Supercritical Fluid Extraction: A Green Method for the Isolation of Valuable Substances from the Various Biological Matrices.

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SUPERCRITICAL FLUID EXTRACTION: A GREEN METHOD FOR THE ISOLATION OF VALUABLE SUBSTANCES FROM THE VARIOUS BIOLOGICAL MATRICES

Topiar M.[1], Sajfrtova M.[1], Solcova O.[1], Hradecky J.[2], Hurkova K.[2], Hajslova J.[2]

[1]*Institute of Chemical Process Fundamentals of the CAS, Rozvojova 135, 165 02, Prague 6*
[2]*University of Chemistry and Technology, Prague, Technicka 5, 166 28, Prague 6*
topiar@icpf.cas.cz

Abstract

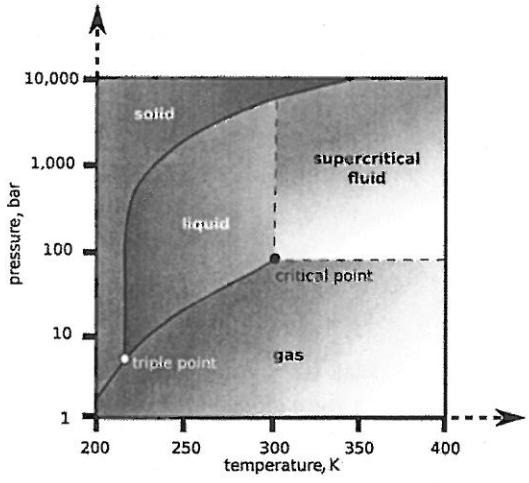
Supercritical fluid extraction (SFE) using carbon dioxide is a sophisticated technology suitable for isolation of un-polar, high value biological active substances from the various biological matrices. In this study, it was used for the extraction of *Magnolia x pruhoniciana* flowers and *Japonochytrium marinum* fungus. Results obtained from the SFE were compared with those from conventional extraction methods. A total number of 37 compounds were identified in the magnolia isolate, from which α-pinene (18 % of peak area), cymene (14 % of peak area), β-pinene (13 % of peak area) and humulene (6 % of peak area) were its main components. Yield of valuable free fatty acids DHA and EPA obtained by the SFE of *Japonochytrium marinum* was more than 1.5 times higher in comparison with organic liquid extraction using mixture of methanol and dichloromethane.

Introduction

SUPERCRITICAL FLUIDS APPLICATIONS

Supercritical fluid extraction (SFE) is a sophisticated separation technique, which uses selected solvents at temperatures and pressures above their critical point. The most frequently applied solvent in this process is carbon dioxide (sc-CO_2) because of its convenient properties, see Fig. 1. A significant advantage of the SFE, over the conventional extraction techniques, is its ability of an easy change of the process selectivity, which is possible by adjusting of the sc-CO_2 density. Even then, the isolate is often a mixture of various substances. A further concentration of target compounds can be solved by using of one or several fractionation techniques, such as time fractionation, using of an additional separator or fractionation on an adsorbent[1].

Apart from the SFE[2], sc-CO_2 finds application in various lab-scale processes, such as supercritical fluid chromatography[3], spray drying[4], impregnation of aerogels[5], TiO_2 nano-crystallization[6] or pressure induced foaming[7]. Because of quite extensive research, sc-CO_2 is now widely used in several special applications requiring high quality products without traces of organic solvents such as hop[8], cork[9] or caffeine extraction[10], vine dealcoholisation, wood impregnation[11] or high-pressure sterilization[12].



+	-
T_c = 31 °C p_c = 7.38 MPa	Weak solvent power = higher consumption
Non-toxic	Using of co-solvents for the extraction of a polar substances
Non-flammable	
Explosive proof	
Cheap in a pure quality	

Figure 1. Carbon dioxide: Diagram of state with supercritical area and pros and cons of sc-CO_2

EXTRACTED MATERIAL

Magnolia x pruhoniciana is a hybrid of *Magnolia obovata* and *Magnolia tripetala* planted from 1952 in Průhonice park area. Among that, nowadays we can find this specie in Lednice park area (Czechia) and arboretum Herkenrode (Belgium). SFE extract of magnolia species has antioxidatant activity[13] among the others. Major bioactive substances found in magnolia isolates are honokiol and magnolol[14].

Japanochytrium marinum belongs to the order of Thraustochytriida, as described in the World Register of Marine Species. Thraustochytriida can utilize various sources of carbon such as glucose, glycerol, mannose, galactose, and fructose[15], and their capacity to accumulate lipids, especially docosahexaenoic acid (DHA, C22:6n-3) is very much relevant for biotechnological applications[16]. Biomass rich in DHA may be valuable in nutritional and pharmaceutical applications[17].
This work focuses on the identification of volatile compounds in the SFE isolate of magnolia hybrid flowers (*M. x pruhoniciana*) and evaluation of free fatty acids profile in *J. marinum* SFE extract and its comparison with conventional organic solvent extraction methods.

Experimental

SUPERCRITICAL FLUID EXTRACTION
Scheme of the SFE experimental apparatus used for the extraction experiment is listed as a Fig. 2. The SFE experiments were carried out using a 25 mL extraction column (I.D. 15 mm) filled with biological material (10 g of J. *marinum* and 17.2 g of *M. x pruhoniciana* flowers) between layers of glass wool and glass beads (I.D. 2 mm) serving as solvent flow distributors. The extractor was immersed in a temperature-controlled water bath (temperature 40 °C). Carbon dioxide, pressurised by compressor NovaSwiss 560.0007 and controlled by pressure regulator unit NovaSwiss 560.0009 to operating pressure (*j. marinum* 30 MPa, magnolia 25 MPa), flowed through the extractor. The flow direction was chosen from the top to the bottom because it accelerates the extraction, in particular at lower Reynolds numbers and for conditions near the critical point of CO_2 where natural convection is dominant[18]. The flow rate was adjusted to 3.5 g min^{-1}. The extractor was connected to a heated micrometre valve where the solution expanded to ambient pressure. Extract was collected in a glass vial. The amount of passed solvent was measured at ambient pressure behind the vial where the extract was collected, using a gas meter. The extract was weighed and closed vials were stored in a freezer.

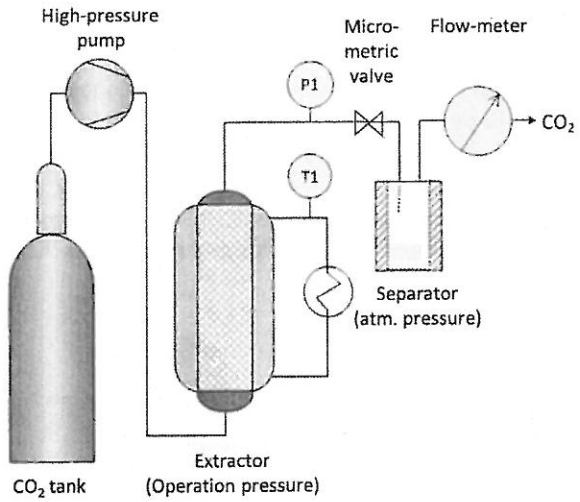


Figure 2. Scheme of the experimental apparatus used for the SFE

CHEMICAL ANALYSIS OF ISOLATES
M. x pruhoniciana analysis (SPME-GC TOF MS)
Volatile substances from magnolia flowers were analysed by HS-SPME technique. For the headspace analysis, 50 µl of isolate from organic liquid extraction and 3 mg of SFE isolate were placed into 10 ml glass vial. Emitted compounds were adsorbed to string with using of automatic SPME technique (PDMS/CX/DVB (30/50 µm)) during 1 min at temperature 40 °C followed by their thermal desorption into injection port of a gas chromatograph (GC HRTOF MS; Pegasus GC-HRT; LECO; USA). For identification of volatile components of the isolates, ChromaTOF for HRT (LECO,USA) software was used. Analyzed spectra were compared with NIST 11 MS spectra library, which contains large database of different compounds.

J. marinum analysis (U-HPLC-HRMS/MS)
Samples were extracted with the mixture of isopropyalcohol:methanol (65:35) to isolate as broad spectrum of lipids as possible. Aliquots of extracts were filtered and examined using non-targeted screening approach. Ultra-high performance liquid chromatograph UHPLC DionexUltimate 3000 (Thermo Fisher Scientific) coupled

to high-resolution tandem mass spectrometer TripleTOF 6600 (SCIEX) was used to determine lipidomic profiles of extracts. 2 µl of extracts were injected into the system using auto sampler, separation was carried out on the reverse phase column. Ionization was performed in both positive and negative mode by electrospray (ESI).

Software package PeakView 2.0 (SCIEX, Concord, ON, Canada) equipped with FormulaFinder was used for molecular formula estimation, structural elucidation and subsequent tentative identification of compounds based on MS and MS/MS accurate mass spectra. For identification of lipids, software LipidView 1.3 beta (SCIEX, Concord, ON, Canada) which contains large database of different lipid species was used.

Results and discussion

Magnolia x pruhoniciana

Total yield of the SFE isolate obtained at 25 MPa and 40 °C with the CO_2 consumption equal to 10.3 g g_{feed}^{-1} was 23.3 mg g_{feed}^{-1}. It was possible to do only one SFE extraction experiment because of the limited amount of dried magnolia flowers. Chemical composition of SFE isolate represent Fig. 3. In total, 37 different chemical compounds were identified in the SFE isolate. Major compounds found in magnolia SFE isolate were α-pinene, cymene and β-pinene, which creates almost 50 % of cumulative frequency. These particular terpenes we can found in many types of essential oils. Unfortunately, no magnolol and honokiol were analysed in our sample. Isolates obtained by another extraction techniques contains mostly the same compounds but with their different ratio and low concentrations of volatile substances.

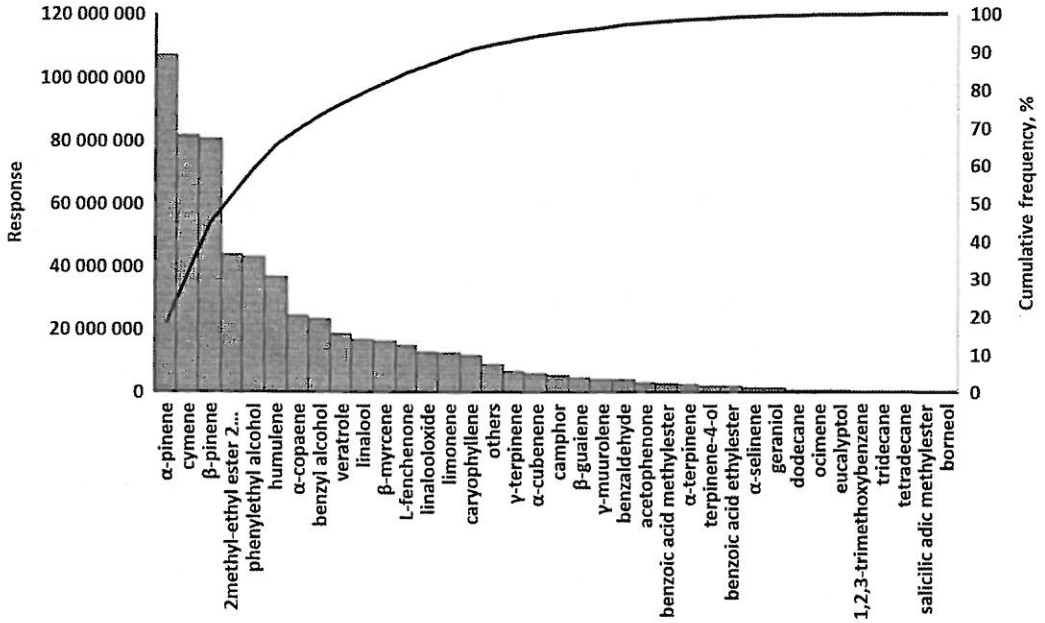


Figure 3. Chemical compounds found in SFE sample from *magnolia x pruhoniciana* expressed by Pareto chart

Japonochytrium marinum

Fig. 4 expresses extraction curves for the SFE with pure CO_2 and total yields for experiments when modified CO_2 was used. Optimum CO_2 consumption for the extraction of *J. marinum* with a pure CO_2 seems to be usage of 25 g_{CO2} g_{plant}^{-1}, when isolate yielded at 97.3 mg g_{plant}^{-1}. Another increase in a CO_2 consumption resulted in a low increase of yield, when total yield with using of 35 g_{CO2} g_{plant}^{-1} was 105.1 mg g_{plant}^{-1}. During two experiments, ethanol was used as a polar modifier together with a sc-CO_2. Increase in concentration of ethanol in a CO_2 flow led into the increase of total extraction yield, because of the isolation of polar substances, which are not extractable with a pure CO_2. Despite of the higher total yield when 10 wt.% of ethanol was used, 5wt.% concentration seems to be a better option in case of extraction of higher feed amounts. When high ethanol concentration (10 wt.%) was used, clogging observed. This was able to solve only by short-term increase in operation pressure.

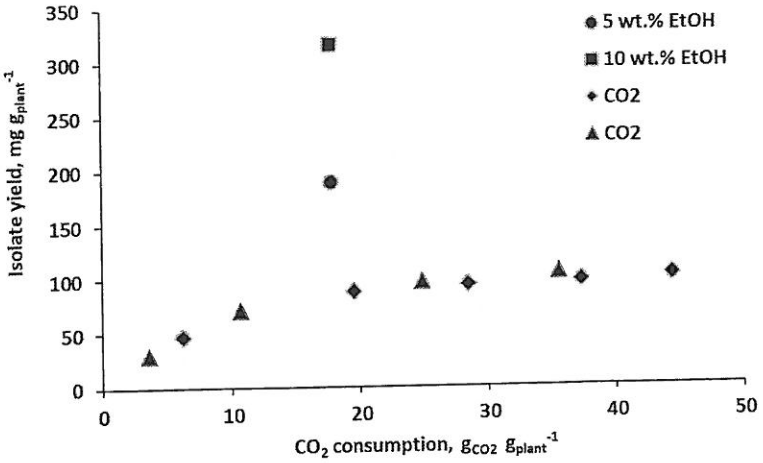


Figure 4. Extraction yields from *J. marinum* with using of pure CO_2 and modified CO_2 by ethanol as a solvent during SFE

Chemical composition of *J. marinum* isolates obtained by several separation methods in terms of their free fatty acids representation in samples shows Figure 5. Our main interest was to isolate high yields of ω-3 fatty acids, namely DHA (docosahexaenoic acid, FFA 22:6), DPA (docosapentaenoic acid, FFA 22:5) and EPA (eicosapentaenoic acid, FFA 20:5). High yields of DHA and EPA were found in isolates from organic liquid extraction using mixture of methanol and dichloromethane and SFE using pure sc-CO_2. The undisputed advantage of SFE is high yield of ω-3 fatty acids with no occurrence of unhealthy organic solvents in the isolate and no need of its energy-intensive subsequent vaporization. Isolate obtained by SFE contained also the highest yield of palmitic acid (FFA 20:5) in comparison with other studied extraction techniques.

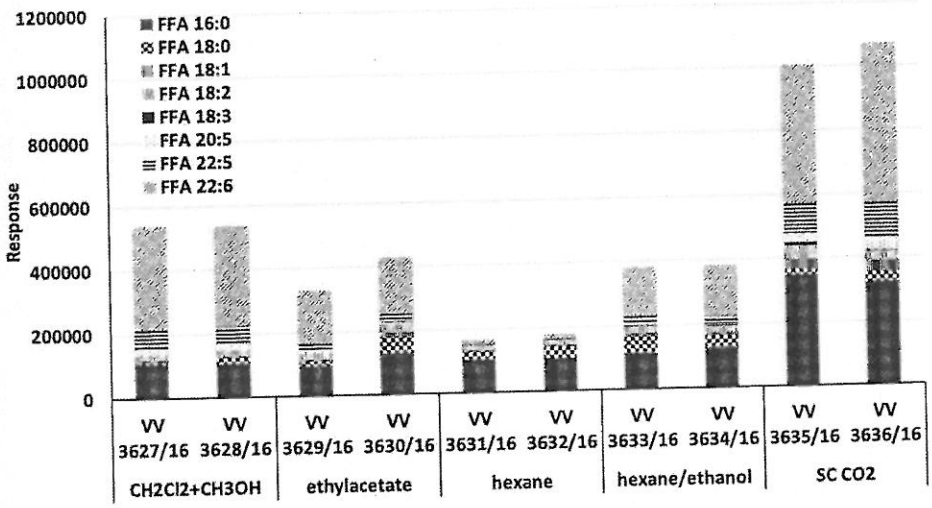


Figure 5. Proportionate representation of free fatty acids in isolates from *J. marinum* obtained by different separation techniques

Conclusion

Supercritical fluid extraction is a green extraction method suitable for special applications. We were able to identify 37 different compounds in the *M. x pruhoniciana* flower extract obtained by SFE with α-pinene, cymene and β-pinene as major components. SFE provided the best results in terms of ω-3 fatty acids isolation from *J. marinum* in comparison with traditional organic liquid extraction. Yields of EPA and DHA were almost 1.5 times higher than with using of organic extraction with a mixture of dichloromethane and methanol as a solvent. Based on that, subsequent SFE experiments with ethanol as modifier were done, but until now we have not obtain results of HPLC analysis. Our results prove SFE can provide higher yields of volatile compounds (*M. x pruhoniciana*) and free fatty acids (*J. marinum*) in comparison with traditional organic liquid extraction.

Acknowledgement

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