigated first the potential of this extraction method at certain species.

Material and methods

Plant material

Plant materials grown at the Experimental Station of Soroksár, Budapest (2006-2008) were used in our SFE investigations, with the exception of lemon balm, where a commercial source was available. The flowering shoots of plants were harvested and dried in shade, in order to obtain specific drugs. Further information concerning plant materials can be found in **Table 1**. Pulverization of drugs was carried out directly before SFE and conventional extraction methods.

Table 1: Origin, year of collection and main characteristics of the drugs of species investigated

Species	Origin	Year of collection	Drug specifications (according to Ph.Hg.VIII, 2004)
Melissa officinalis	Herbária Zrt. Hungarian cultivated population	2007	*Melissae folium* Ph.Hg.VIII. Dried, crumbled
Ocimum basilicum	Soroksár Experimental Station of BCE Cultivated variety: 'A1'	2006	*Basilici herba* (MSZ 20687:1985) Dried, crumbled
Satureja hortensis	Soroksár Experimental Station of BCE Cultivated population	2006	*Saturejae herba* (MSZ 20047:1984) Dried, crumbled
Satureja montana	Soroksár Experimental Station of BCE Cultivated variety: 'Bokroska'	2007	*Saturejae montanae herba* Dried, crumbled
Thymus pannonicus	Soroksár Experimental Station of BCE Cultivated population (wild origin of Ceglédbercel)	2008	*Thymi pannonici herba* Dried
Thymus vulgaris	Soroksár Experimental Station of BCE Cultivated population (origin of Kalocsa)	2006	*Thymi herba* Ph.Hg.VIII. Dried, crumbled

Method of hydro-distillation

The hydro-distillation was carried out with a Clevenger-type apparatus, according to the Ph. Hg. VII. (1986). Drug quantity of 20 g was used, it was distilled with 500 ml of water for 3 hours. The amount of essential oil obtained was expressed in mL/100 g calculated on the basis of dry matter content. For the sake of the comparability of essential oil contents (mL/100g=V/m%) with SFE yields (g/100g=m/m%), specific gravity (g/cm^3) of volatile oils was used to convert the essential oil content to m/m%.

Method of supercritical fluid extraction (SFE)

Our experiments were performed in an **Isco SFX 2-10™** type (ISCO, Lincoln, Nevada, USA) laboratory scale extractor equipped with an **Isco Model 260 D** pump filled with CO_2.

Second 260 D pump filled with modifier solvent was used when non volatile substances were extracted. The extraction was carried out with analytically pure (99,995 %) carbon dioxide (Linde, Répcelak), at constant pressure mode when flow rate has changed around 1.2 ml/min. Approximately, 3.5-5.0 g dried, pulverized drug was weighed for extraction tests. The ratio of co-solvent (MeOH %) was also programmed by using a mixing valve supplying modified fluid solvent passing toward the extractor space.

During optimization of extraction parameters adjusted separately, one was tested (extraction pressure, time or temperature), while the other two parameters were set as constant. In the course of the **pressure optimization experiment of volatile fraction, pressure values were varied between 8 and 30 MPa** (increasing stepwise by 1 MPa) by setting constant 40 ^{o}C and 30 min. In order to find the most appropriate **extraction time, 10, 20, 30, 40, 50 and 60 min** extraction periods were tested by using constant 10 MPa and 40 ^{o}C. When determining the optimal as well as harmless **extraction temperature applying 30, 35, 40, 45 and 50 ^{o}C**, constant pressure of 10 MPa and time of 30 min were set. When studying the yields and proportions of the extractable non-volatile compounds, SFE was performed either by fluid carbon dioxide or by using modifier. In the former case, the effect of **extraction pressure** between **30-50 MPa** was tested, while in the latter case the **combined effects of pressure (30, 40 or 50 MPa) and modifier ratio (methanol: 5-50 %)** were evaluated (methanol of HPLC purity, Merck, Germany). Extraction tests were performed in four replicates, then average values were calculated and compared.

Extracts obtained by SFE-CO_2 extraction were dissolved in 4 ml n-hexane (purity of HPLC, Merck, Germany). Solvent was evaporated at room temperature and extracts were stored in refrigerator before analysis. The extract yields were expressed in m/m % calculated on the basis of dry mass of the drug. Later on, diluted extracts were subjected to gas chromatographic analysis.

Method of Soxhlet extraction

Accurately measured 1 g of dry, homogenized and powdered plant material was boiled with 25 ml ethanol for 6 hours, thereafter the solvent was evaporated in vacuum. After had calculated the extract yields, samples were analysed by HPLC method of gradient elution. These extracts served as controls to the SFE extracts of non-volatile compounds. The extract yields were given in m/m % (DW).

Method of gas-chromatographic analysis

The compositions of the volatile oil rich extracts and distilled volatile oils were determined by gas chromatographic method (GC-FID). The distilled volatile oils were directly injected to the colonna, while SFE-extracts were diluted with 0,1 ml n-hexane (HPLC pure, Merck, Germany) before analysis.

A GC 6890 N (Agilent Technologies, United States) gas chromatographic system was applied. Gas chromatographic circumstances were as follows: injector temperature: 250 °C, split ratio: 30:1, injection: automata injector 7683B (Agilent Technologies, United States), injected amount: 0.2 μl (in 10 % n-hexane solution), carrier gas: helium (Linde, Répcelak), flow rate: 1 ml/min (constant), colonna: HP-5 (5 % phenyl-methyl-siloxane) (length: 30 m, d= 250 μm, film thickness: 0,25 μm), temperature programme: 60-240 °C: 3 °C/min, 240 ^{o}C/ 5 min, detection: at 250 °C, with flame ionization detector (FID).

Identificacion of the compounds of SFE-extracts and of distilled oils was done by comparing their retention times and retention indices to those of the standards.

Method of HPLC analysis

SFE fractions rich in non-volatile (phenolic and triterpenic) compounds were analysed by HPLC (High Performance Liquid Chromatography) method. After diluting, cleaning and filtering (MILEX SLCR 013NL type (Millipore, United States), 20 μl was injected into the system from each supercritical samples.

Operating circumstances of HPLC (Waters, United States): Waters 717 plus type automata injector, Waters 1252 type pump, Waters 2487 type absorbancy detector (wavelength: λ=350 nm), flow rate: 1 ml/min. Chromatographic circumstances: colonna: SYMMETRY RP C18 (5 μm, 4.6 x 150 mm) (Waters, United States), mobil phase: 2.5 % acetic acid (dissolved in 350 ml micro prefiltered water); MeOH (50 ml); acetonitril (100 ml) (Merck, Germany), elution: gradient. Identification of the compounds was done by using standards and retention times.

Method of statistical analysis

Data obtained in 4 repetition, were entried by using Microsoft Office Excel 2003, while their statistical analyses were performed by Statistica 8.0.

Effects of the extraction parameters (pressure, temperature, time and ratio of modifier) on the yield and proportion of the main compounds were evaluated by one-way analysis of variance (ANOVA). The significance level was 95 % in all cases ($p<0.05$).

New scientific results

The most important outcomes obtained by taxa can be summarized as follows:

In our experiments, the amount and composition of supercritical extracts of six species belonging to the *Lamiaceae* family were analysed in details, in order to determine the effects of extraction parameters investigated – extraction pressure, temperature, time and ratio of the modifier - on the quality as well as on the proportion of certain compounds within the extracts.

Melissa officinalis

As a result of the pressure optimization experiment, it was established that the ratio of β-caryophyllene within the essntial oil rich fraction of *Melissae folium* was the highest at 13 MPa, while the proportion of other important compounds (citronellal, caryophyllene oxide and geranial) was outstanding at 17 MPa or higher pressure values.

In the pressure optimalization of non-volatile fraction, the effect of pressure on the yield was statistically proven ($p=0.000383$). 31 MPa have been considered as optimal value from the point of view of yield (0.27 %). It was established that the pressure of 40 MPa resulted in the highest amount of rosmarinic acid (0.54 %), while the highest ursolic acid ratio (38.64 %) could have been measured at 45 MPa. By using 20 % methanol modifier at 40 MPa, outstanding amount of rosmarinic acid (19.93 %) have been detected. Eriodictiol and luteolin were also identified in the supercritical extracts. The latter one have been appeared in the extracts made with or without modifier, however, the highest proportion (4.08 %) have been reached at pressure of 50 MPa and 40 % methanol modifier.

Ocimum basilicum

The amount of the volatile-rich extract of *Basilici herba* was proven to be optimal (0,44 %) at the extraction pressure of 15 MPa. The extraction yield and the volatile composition could be influenced by the duration of the extraction. We have found that the 40 minutes' extraction resulted in 50 % higher yield (0.33 %) than the 30 minutes' one, recommended by the literature. Extraction possibilities of phenolic compounds of basil by SFE method were investigated for the first time in our experiments. The extract yield of the non-volatile fraction of basil was found to be optimal (0,71 %) at the pressure value of 40 MPa, when using SFE-CO_2 as a solvent. In these SFE extracts outstanding amounts of phenoloids have been detected at certain pressure values as follows: rutin (35 and 45 MPa), chlorogenic acid (45 MPa), apigenin (50 MPa), apigenin-7-glycosid (35 MPa) and eryodictiol (45 MPa). The highest proportion (29,31 %) of luteolin have been measured at the pressure of 45 MPa. The combined effect of extraction pressure and

modifier on the yield was verified by ANOVA. In the SFE extracts produced by adding methanol modifier, apigenin, apigenin-7-glycoside, eryodyctiol as well as ursolic acid were revealed. The amount of luteolin did not changed significantly, comparing its highest ratio (27,85 %: 40 MPa+40 % methanol) to those of the SFE-CO_2 extracts.

Satureja hortensis:

During extraction pressure optimization of the volatile fraction of *Saturejae herba* with SFE-CO_2, 27 MPa was proven to be optimal, resulted in the highest extract yield (0,78 %). The effect of the extraction pressure on the most important compound, carvacrol, was supported by statistical data. Besides, it has been verified that the extraction temperature of 50 °C resulted in the highest extract yield (0.37 %), as well as in the highest proportion of γ-terpinene (16.70 %). However, the most important volatile compounds reached their maximal ratio at 40 °C (carvacrol: 75.37 %) and at 35 °C (p-cymene: 10.00 %), respectively. Regarding time parameter, even the 15 minutes' extraction resulted in optimal yield (0.34 %), while the highest ratio of carvacrol has been reached at 30 min. The effect of time modification on the yield was found to be significant.

When extracting the non-volatile fraction of savory with SFE-CO_2, the extraction pressure of 39 MPa was found to be optimal concerning extract yield (0.71 %). The effect of pressure modification on the yield was verified statistically. In these extracts, eryodictiol and ursolic acid have been identified for the first time. With the addition of methanol modifier, as it was expected, higher extract yield have been obtained at 40 and 50 MPa (0.65 % and 0.63 %, respectively), than with fluid CO_2 extraction. The collective effect of pressure and modifier on ursolic acid content was significant. It was also established that it worth utilizing lower pressure values with higher modifier ratio and vice versa.

Satureja montana

The significant effect of the extraction pressure as well as the extracion time on the yield of volatile fraction of *Saturajea montanae herba* was confirmed. When analysing the results of the extraction time and temperature optimization experiments, it was proven that the change of these parameters had significant effect on the proportion of certain compounds (extraction time on thymol, β-bisabolen and linalool; extraction temperature on linalool). In our experiment, the extraction temperature of 45 °C was found to be optimal, concerning extract yield of volatiles (0.93 %). However, the highest ratio of carvacrol and of p-cymene have been revealed at different values (at 40 and at 60 °C, respectively). Regarding extraction time, the optimal yield

of volatile fraction have been obtained during 35 minutes, however, in the case of geraniol and of carvacrol, another value (15 min) was considered to be the most appropriate.

When investigating the effects of the pressure on the non-volatile fraction of winter savory, the following results have been obtained: with fluid carbon dioxide, the extract yield was optimal at 36 MPa and the pressure had significant effect on extract yield. In the SFE-CO_2 extract, rutin, luteolin as well as rosmarinic acid were detected for the first time in our experiments. The pressure of 40 MPa with 50 % methanol modifier was determined as extraction optimum. The effects of both pressure and modifier have found to be significant. By adding co-solvent, rutin, quercitrin, apigenin, luteolin and quercetin have been identified in the extracts, proportion of which sometimes exceeded even those of the Soxhlet extracts.

Thymus pannonicus

Regarding this species, all the results obtained in our studies can be considered as new, because no SFE research have been conducted previously on the SFE extraction of its herba drug.

Considering the data obtained during SFE-CO_2 extraction of volatile fraction, it has been proven that the effect of pressure on the yield was statistically significant. Extract yield at 13 MPa was found to be optimal, however, the highest proportion of thymol (60.47 %) were obtained at 19 MPa. In the case of the thymol methyl ether, the highest level (7.52 %) could be measured at 12 MPa, while the optimal level of β-bisabolene was isolated by 28 MPa. During testing temperature parameter it has been established that the best result for extract yield was achieved at 50 °C, while the ratio of thymol was the highest at 45 °C. The effect of the extraction temperature was statistically proven only on the proportion of geraniol and on the ratio of spathulenol. Among temperature values investigated, the 50 minutes' extraction resulted in optimal yield (0.18 %). The proportion of thymol reached its maximum at 35 minutes, while the highest ratio of β-bisabolene could have been detected after a 10 minutes' extraction. The significant effect of the extraction time was proven on the extract yield and on the ratio of thymol, either.

After analysing the results of the investigations of non-volatile fraction obtained by SFE-CO_2, it was established that the optimal pressure was 36 MPa, from the point of view of extract yield. In SFE-CO_2 extracts, luteolin was detected for the first time, with an outstanding value obtained at 50 MPa. This compound was absent from all the Soxhlet-extracts. According to our results obtained, it could be determined that the effect of pressure on the yield of non-volatile fraction was significant. It have been proven, that the extraction using 20-40 % of modifier at 40 MPa pressure resulted in the optimal extract yield (1.59-2.21 %). Similarly to the SFE-CO_2

experiment of non-volatile compounds, samples obtained by modifier contained significant amount of luteolin.

Thymus vulgaris

From the point of view of yield, the significant effect of the extraction pressure and time on volatile fraction extracted by SFE-CO_2 could have been confirmed. It was also proven that the extraction temperature of 55 °C resulted in the highest extract yield (0.99 %), while thymol and carvacrol reached their highest proportion at 40 °C within the volatile fraction. It was established, that the 60 minutes extraction time was optimal regarding either the extract yield (1.04 %), or the ratio of thymol (67.49 %) and of carvacrol (5.48 %).

When extracting non-volatile fraction by SFE-CO_2, pressure range of 34-38 MPa was regarded as optimal. It was verified, that extraction with modifier resulted in the highest yields at 50 MPa by adding 25-35 % of methanol entrainer. Quercetin – with or without modifier – was generally present in garden thyme extracts in outstanding amount.

Conclusions and recommendations

Significant differences have been experienced, when supercritical extracts of six species belonging to the *Lamiaceae* family were compared with those obtained subsequently by the conventional extraction methods (Clevenger type distillation, Soxhlet extraction). The primary reason of differences between volatile-rich extracts is the high temperature of hydro-distillation (about 100 °C), because the possibility of formation of artefacts increases at this temperature. Moreover, the thermally unstable compounds may get damage or transform to undesirable structures. In the case of traditional Soxhlet extraction applied to extract the non-volatile, polar substances of higher molecular weight, extraction temperature as well as solvents with distinct polarity and solvent power cause differences as compared to SFE. We expected the same as it is known from the literature that the composition of SFE extracts is more similar to the composition existing in the corresponding plants and drugs than in the extracts obtained by other extraction methods. This phenomenon can be predicted first of all in the case of volatile oil rich extracts, which can be produced by fluid carbon dioxide having low critical temperature solvent.

Conclusions drawn from our results regarding some species examined can be summarized as follows:

-two main compounds of the volatile rich SFE extract of *Melissa officinalis*, citronellal and geranial can be obtained with optimal amount by using 13-14 MPa and 17 MPa extraction pressures, respectively (at 40 °C and during 30 min). From non-volatile compounds, ursolic acid can be extracted successfully by SFE-CO_2, however, in the case of caffeic acid, Soxhlet extraction can be suggested instead. For the extraction of rosmarinic acid, luteolin and eryodictyol, the appropriate method is supercritical fluid extraction using modifier.

-in the course of the SFE-CO_2 extraction of the volatiles of *Ocimum basilicum*, optimal linalool ratio can be achieved at 18 MPa extraction pressure, 50 °C temperature and 15 min, while that of the estragol can be realized by setting 11 MPa, 40-45 °C and 60 min. The best choice of the extraction of rutin and rosmarinic acid can be Soxhlet extraction, while luteolin, ursolic acid and apigenin can be efficiently extracted by SFE-CO_2. The latter method by adding modifier is adequate when apigenin-7-glycoside and eryodyctiol are the subjects of extraction.

-during SFE-CO_2 extraction of volatile fraction of *Satureja hortensis*, carvacrol rich extract can be obtained at 13-14 MPa extraction pressure, at 40 °C extraction temperature during 30 min. Rutin, luteolin and rosmarinic acid can be effectively retrieved by fluid carbon dioxide in

appropriate amount, while in the case of apigenin, quercetin and quercitrin, modifier is necessary to attain considerable rates. Satisfactory yields of caffeic acid and chlorogenic acid can be obtained only by Soxhlet extraction.

-SFE was proven to be an effective method for extracting essential oil compounds from the drug of *Thymus pannonicus*: thymol reached the optimal ratio at 19 MPa, at 45 °C and 35 min, while β-bisabolene at 28 MPa, 60 °C and 10 min, respectively. For the extraction of luteolin, caffeic acid and apigenin, Soxhlet extraction was the most efficient method. Fluid extraction with modifier can be proposed for extracting luteolin, caffeic acid as well as apigenin.

-SFE-CO_2 extraction was prosperous in obtaining thymol-rich volatile fraction of *Thymus vulgaris* at 18 MPa, 40 °C and 60 min. In the case of luteolin, rosmarinic acid and quercetin, utilization of SFE with methanol modifier is recommended. However, rutin, caffeic acid and chlorogenic acid should be extracted by the traditional Soxhlet extraction, resulting appropriate extract yields.

We consider that important results have been obtained in our experiments, where the following active substances with remarkable therapeutic effects were successfully extracted by SFE with or without modifier:

- rosmarinic acid from phenolic acid group
- flavonoids: apigenin and its glycosides, quercetin and luteolin
- ursolic acid of triterpenic structure

We have established that the optimal parameters of yields and of certain compounds usually do not coincide, however, certain extraction parameters can be selected, resulting in the increase of the ratio of some compounds. This possibility has occurred especially in the case of phenolic monoterpenes (thymol and carvacrol) involved in volatile fraction of thyme an savory species, though, proportion of other important aromatic compounds (e.g. linalool at basil) could also be augmented.

During our experiments, four parallel samples have been prepared at the same extraction parameters. In our opinion, it is worth increasing the number of repetitions or determining the least sample number with acceptable level of variance. In some cases, the low sample volume can also be the origin of faults, therefore, a larger scale extraction would be reasonable to solve

the problem. Literature data are not consistent regarding particle size: its optimization can also be a purpose of further studies, either on yields or on certain compounds.

Our results obtained by an analytical scale extractor, in some cases only indicate the presence of volatile or non-volatile compounds in plant sources, expressed in GC/HPLC area %. A more exact evaluation would be possible after a larger scale extraction of drugs of the species involved, when significant plants sources of effective compounds could have been selected for industrial (food, cosmetically and household chemical) and for therapeutic application.

Furthermore, it should be emphasized, that SFE method provides an opportunity to the application of solvent free plant extracts produced by environmentally friendly methods in more and more industrial sectors and also in households, where the utilization of safe and harmless products are of increasing importance.

Publications connected with the subject of the doctoral thesis

Scientific papers (without IF)

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