Supercritical Fluid Extraction of Biologically Active Compounds From Herbs Applicable in Pharmaceuticals, Cosmetics and Dietary Products

Ph.D. Thesis

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1. Introduction and Theoretical Background

Recently more attention has been paid to the healthy way of life, mainly to the importance of healthy nutrition intake. That means the general use of natural products, food supplements and different natural medical preparations at homes.

The production of solvent residual-free natural products has become important point of view in dietary, medical industry and in cosmetic industry as well. As the consumption of the natural products become more general around the world, the quality standards of these products become more strict that concern to the plant raw materials and to the processing. The official regulations motivate the producers to manufacture products less harmful to the people and environment, and to initiate new environment friendly technologies. The authoritative FDA (Food and Drug Administration, USA), EMEA (European Agency for the Evaluation of Medicinal Products, EU) and EPA (Environmental Protection Agency, USA) regulations promote the spreading of the techniques, which do not use organic solvents. The supercritical fluid extraction (SFE) substitute the traditional organic solvent extraction in an increasing area. By comparing the traditional organic solvent extraction with the SFE, which uses CO$_2$ as solvent for extraction of active substances from plants, we see that one of the most significant advantages of SFE is to separate the solvent from the extract without leaving any residue. This way high value extract can be achieved, which does not contain any solvent even in trace amounts. Thus the obtained extract can already be either an end product after confectionation, or can be further processed to a basic substance of any natural product, food supplements, cosmetics or food. An other important advantage of SFE using CO$_2$ is given by a relatively low extraction temperature. This attribute especially useful for the extraction of heat sensitive, biologically active compounds. It should be noted that the two main operational parameters of SFE are the extraction pressure and temperature. The change in these parameters influence the solvent power making the fractionated extraction also viable. The aim of this work was to extract active substances from plants applicable in pharmaceuticals, cosmetics or dietary products. The valuable biologically active compounds were extracted from three domestic herbs (sea buckthorn /Hippophae rhamnoides L./, chaste tree /Vitex agnus castus L./, St. John’s wort /Hypericum perforatum L./) and from a mushroom (shiitake /Lentinus edodes/) that originates from Far-East. My goals were to investigate and establish a new extraction method with supercritical CO$_2$ (sc-CO$_2$), which has less impact on the environment compared to the traditional solvent extraction technique. In this study, the extraction of the plant raw materials were carried out with sc-CO$_2$ and organic solvents as well and the obtained yield results were compared. The operational parameters (pressure and temperature) were
optimized according to the extraction yields and recoveries of the valuable minor components.

2. Experimental methods

The Soxhlet extraction and supercritical fluid extraction were carried out in laboratory and pilot scales. Five different solvents (ethanol, 2-propanol, ethyl-acetate, n-hexane, carbon dioxide) were applied in the experiments, all of them are permitted extraction solvents in the production of food and food ingredients in European Union (88/344/EEC).

![Diagram of supercritical fluid extraction](image)

Figure 2-1.: The equipment of supercritical fluid extraction in pilot plant scale

The operating method of the extraction unit depicted in Figure 2-1. is the following. The carbon dioxide is supplied from the tank (1) through a cooler (2). The pump (3) and the heat exchanger (4) provide the supercritical conditions. The soluble compounds of herbs are dissolved in the extractor (5) by the sc-CO$_2$. After the pressure reduction the dissolved components are precipitated in the separators (6).

The effects of the operational parameters (pressure and temperature) on the extraction yield and the recoveries of the biologically active minor compounds were studied using a full factorial experimental design. The evaluation of the effects were performed by Pareto diagrams and fitted response surfaces using Statistica software.

The obtained extracts were analysed by various analytical methods like TLC-densitometry, GC, GC-MS, HPLC, UV-Vis spectrophotometry.

The extracts of the studied herbs were applied in product development experiments.
3. Results
The experimental work of the present theses can be divided into four main sections due to the studied herbs.

3.1. Sea buckthorn (*Hippophae rhamnoides* L.)

The effects of the operational parameters of the supercritical fluid extraction were studied in the ranges of 300-460 bar and 40-80°C in the sea buckthorn pomace extraction. The results obtained in the $3^2$ full factorial design were compared to the results of the extracts obtained with *n*-hexane and ethanol (96% v/v). The extraction yields of the supercritical fluid extraction varied between 142-164 g/kg according to the pressure and temperature. These results are similar to the values obtained by *n*-hexane in laboratory scale Soxhlet extraction.

The fitted response surface on the measured points of the extraction yields indicated that high values can be achieved when the temperature of 60±5°C and the pressure above 350 bar are applied. Variance analysis highlighted the significant effect of pressure on the yield at 5% significance level. The optimal recoveries of the studied minor compounds were different. For β-sitosterol and ursolic acid, optimal recoveries were achieved at 380 bar extraction pressure and 80°C extraction temperature, while the best conditions for carotenoid and tocopherol extraction were at 460 bar and 60°C. Besides the linear terms of pressure and temperature, the interactions of these SFE parameters were significant on the recovery of total-tocopherol at 5% significance level, within the ranges investigated. The extraction yield obtained with ethanol was more than two times higher, than those obtained with sc-CO$_2$, however, the β-sitosterol concentration of the supercritical extract was more than 1.5 times higher and the ursolic acid concentration was almost four times higher than measured in the extracts obtained with ethanol and *n*-hexane. The β-carotene and the total tocopherol concentrations of these extracts were similar with the three different solvents.

It can be concluded that supercritical fluid extraction is a suitable method for the valorisation of sea buckthorn pomace, a by-product from the processing of sea buckthorn berries. The suggested operational parameters of the SFE process are $P\geq460\text{ bar}$ and $T=60\pm5°C$. Under these conditions, vitamin E (α-tocopherol) and proretinol (β-carotene) were obtained in the highest amounts. To ensure the 100% daily dose of vitamin E and β-carotene, four to five gramms of the extract should be used in organic products, retrieved after the extraction at 460 bar and 60°C. The results obtained in this study prove that the recycling of sea buckthorn pomace into the process is possible.

In a part of my research work, I have been involved in the development of a natural gel based cosmetical product with sea buckthorn extract content.
3.2 Chaste tree (Vitex agnus castus L.)

The effects of the extraction pressure and temperature on extraction yield and recovery of the biologically active components were studied using a $3^2$ full factorial design. A pilot plant extractor was used to perform these experiments. The pressure and temperature values were varied over the ranges of 100-450 bar and 40-60°C, respectively. The yields and recoveries were compared to those obtained in $n$-hexane and ethanol (96% v/v) extractions. According to the statistical evaluation of the measured full factorial design points, it was found that high yields can be achieved at pressures above 300 bar and temperatures of 45±5°C, within the ranges investigated. The Pareto charts showed the significant effects of the linear and quadratic terms of the pressure and temperature, as well as the major influences of the interaction terms of the linear and quadratic pressure and temperature on the yield values at the 5% significance level. The extraction yields of the supercritical fluid extraction are comparable to the yield of the extract, obtained with $n$-hexane according to their similar solubility parameters. The yield of the ethanolic extract was almost three times higher than the yield obtained with sc-CO$_2$. The minor components of the fruit of the chaste tree, namely the rotundifurane, β-sitosterol, β-amyrin and casticin, were retrieved together in high amounts at 450 bar extraction pressure and 45±5°C extraction temperature. The linear and quadratic terms of extraction pressure were found to be significant on the recoveries of minor compounds, while the rotundifurane and β-sitosterol retrieval were mainly influenced by the interaction effects at 5% significance level. The suggested operational parameters of the SFE are 450 bar and $T=45±5°C$. At these conditions, the concentrations of the minor components were 3-4 times higher in the sc-CO$_2$ extracts compared to those obtained from the ethanol extracts.

The extract obtained with sc-CO$_2$ contains valuable components like essential oil (1,8-cineol, β-caryophyllene, etc.), diterpenes (rotundifurane), triterpenes (β-sitosterol, β-amyrin), flavonoids (casticin). These compounds can be extracted in high amounts with sc-CO$_2$.

I participated in the product development experiments in order to produce chaste tree tincture as well as capsules containing agni casti fructus dry extracts, which can be used in gynaecology.
3.3. St. John’s wort (*Hypericum perforatum* L.)

The extractions of St. John’s wort were studied by using three different, dried raw materials, SJW 1., SJW 2. and SJW 3. Data from the SJW 3. sample SFE extraction were evaluated by a $3^2$ full factorial design to determine the effects of the operational parameters in the SFE (pressure and temperature) on the extraction yields and the recovery values of hyperforin, a compound, which can be extracted by the non-polar sc-CO$_2$.

The laboratory scale Soxhlet extraction of the SJW 1. and SJW 2. were carried out using four different solvents: ethanol, 2-propanol, ethyl-acetate, $n$-hexane. Increasing the polarity of solvents resulted in higher extraction yields. Pilot plant experiments were also performed in a Soxhlet extractor with $5 \cdot 10^{-3}$ m$^3$ volume using ethanol and in a high-pressure vessel of the same volume at $40^\circ$C and 450 bar using supercritical CO$_2$. The hypericin and hyperforin content of the extract samples were analysed by HPLC. Since the hypericin is the most polar component, the highest dissolution was obtained in ethanol as the most polar solvent used in the course of experiments. Decreased dissolutions of hypericin were achieved by applying solvents with lower polarity. Any recovery of hypericin could not be detected in case of $n$-hexane and sc-CO$_2$.

In accordance with the non-polar caracteristics of the hyperforin, the non-polar sc-CO$_2$ provided the best solubilization for this solute. It was expected that the solubility of hyperforin in $n$-hexane is similar to that in sc-CO$_2$ because of the approximately same solubility parameters of these solvents. Nevertheless, the recovery of hyperforin was negligibly low in the extracts obtained with $n$-hexane. A possible explanation for this phenomenon is that the hyperforin is extremely unstable in $n$-hexane solution. The two main degradation products are the isomers of furohyperforin.$^1$

In my experiments, effective extractions of both the main compounds (hypericin and hyperforin) were achieved. The SJW 1. sample was collected at the end of the blossoming period, using supercritical carbon dioxide at mild temperature (~$40^\circ$C) results in stable hyperforin rich extract. The SJW 2. sample was collected at the beginning of the blossoming period, using a polar solvent (ethanol) at mild temperature (~$40^\circ$C) results in stable hypericin rich extract.

Due to the different polarity/affinity of the two main compounds, it is worth to use different solvents in separate extractions to obtain extracts rich either in hypericin or

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hyperforin. A subsequent standardized mixing procedure will provide the required products from these extracts.

The extractions starting from the SJW 3. sample were carried out under 100, 275, 450 bar extraction pressures and 40, 50, 60°C extraction temperatures with carbon dioxide, respectively. The evaluation of the extraction yield values by a full factorial design revealed the phenomena of the crossover pressure and indicated that the pressure possesses more significant effects on the yield between 100 and 300 bar than above 300 bar. According to the Pareto chart, the linear and quadratic terms of extraction pressure, the interactions of the temperature, the linear and quadratic terms of extraction pressure, and the interactions of the quadratic terms of temperature and extraction pressure as well had significant effects on the extraction yield values at the studied 5% significance level. I found that the linear and quadratic terms of the extraction parameters and their interactions had significant effects on the recovery of hyperforin, although above 300 bar the effects of both the pressure and temperature decreased on the yield of hyperforin. In case of the SJW 3. sample, the best extract was achieved at 450 bar and 60°C.

In connection with the aforementioned experiments, I have been working in a project, which aimed to develop an antidepressant product containing St. John’s wort dry extract.
3.4. Shiitake mushroom (Lentinus edodes)

The extraction of shiitake mushroom was studied by a laboratory scale Soxhlet extractor using ethanol (yield: 308 g/kg), 2-propanol (158 g/kg), ethyl-acetate (57 g/kg) and n-hexane (10 g/kg) solvents. Pilot plant experiments were also performed in a Soxhlet extractor using ethanol (280 g/kg). Extractions with supercritical CO$_2$ in a $5 \cdot 10^{-3}$ m$^3$ volume high-pressure vessel were performed at 50°C and 450 bar, resulting 11 g/kg yield. The extract samples obtained with SFE were analysed by GC-MS method. The following five aroma compounds of the mushroom were identified by a spectrum library and literature survey: dimethyl trisulfide, 1,2,4-trithiolane, 2-methylthio-4,6-pirimidinediamine, 1,3-dithiolane, lenthionine. These are the main organosulfur aroma compounds of the shiitake, which are primarily responsible for the characteristic flavour of the mushroom. Besides giving flavour to the shiitake mushroom, lenthionine inhibits platelet aggregation, which may be useful in treatment of thrombosis via similar effects of other organosulfur compounds (diallyl-disulfides) found in garlic. The supercritical fluid extraction was capable to extract the main organosulfur aroma compounds from the mushroom. The flavour compounds of shiitake are utilizable in the production of different food products like dehydrated stock, mushroom paste etc.

In the other experiments the studied raw material was wet (not dried) shiitake. Pilot plant water extractions were achieved in an autoclave. The valuable polysaccharide fraction of shiitake was extracted with water. To enhance the precipitation of the lentinane, which is the most valuable polysaccharide of the Lentinus edodes, ethanol was added to the mushroom juice. The yield of the white-grey coloured precipitates, obtained of the lentinane fraction, was 2.4 g/100 g dry shiitake.

Further experiments are required to develop the analytical method of lentinane determination and thus to improve the extraction of the mushroom. This would be an important step towards the exploitation of the shiitake juice, which is currently just a by-product of the shiitake process.

I contributed to the development of a food supplement product that is based on shiitake mushroom, enriched in vitamin E and wheat germ.

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4. Theses

1. The effects of the extraction pressure and temperature during supercritical fluid extractions, were studied in case of the sea buckthorn (*Hippophae rhamnoides* L.) pomace. The yield values and recoveries of the minor compounds obtained in the $3^2$ full factorial design were compared to the results of the extracts obtained with $n$-hexane and ethanol (96% v/v).

   a) In case of the supercritical fluid extraction of the sea buckthorn pomace, I found that the optimal recoveries of the studied minor compounds are different from each other within the investigated P and T ranges. The optimal recoveries for the $\beta$-sitosterol and ursolic acid (triterpenes) are achieved at the applied medium pressure and high temperature, while the optimal values during the extraction of the carotenoid and tocopherol are achieved at the applied maximum pressure and medium temperature. It is possible to determine a set of the operational parameters that lead to a relatively high extractions of the valuable minor compounds.

   b) I found that the concentrations of the $\beta$-sitosterol and ursolic acid in the extracts obtained by supercritical fluid extraction under the suggested operational parameters are several times higher compared to those obtained with ethanol or $n$-hexane. The $\beta$-carotene and tocopherol concentrations of the extracts achieved with the three different solvents are almost the same. [3, 12]

2. I conducted the first and foremost experiments for the supercritical fluid extraction of the fruit of the chaste tree, according to the few traceable literature sources. The effects of the supercritical fluid extraction conditions (pressure and temperature) on the extraction yield values and recoveries of the biologically active components were studied in a pilot plant extractor using a $3^2$ full factorial design. The yield and recovery data were compared to those obtained with $n$-hexane and ethanol extractions.

   a) I concluded that the linear and quadratic terms of extraction pressure in case of rotundifurane and $\beta$-sitosterol and the interactions of the SFE parameters as well have significant effects on the recoveries of the minor compounds at 5% significance level, within the ranges investigated. Exploiting the statistical evaluation, it is possible to determine a set of the operational parameters, which result in high yields of the important minor compounds such as, the
rotundifurane (diterpene), \( \beta \)-sitosterol, \( \beta \)-amyrin (triterpenes) and casticin (flavonoid).

b) I determined that the concentrations of the minor compounds in the extracts obtained with supercritical carbon dioxide are multiple compared to the extracts of ethanol. The supercritical fluid extraction is a suitable method for the valorization of the chaste tree fruit. [2, 4, 7, 8, 10, 11]

3. During the investigation of St. John’s wort (*Hypericum perforatum* L.), three different dried raw materials were studied. In case of two samples, the used solvents were ethanol, 2-propanol, ethyl-acetate, \( n \)-hexane and carbon dioxide that extracted the main compounds and produced valuable extracts. The third sample was used in a 3\(^2\) full factorial design in order to study the effects the operational parameters of the supercritical fluid extraction on the extraction yield and the recovery values of the hyperforin.

a) I proved that the extracion of the sample, collected at the end of the blossoming period, using supercritical carbon dioxide at mild temperature results in stable hyperforin rich extract.

b) I proved that the extracion of the sample, collected at the beginning of the blossoming period, using a polar solvent (ethanol) at mild temperature results in stable hypericin rich extract.

c) A standardized mixing of the extracts, which are rich in the different compounds (hypericin and hyperforin) provides an outstanding basic substance for a natural product applicable in the treatment of mild to moderate depression.

d) During the supercritical fluid extraction of the sea buckthorn, I found that the operational parameters have significant effects on the recovery of the hyperforin, although their influence decrease over 300 bar. [1, 5]
4. The investigation of the shiitake mushroom (*Lentinus edodes*) extraction was carried out with ethanol, 2-propanol, ethyl-acetate, n-hexane, carbon dioxide and water solvents in laboratory and pilot plant scale.

   a) The supercritical extract of the shiitake mushroom was analysed by gas chromatography and mass spectrometry and the main organosulfur aroma components were identified by a spectrum library and literature sources. These compounds are the followings: dimethyl trisulfide, 1,2,4-trithiolane, 2-methylthio-4,6-pirimidinediamine, 1,3-dithiolane, lenthionine.

   b) I determined that the supercritical fluid extraction is suitable for the extraction of the main organosulfur aroma compounds from shiitake. [6, 9, 13-15]

5. Applications

The extracts collected during supercritical fluid extractions using carbon dioxide as solvent can be primarily used in the food industry, pharmaceutical and in the cosmetic sector. An other example for the exploitation of the SFE method is the production of the sea buckthorn pomace extract, which can be used as food supplements or cosmetic products. The extract of the chaste tree can be used also in food supplements or in beverages, moreover in various essential oils for aromatherapy. It would be practical to combine the supercritical and the ethanolic extracts of the St. Johns wort to produce an end product, standardized to the two main compounds: hypericin and hyperforin. This product could be used in the treatment of mild to moderate depression. The shiitake mushroom has strong immune stimulant effect thus can be used in food supplements. Furthermore, the aroma components of this mushroom could have a potential market as spice.

During my Ph.D. work, I cooperated with a product developer company, the Gradiens Ltd. As a result, several products were developed and finalized from the studied herbs. The name of the products are the followings: Vitagold, Klimovit, Deprovit, Shibu. Two of them (Vitagold and Shibu) are commercialized, while the others are already in the registration procedure.
6. Publications

Communications in international journals:


  *impact factor: 0.656  citation: 0 independent*


  *impact factor: 2.428  citation: 1 independent*


  *impact factor: 1.304  citation: 1 independent*


  *impact factor: 1.708  citation: 4 independent*

Communications in international conferences:

[5] Cossuta, D., Baffi, B., Mészáros, Á., Kiss, K., Fekete, J., Keve, T., Simándi, B., Supercritical fluid extraction of St. John’s wort (*Hypericum perforatum* L.) and marigold (*Calendula officinalis* L.), *12th European meeting on Supercritical Fluids (International Society for Advancement of Supercritical Fluids – ISASF Event)*, Graz, Austria 9th-12th May 2010, accepted *Poster*


**Communications in Hungarian journals:**


**Communications in Hungarian conferences:**


Other communications:
