Theses of Ph. D. dissertation of

SUPERCRITICAL FLUID EXTRACTION OF PLANTS AND THE FUNCTIONAL PROPERTIES OF THE EXTRACTS

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Introduction

Our developed World has a strong demand for creation healthy and less polluted environment using clean technologies and producing solvent residue free, nutritional foods. These food products must possess with natural colour, taste and self-live extensive properties as well as must contain biological active, health preventive compounds (e.g. antioxidants, vitamins).

Supercritical fluid extraction is one of the desirable technologies, which uses carbon dioxide for extraction of essential oils, fatty oils, pigments, and natural waxes from natural sources, mainly from herbs, spices and medical plants. According to the physico-chemical properties of supercritical CO_2, the extraction is carried out at moderate temperature (mainly between 31 - 60°C), therefore thermo-labile compounds can be obtained without any decomposition. The extract is absolutely solvent residual free as the CO_2 is in gaseous state at room temperature. Numerous, mainly apolar compounds can be extracted and/or fractionated as the solvating power of supercritical CO_2 changes within wide ranges with changes of the pressure and temperature during extraction. The extraction is carried out at relatively high pressure (between 74-400 bar) therefore the investment cost of such a plant is higher than that of the conventional units, although the process is easily controlled with low operating costs.

My goals were applying supercritical CO_2 extraction to obtain clean and residual-free plant extracts in which high valued compounds were accumulated. With the aim of further application, the physical-chemical

and biological properties of these high valued products were widely mapped. The volatile oil, fatty acid and pigment (carotenoids and cholorphylls) compositions and the antioxidant, antimicrobial properties of the extracts obtained from marjoram (*Origanum majorana* L.), thyme (*Thymus vulgaris* L.), and industrial waste, tomato pomace (*Lycopersicon esculentum* Mill.) were revealed.

Materials and methods

The raw materials used for the experiments were obtained from distributors or local merchants. The CO_2 used was 99.5 % (w/w) pure. Reagent-grade solvents were used for conventional Soxhlet extractions. Analytical grade reagents were used for chemical analysis.

The characteristic particle size and the size distributions of the ground raw materials were investigated by Rosin-Rammler-Sperling-Bennet (RRSB) model.

Supercritical fluid extraction was carried out in a high-pressure apparatus equipped with a 5 L volume extractor vessel. The effects of process parameters (temperature and pressure) on the extraction yields, and on the yields of certain biological active compounds were revealed. Standard methods described in the Hungarian Pharmacopoea Ed. VII. were applied for the determination of the essential oil (by hydrodistillation), oleoresin (by Soxhlet extraction), and the moisture content of the samples. Soxhlet extraction was also carried out in a pilot plant apparatus (5 L volume) using 96% ethanol as a solvent.

The volatile oil compositions in essential oils and solvent extracts were quantified by GC and GC-MS methods. The fatty acid compositions were measured by GC. For

determination of natural pigments like chlorophylls, carotenoids and the antioxidant compounds (triterpenoids, diterpenes and tocopherols) from marjoram and tomato pomace HPLC methods were used.

The antioxidant properties of marjoram herbs and extracts were revealed by using *in vitro* screens in apolar and polar systems. In polar system the hydrogen-donating abilities of the samples were obtained in the presence of 1,1-diphenyl-2-picrilhydrazyl (DPPH) free radical and in the presence of instable $^{\bullet}OH$ radical in $H_2O_2/^{\bullet}OH$ – luminol systems. The antioxidant capacities of ethanolic and supercritical extracts were investigated in apolar system by Rancimat method.

The antimicrobial activities of marjoram and thyme essential oil and solvent extracts were revealed against food-poisoning fungi (*Trichoderma viride*, *Aspergillus niger,* and *Penicillium cyclopium*) and bacterial strains (*Escherichia coli, Pseudomonas fluorescens* and *Bacillus cereus*). Modified agar-diffusion and dilution methods were used for determination of antimicrobial properties of the highly viscous supercritical extracts.

Summary of theses

1. Extraction of plant materials

Different extraction methods carried out with different solvents, in laboratory and pilot-plant sizes were compared according to the yields, the effects of process parameters and the compositions of extracts using the three test plants, marjoram, thyme and tomato pomace.

1.1. The particle size distribution of ground plant materials is well described by applying the Rosin-Rammler-Sperling-Bennet (RRSB) distribution model. The characteristic particle size (x_0) and the distribution coefficient (n) can be established. The Hungarian **marjoram** was finely ground (x_0 = 0.36 mm), while the Egyptian crushed and ground herb was characterised with x_0 = 0.52 mm. In this range of particle sizes, it can be concluded that the particle size do not influence the yield of extraction, as applying all the extraction methods and solvents higher yields were obtained with Egyptian marjoram than the Hungarian one. On the other hand for the total extraction of the crushed **thyme** herb with characteristic particle size of 1.15 mm, double amount of CO_2 was used than that used for the extraction of marjoram. The P1-P3 samples of **tomato pomace** were ground on hammer grinder, while P4 sample obtained as finely ground powder. P4 sample had the smallest characteristic particle size (0.31 mm), while the particle sizes of the other samples were ≥ 0.44 mm. General conclusion cannot be drawn among the characteristic particle sizes and the yields of SFE, because of the differences in the ratios of seeds/skins among the different samples. Shorter extraction times were determined with the samples,

which contained higher amounts of apolaric compounds (probably contained more seeds), meanwhile sample P4 contained lycopene in higher amount, which was proved with the lower extraction yields; therefore the skin fraction was higher in this sample compared to the others.

1.2. The yields of extraction depend on the sources and the qualities of raw materials, on the applied solvents and methods.

Two different originated **marjoram** (*Origanum majorana* L.) herbs (Hungarian and Egyptian) were compared. Small amounts of essential oils (0.78 and 0.97 ml/100 g dry material)*, and increased amounts of solvent extracts were obtained with *n*-hexane (5.0 and 7.3 g/100 g d.m.)* with ethanol (13.4 and 29.0 g /100 g d.m.)*, respectively. Pilot plant extractions were carried out with 96% ethanol and supercritical CO_2 solvents in 5 L extractors. Due to the less soluble properties of $scCO_2$ smaller amounts of dark green, highly viscous extracts (3.8 and 5.4 g/100 g d.m.)* were obtained at 450 bar pressure and 50°C temperature than with organic solvents. Ethanol has better soluble properties, therefore high amounts of extracts (9.1 and 26.0 g/100 g d.m.)* were produced, which are comparable to those obtained in laboratory scales. The effects of SFE parameters (temperature and pressure) on the yield of Hungarian marjoram were investigated. Applying a 3^2 factorial design the experimental results demonstrated that the linear and quadric terms of pressure and the relation between temperature and pressure terms were highly significant on 95% significance level. The effect of

* The first data refers the Hungarian marjoram sample, while the second data refers the Egyptian sample.

temperature was insignificant. The highest yield (3.8 g/ 100 g d.m.) was obtained at 400 bar and 60°C. Fractionated separation was carried out at 450 bar and 50°C extractor parameters with two separators connected in series. In the first separator higher molecule weighted compounds, pigments, di-, tri-, tetra-terpenes, fatty acids and oils were collected (2.9 g/100 g d.m.), while the volatile compounds and linear alkanes, waxes were separated into the second separator (0.8 g/100 g d.m.) at its lower pressure.

From medicinal herb, **thyme** (*Thymus vulgaris* L.) 1.94 ml/100 g d.m. essential oil was hydro-distilled, while applying solvent extractions with *n*-hexane 3.9 g/100 d.m. and with ethanol 32.5 g/100 g d.m. products were obtained. With $scCO_2$ strongly scented, dark brownish-green creamy product (4.9 g/100 g d.m.) was obtained at 400 bar and 60°C. Among the examined effects of parameters on the yield of SFE, the linear and quadric terms of pressure, the linear term of temperature and the interactions between these two parameters were significant on the 95% significance level. The yield of extraction increases with increasing the temperature and pressure of SFE.

The extraction of **tomato pomace**, an industrial by-product was also mapped. Four samples were widely studied. Two of those had same origin, were stored at air-dried (P1) and deep-frozen (P2) states and the effect of storage on the yields and the compositions were recovered. The other two samples (P3, P4) possessed with different origins as they were obtained from different sources. The extraction yields were obtained within wide ranges. It was observed that from a raw

material with higher quality, lower amounts of extracts were recovered. With apolar solvents (with *n*-hexane: 3.4 – 15.3 g/100 g d.m.) oils, while with ethanol high amounts of highly viscous, dark red coloured products were obtained (between 12.6 – 57.9 g/100 g d.m.). Among the examined parameters, only the effect of temperature was significant on 95% significance level. The highest amount of extract was obtained at 460 bar and 80°C without the degradation of the highly valued compounds.

2. Quantification of biological active compounds

The main biological active compounds were qualified and quantified in the examined plants. In the marjoram the essential oil compositions, the carotenoids and chlorophylls and antioxidant molecules such as diterpenes and triterpenoids were determined. In tomato pomace the characteristic fatty acid compositions and carotenoids and tocopherols were identified.

2.1. The ***volatile constituents*** in the Hungarian and the Egyptian **marjoram** extracts were similar. The main components were terpinen-4-ol and γ-terpinene in the marjoram essential oils, in the alcoholic- and in the supercritical extract. The other characteristic compounds were determined in smaller amounts and with slight differences like γ-terpinene, linalool, α-terpineol, α-terpinolene, α-terpinene, β-caryophyllene and spathulenol. *cis*-Sabinene hydrate was found only in supercritical extract in low amount (1.1%). The composition of essential oils had similar chemovariety to Central and Eastern European spices of marjoram. In the extracts obtained with apolaric solvent and $scCO_2$

palmitic acid was identified by GC and GC-MS methods in higher amounts.

2.2. The ***fatty acid*** composition of **tomato pomace** extracts were revealed. The ethanolic and apolaric (*n*-hexane and $scCO_2$) solvent extracts had similar fatty acid compositions, with main components of linoleic- (45.1-51.6%), oleic- (19.1-21.5%) and palmitic acids (16.6-23.5%). Minor compounds such as stearic acid (5.6-8.3%) and linolenic acid (3.2-4.1%) were also quantified. The extracts obtained with apolaric solvents contained palmitoleic acid (0.3%), arachidic acid (0.5-0.6%) and behenic acid (0.2%) in small amounts.

2.3. ***Chlorophyll and carotenoid pigment***s were examined in the extracts of Hungarian **marjoram**. Chlorophyll-a and –b and theirs decomposed compounds such as pheophytin-a and –b green pigments were found in high amounts in the ethanolic extracts. In this polar extract smaller amount of cholorpyll-a (6 mg/100 g d.m.) and cholorophyll-b (54 mg/100 g d.m.) were found, meanwhile high amounts of pheophytin-a (360 mg/100 g d.m.) and pheophytin-b (201 mg/100 g d.m.) pigments were obtained. Among the carotenoids, marjoram contained only β-carotene and lutein in higher amounts. These compounds were found in similar quantities in the ethanolic and the apolar (with *n*-hexane and $scCO_2$) extracts. In the extracts obtained with $scCO_2$ 5.7 mg lutein /100 g d.m. and 6.1 mg β-carotene/100 g d.m. were found, meanwhile with ethanol 9.5 mg lutein/100 g d.m. and same amount of β-carotene were recovered. Examination of the effects of pressure and temperature of SFE on the yield of cholorphylls and carotenoids revealed that the effect of the linear term of pressure was the most

significant. Among chlorophylls the effect of temperature was also significant on the yield of green pigments.

In the **tomato pomace** extracts carotenoids were identified and the health preventive lycopene was found in the highest amount in the extracts. The extracts obtained from the deep-frozen stored sample contained ten times higher amount of carotenoids than those obtained from the sample stored in dried form. The lycopene was concentrated in the extracts obtained with apolar solvents (with *n*-hexane and $scCO_2$). The effects of the process parameters of SFE were examined with a 3^2 full factorial experimental design. High amount of carotenoid-rich extract (34.9 mg carotenoid/100 g d.m.) can be achieved applying high pressure and high temperature during SFE. In the solvent residual-free extract obtained at 460 bar and 80°C the carotenoid-rich extract contained 90.1% of pure lycopene.

2.4. The ***antioxidant compounds*** in the Hungarian and Egyptian **marjoram** herbs and extracts were identified and compared. In the raw materials and their extracts ursolic acid, carnosol and rosmarinic acid (< 1 mg/100 g d.m.) were quantified. These diterpene and triterpenoid compounds possess with antioxidant properties were found in higher amounts in the Hungarian herb, than in the Egyptian one. The herbs contained ursolic acid in higher amounts (708-907 mg/100 g d.m.) and carnosol in smaller amounts (56-73 mg/100 g d.m.). Ursolic acid was found mainly in the ethanolic extracts, meanwhile the carnosol was concentrated in the extracts obtained with *n*-hexane and $scCO_2$. The effects of the linear and quadric terms of pressure on the yield of carnosol in the SFE extracts were significant. An extract containing high

amount of carnosol (18.5 mg carnosol/100 g d.m.) was obtained at high pressure (450 bar) and 50°C temperature. In the **tomato pomace** extracts the compositions of antioxidant tocopherols were examined, and as well as the effects of pressure and temperature on the yield of tocoperols were mapped. The tocopherols were concentrated in the apolar extracts (3.7-22.2 mg/100 g d.m.), while with ethanol the amount of the recovered tocopherols slightly increased (6.4-19.6 mg/100 g d.m.). The highest tocopherols-rich extract was obtained with SFE (22.2 mg/100 g d.m.) at 300 bar pressure and 80°C temperature. The effects of linear terms of temperature and pressure were the most significant on the yield of tocopehrols. The term of pressure was negative at 95% significance level, therefore the lower pressure was desirable (in the range of design) to recover high amount of tocoperols in concentrated forms. The compositions of tocopherols in the extracts were different due to the changes in the raw materials. Samples P1 and P2 contained γ-tocopherol in high amounts (51-80%[◆]), however the amounts of α- and β-tocopherols in the extracts from the deep-frozen stored sample were twice as high as in the dried stored sample. This can be explained by the better storage that prevented the more sensitive molecules. The sample P3 contained high amount of γ-tocopherol (83-90%[◆]) and in smaller amounts α- and β-tocopherols (< 14%[◆]) were identified. In sample P4 the α-tocopherol (68-73%[◆]) was found in the highest amount, with other compounds such as γ-tocopherol (24-29%[◆]) and in the smallest amounts

[◆] % refers to the amount of all tocopherols.

(<2.7%◆) δ-tocopherol and γ-tocotrienol. High quality products were isolated from sample P4 with supercritical CO_2 extraction.

3. Antioxidant properties of the extracts

The antioxidant properties of Hungarian and Egyptian marjoram herb and its extracts were revealed in aqueous and lipophilic *in vitro* systems.

3.1. The aqueous extracts in higher concentrations (0.04% w/w) possessed significant hydrogen-donor activities in the presence of 1,1-diphenyl-2-picrylhydrazyl free radical. The scavenging activities of these aqueous extracts obtained by chemiluminescence method in the $H_2O_2/OH^{\bullet}$-luminol-microperoxidase system were also concentration dependent.

3.2. Applying the lipid oxidation assay (Rancimat method) the antioxidant properties of the extracts dissolved in sunflower oil (test system) were determined. The ethanolic extract at the concentration of 1% showed comparable antioxidant activities to a synthetic antioxidant (0.1% BHT). It was concluded that the Egyptian marjoram herb and its extracts possessed stronger antioxidant properties in all three polar and apolar systems than those of the Hungarian sample.

4. Comparison of the antimicrobial properties

The antimicrobial properties of marjoram ethanolic and SFE extracts and the essential oil, ethanolic and SFE extracts of thyme were revealed and compared in self-developed antibacterial and antifungal tests against food borne and health indicator microbes.

4.1. The antifungal activities and the minimal inhibition concentrations (MICs), in which no fungal growth was observed, were obtained by agar diffusion method against three filamentous fungi *Trichoderma viride*, *Aspergillus niger,* and *Penicillium cyclopium.* The SFE extract of marjoram showed significantly stronger antifungal activities (MIC_{SFE} = 0.4-0.5 g extract/100 g medium) than the ethanolic extract (MIC_{EtOH} = 5 g extract/100 g medium) against the three tested fungi strains. Among the thyme essential oil and solvent extracts, the essential oil showed the strongest antifungal activity, even at the concentration of 0.025 g essential oil/100 g medium totally inhibited the fungal growth of the three strains. The SFE extract at the concentration of 0.04% and the ethanolic extract at the concentration of 1% presented total inhibition against the three food borne fungi.

4.2. The antibacterial properties of marjoram and thyme extracts against three health significance and food poisoning bacteria (*Escherichia coli, Pseudomonas fluorescens* and *Bacillus cereus*) were revealed by dilution method following the bacterial turbidity. The SFE and ethanolic extracts of marjoram were examined at the concentrations of 0.2 and 0.4% (g extract/100 g medium). The antibacterial activity of ethanolic extract was insufficient, meanwhile the SFE extract at the concentration of 0.4% showed strong antibacterial activities (> 85% inhibition) against the three bacteria. In the tests carried out with thyme essential oil and solvent extracts wider concentration ranges were applied and the minimal inhibition concentrations (MICs) were established. The essential oil at the concentration of 0.1% totally inhibited the three bacteria, while SFE extract at

the concentration of 0.1% showed similar inhibition properties except in the presence of *P. fluorescens*. For total inhibition against this species, the SFE extract was used at the concentration of 0.2%. The ethanolic extract showed slight antibacterial activity, at the concentration of 0.4% only 12-40% of inhibitions were revealed.

Industrial applications

On the basic of the results above it can be concluded that the products obtained by $scCO_2$ extraction possess high biological values; therefore the usage is reasonable for well-defined purposes (food-, cosmetic or pharmaceutical industries). Natural oils, waxes, colours, aromas and flavours can be extracted and due to their antimicrobial and antioxidant properties longer shelf-live can be achieved.

In the marjoram extracts obtained by SFE high amount of green pigments can be determined; therefore it can be used as colouring agents in pastry or other food products. According to the antioxidant and antimicrobial properties of marjoram extracts shelf-live stability of food products such as cheeses, creams and salads can be enhanced.

The thyme extract with strong antimicrobial stability can increase the shelf-life of meats and meat products.

The extracts rich in lycopene obtained from tomato pomace by-product are highly demanded products on the market nowadays. Lycopene is one of the mostly recommended natural food additives in functional and health-related products. As it has strong red colour; therefore it can be used as natural colouring agent.

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