Extraction and Fractionation of Bioactive Compounds from Aromatic Plants

P. R. Venskutonis[1], G. Miliauskas[1], B. Sivik[2]*
[1]Kaunas University of Technology, Radvilenu pl. 19, LT-50015, Kaunas, Lithuania
**e-mail: rimas.venskutonis@ktu.lt; fax: +37037456647*
[2]Lund University, Box 124, S-221 00 Lund, Sweden

Abstract

Extracts were isolated from aromatic and medicinal plants by supercritical fluid extraction with carbon dioxide (SFE-CO_2), with and without using co-solvent ethanol and Soxhlet extraction with pentane. The following plants were extracted: lemon balm (*Melissa officinalis*), hyssop (*Hyssopus officinalis*), catnip (*Nepeta cataria*), stinging nettle (*Urtica dioica*), sage (*Salvia officinalis*), summer savory (*Satureja hortensis*), gum plant flowers (*Grindelia robusta*) and tubers of tigernut (*Cyperus esculentus*). The highest yields in most cases were obtained by Soxhlet extraction, except for gum plant flowers. Medium yields were obtained by SFE-CO_2 with co-solvent, and the lowest ones (except for summer savory and lemon balm) by SFE-CO_2 using pure CO_2. The effect of ethanol on the extract yield and composition was assessed by using two separators operating at 40°C, 20 and 5 MPa, respectively (extraction was performed at 300-315 bar and 60 °C). The total amount of extracted substances in the first separator was remarkably lower than in the second one. The yield in the first separator increased when the concentration of ethanol was higher. Preliminary screening of extract composition by GC/MS and HPLC/UV/MS revealed differences in the distribution of individual compounds in extracts. For instance, in case of pure CO_2 phenolic compounds were precipitated mainly in the second separator (5 MPa), while in case of added ethanol the fraction distributed more evenly between the first and the second separators.

Introduction

In general, two conventional methods are most frequently used for the isolation of natural substances from the plants: (i) extraction using organic solvents (e.g., hexane, acetone, methanol, ethanol, methylene chloride), and (ii) distillation with water. The extracted substances usually are concentrated by removing the excess of solvent and depending on solvent properties different products, such as oleoresins, absolutes are obtained. A distillation enables to obtain a concentrated volatile fraction, which is called essential or volatile oil.

Several shortcomings are characteristic to the traditional extraction methods. Firstly, the majority of organic solvents are toxic and their presence in foods are regulated by laws; e.g. the concentration of solvents should be reduced in the final product to 25–30 ppm. Secondly, valuable volatile compounds can be partially lost during the evaporation of solvent. And finally, the products obtained with the use of hazardous chemical solvents cannot be labelled as ”natural”, which reduces their consumer acceptance and market value.

Supercritical fluid extraction with carbon dioxide (SFE-CO_2) is a promising and challenging method for the isolation of valuable phytochemicals with such advantages as safety and easy removal of solvent, low extraction temperature, low energy consumption and extraction selectivity depending on pressure and temperature [1]. The main disadvantages of SFE-CO_2 are related to rather high equipment costs and low polarity of CO_2 that makes extraction of polar hydrophilic components rather problematic. The majority of plant origin antioxidants are polar compounds and it was reported that the solubility of antioxidants in CO_2 is very low. For instance, one of the strongest antioxidant compounds in various Labiatae family plants, carnosic acid was found to be almost insoluble in supercritical CO_2 below 30 MPa [2]. Fractional extraction at high pressures is very effective in obtaining carnosic acid

with a low content of undesirable compounds [3]. The method describing isolation of antioxidants by SFE at high pressures was patented in USA in 1991 [4]. A number of reports on the use of SC–CO_2 to isolate natural antioxidants from Labiatae species have remarkably increased during last decade. For instance, SFE techniques were applied for the deodorization of rosemary extracts [5] and for reextracting antioxidants from ethanol extract of sage [6]. Most recently it was reported that applying SFE–CO_2 the highest value of antioxidant carnosol was obtained at 40 MPa and 60 °C [7].

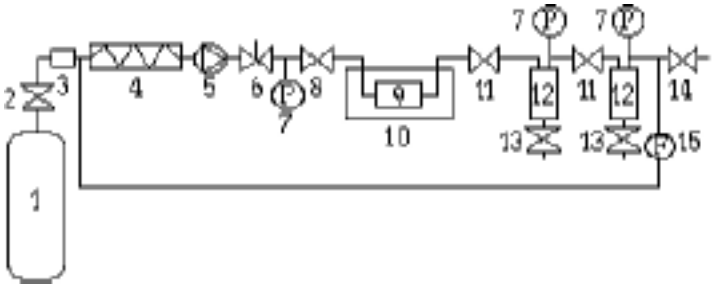
The solubility of polar compounds in supercritical CO_2 can be increased by the use of a co-solvent, e.g. ethanol, however in this case an extra step is usually required to remove the excess of the co-solvent at the end of the extraction process. Boiling temperature of a co-solvent is usually much higher than that of pressurised gasses and lower than that of extractable plant components. Therefore, to obtain similar extract yields extraction with an entrainer can be performed at lower pressures.

I – Materials and Methods

The following plants were obtained from Lithuanian Institute of Horticulture and Kaunas Botanical Garden: lemon balm (*Melissa officinalis*), hyssop (*Hyssopus officinalis*), catnip (*Nepeta cataria*), stinging nettle (*Urtica dioica*), sage (*Salvia officinalis*), summer savory (*Satureja hortensis*), gum plant flowers (*Grindelia robusta*) and tubers of tigernut (*Cyperus esculentus*). The plants were dried and ground in a laboratory mill (Siemens SK-915-I for aerial parts and KNIFETEC 1095 Sample Mill for tubers) to pass 0.8 mm size sieve. Carbon dioxide was from AGA (99.99%, Sweden), ethanol from Kemetyl (99.5%, Sweden) and pentane from Sigma-Aldrich (99+%, Germany).

Raw material and extracts were weighed by Mettler AE 163 (Switzerland) analytical balances. SFE setup was equipped with a high pressure pump Dosapro Milton Roy, (Milroyal B-C, France) operating up to 40 MPa.

Extraction pressure in the extractor was 30-32 MPa, temperature 90°C and flow rate of supercritical CO_2 (SC–CO_2) 0.025 kg/min. When ethanol was used as an entrainer its content in CO_2 was 1% and 5 % and the temperature was 60°C. Extraction was completed after passing 10 kg of CO_2. The extracts were collected in the two 200 ml volume separators operating at 40°C and different pressure in order to obtain two extract fractions. It was expected that less soluble in liquid CO_2 components would precipitate in the first separator operating at 20-21 MPa, while other fractions will be collected in the second separator operating at 5 MPa. Two replicate extractions were performed for every plant sample.



1 - CO_2 container; 2, 6 – safety valves; 3 – gas filter; 4 - ethanol bath for gas cooling (-22°C); 5 – pump; 7 – pressure gauge; 8 – valve; 9 – extractor; 10 – water bath; 11 – micro valve; 12 – separators; 13 – extract removal valves; 14 – extra valve; 15 – flow meter.

Figure 1: Supercritical fluid extraction set

Oleoresins were obtained from 10-40 g ground plants in a Soxhlet apparatus with 200 ml of pentane during 4 h. Three replicate extractions were performed for every plant sample.

Solvents both in SFE and Soxhlet extractions were removed from the extracts in a Büchi rotavapour (Flavil, Sweitzerland) at 40°C temperature.

The HPLC-MS setup for extract analysis consisted of Waters 1525 binary HPLC eluent pump (Millipore, Waters Chromatography, Milford, USA), Merck L-7400 UV detector (LaChrom, Tokyo, Japan) and Waters Micromass ZQ mass detector. The linear binary gradient was used at a flow rate of 0.8 ml/min. Solvent A consisted of 10 % (v/v) MeOH and 1 % CH_3COOH (v/v) solution in water; solvent B was 100% MeOH. Gradient conditions were as follows: 0 to 30 min B increased from 30 to 100 % and kept constant till 33 min; 33-36 min B decreased back to 30%. The compounds were separated on a Phenomenex Synergi MAX-RP analytical column, 4 μm, 250×4.6 mm i.d. (Phenomenex, Torrance, USA). UV detector was operating at 254 nm wavelengths. MS detector was operating using electrospray ionization (EI) probe, in positive and negative ionization modes. After HPLC separation eluent flow was split into equal parts using T connection, and only 0.4 ml/min of total flow were transferred to the EI probe. Filtered and soluble in MeOH 1% concentration extract fractions were used for chromatographic analysis.

Volatile compounds in the extracts were analysed by gas chromatography and mass spectrometry. Extract solutions (0.2 %) in diethyl ether (Lachema, Naratovice, Czech Republic) were injected into a HP-5890 (II) gas chromatograph equipped with HP 5971 mass detector and fused silica capillary column HP5 MS (5% phenyl methyl silicone, 30 m length, 0.25 mm i.d.). The temperature was programmed from 30°C (1 min) to 230°C (20 min) at the rate of 4°C/min. Detector was heated at 250°C, injector at 230°C. Helium was used as a carrier gas at 5-psi pressure. Mass spectra were obtained by electron ionisation at 70 eV.

II – Results and Discussion

In general, the highest extract yields were obtained by using Soxhlet method with exception of *Grindelia robusta* flowers, when the highest yield was extracted with pure SC-CO_2 (Figure 2). The use of ethanol in most cases also increased extract yield compared to the yield obtained with pure SC-CO_2. However, in addition to the above-mentioned exception, the yield from *Satureja hortensis* and *Melissa officinalis* was higher when pure SC-CO_2 was used.

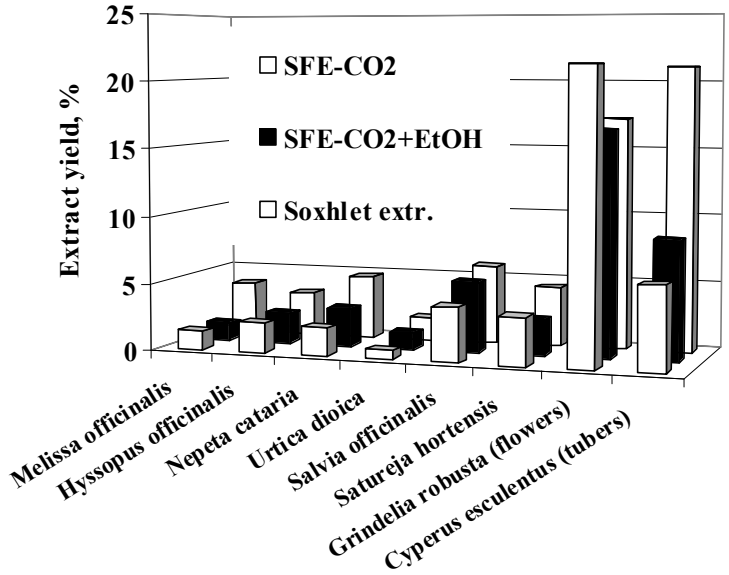


Figure 2. Extract yields obtained by different extraction methods

The yield was further increased by the increase of the entrainer concentration as it is demonstrated in case of *Urtica dioica* (Figure 3). The amount of the extract in the first separator was remarkably lower than in the second one indicating that extracted compounds were still fairly soluble in the SC-CO_2 at 20-21 MPa and 40°C. Comparing the yields obtained from different plants it can be observed that Labiatae family herbs gave lower yields; in most cases they did not exceed 5%. The highest extract yields (>15%) were obtained from gum plant flowers.

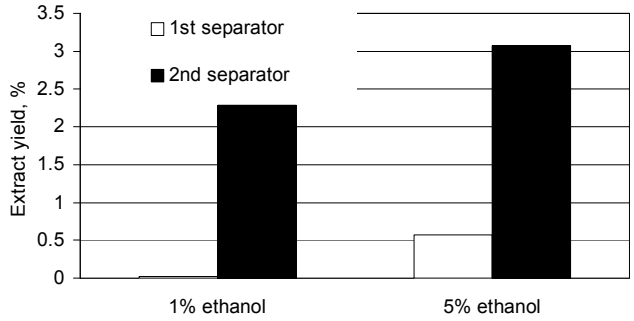


Figure 3. Effect of ethanol on the extract yield of *Urtica dioica* in SFE-CO_2

Lemon balm was selected for the demonstration of the results on the analysis of extract composition. Characteristic HPLC profile of lemon balm extract is showed Figure 4. The biggest peak (UV signal at 254 nm) eluting at 28.5 min has been assigned to kaempferol methyl ether. This compound was identified according to MS (M+H = 301 *m*/*z*) and literature data. Other three compounds, rosmarinic acid methyl ester (M+H = 375 *m*/*z*), carnosic acid (M+H = 333 *m*/*z*), and ursolic acid (M+H = 457 *m*/*z*) were identified in the CO_2 and pentane extracts of lemon balm, except of ursolic acid, which has not been detected in CO_2 extract obtained at 200 MPa without using ethanol.

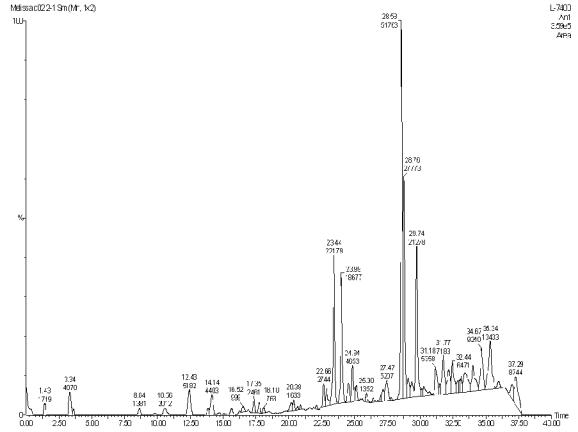


Figure 4. HPLC-UV profile (λ = 254 nm) of *Mellisa officinalis* CO_2 extract separated at 200 MPa

All detected compounds are strong antioxidants. The antioxidant activity of *Melissa officinalis* subsp. *officinalis* and of *Melissa officinalis* subsp. *inodora* extracts, obtained by using SFE-CO_2 was recently reported [8], however other authors did not recommend pressurized solvent extraction and SFE as preparation techniques suitable for polar phenolics exaction from plant material because the yields of all analytes were low in comparison to liquid extraction and SPE, and the cleanness of chromatograms has also been worse than after

SF with OASIS HLB sorbent [9]. The content of compounds present in different extracts was preliminary assessed by the HPLC peak area integrated at 254 nm (Table 1). Judging from the sum of the components collected in both separators SFE-CO_2 with ethanol gave the highest yield of phenolics (344.68 a.u.) detected at the applied HPLC analysis parameters. Reverse phase HPLC combined with UV detector was not able to detect the majority of compounds collected at 20 MPa pressure when pure CO_2 was used; the yields of precipitated compounds at this pressure were significantly lower than those collected at 5 MPa. It was reported that the highest value of phenol compounds was obtained for the extracts of solid residues of supercritical extraction at 10 MPa, 50°C and 30 min. [10]. When ethanol was added the total amount of compounds in the first separator increased more than 34 times, while their yield in the second separator was lower comparing with that collected in case of pure CO_2. The majority of the detected components were high polarity phenolic acids and their derivatives and it is interesting to note that their solubility in SC-CO_2 at high pressure was many times higher than that in liquid CO_2 at low pressure. For instant, total amount of detected compounds in Soxhlet pentane extract was almost 4 times lower than in SC-CO_2 extract.

Table 1. Composition of *Melissa officinalis* extracts, in arbitrary units (HPLC-UV peak area × 10^3)

Compound	CO_2 extracts		CO_2 extracts with ethanol		Soxhlet pentane extract
	20 MPa	5 MPa	20 MPa	5 MPa	
Carnosic acid	0.12	17.09	22.18	11.20	4.78
Rosmarinic acid methyl ester	0.15	6.30	5.21	-	0.76
Kaempferol methyl ether	2.52	20.37	27.77	20.53	8.58
Ursolic acid	-	7.08	7.08	4.83	1.81
Sum of not identified compounds	2.37	213.33	116.94	128.94	61.02
Total	**5.16**	**264.17**	**179.18**	**165.50**	**76.95**

The amount of extract components detected by GC method was approximately 3 times higher in Soxhlet pentane extract than in SC-CO_2 extracts. However, the concentration of such key aroma compounds of lemon balm as citronellal, citronellol, neral, nerol, geranial and geraniol was slightly higher in CO_2 extracts. The content of these compounds in volatile fraction of pentane extract was 3.58 %, while in CO_2 extract fractions it varied from 9.06 to 14.89 %. Consequently it can be reasonably expected that CO_2 extracts should exhibit remarkably stronger citrus-like aroma, which is characteristic to lemon balm. Similar compounds were already reported in SC-CO_2 extracts of *Melissa officinalis* [11]. On the contrary, the content of less volatile components (e.g., ethyl palmitate and linolenate, phytol, heptadecane, sitosterols, squalene, tocopherols) was considerably higher in pentane extracts. However, non-volatile fatty acids (palmitic and linolenic) were better extracted with CO_2.

Distribution of detected compounds between the fractions was somewhat similar to that determined during HPLC analysis. For instance, the concentration of the compounds in the fraction collected at 20 MPa was lower than in the fraction collected in liquid CO_2 (5 MPa); however this difference reduced after adding ethanol. Total concentration of extract components determined by GC for many compounds was almost similar for pure SC-CO_2 and SC-CO_2 + ethanol extracts.

Conclusion

Selected aromatic and medicinal plants can be successfully extracted with SC-CO_2, however, extraction parameters and combination of solvents should be selected individually, depending

on the plant chemical composition. Addition of ethanol to carbon dioxide increases the yield of the majority of plant compounds in the extracts, however, fractionation of extracts by reducing the pressure from 30 MPa (extraction) to 20 MPa (1st separator) and 5 MPa (second separator) resulted in distribution of plant components in both fractions at different ratios.

Table 1. Chemical composition of volatile compounds in *Melissa officinalis* extracts

Compound	Content, GC area %					Arbitrary units from 100g plant material				
	20 MPa	5 MPa	20 Mpa	5 Mpa	Soxhlet	20 MPa	5 MPa	20 Mpa	5 Mpa	Soxhlet
Citronellal	0.74	0.70	0.62	0.99	-	17	84	31	83	-
Nerol	3.26	2.85	1.66	2.31	1.39	75	341	82	194	531
Citronellol	1.73	-	1.36	1.84	-	40	-	67	155	-
Neral	2.16	2.32	1.19	1.87	0.65	50	277	59	157	249
Geraniol	4.63	2.67	2.55	3.41	1.03	107	319	125	287	394
Geranial	2.37	2.85	1.68	2.52	0.51	55	341	83	212	195
β-Caryophyllene	1.64	1.89	1.78	2.35	2.00	38	226	88	198	765
Caryophyllene oxide	2.03	1.08	1.21	1.32	0.72	47	129	60	111	275
Palmitic acid	4.06	2.68	2.94	2.25	-	94	320	145	189	-
Ethyl palmitate	0.51	0.59	-	0.42	2.21	12	71	-	35	845
Phytol	7.65	4.56	4.93	5.61	3.42	177	545	242	472	1307
Linolenic acid	3.43	2.32	2.99	1.74	0.49	79	277	147	146	187
Ethyl linolenate	1.96	1.68	1.43	2.38	3.12	45	201	70	200	1193
Heptadecane	0.94	2.49	0.90	1.88	1.19	22	298	44	158	455
β-Sitosterol	1.22	6.34	-	12.11	7.49	28	757	-	1018	2863
γ-Sitosterol	-	-	14.67	-	9.34	-	-	721	-	3570
Squalene	3.26	2.49	3.95	3.33	3.90	75	298	194	280	1491
Tocopherol (isomer)	2.03	1.96	1.04	1.02	1.43	47	234	51	86	547
Tocopherol (isomer)	1.96	17.91	20.59	16.31	13.17	45	2140	1012	1371	5034
Sum of unidentified compounds	24.56	35.05	27.48	31.58	30.42	569	4189	1351	2653	11629
Total:	**70.14**	**92.43**	**92.97**	**95.24**	**82.48**	**1622**	**11042**	**4571**	**8005**	**31529**

References

[1] STAHL, E., QUIRIN, K.–W., GERARD, D., Dense Gases for Extraction and Refining, Springer-Verlag, Berlin, **1988**.

[2] LACK, E., SEILITZ, H., in Proceeding of the 3rd Congress on High Pressure Chemical Engineering, R. von Rohr, Ch. Trepp (Eds.); Elsevier Science Publisher, **1996**, p. 253.

[3] REVERCHON, E., in Proceedings of the 4th International Symposium on Supercritical Carbon Dioxide, Sendai, Japan, **1997**, p. 839.

[4] NGUYEN, U., FRACKMAN, G., EVANS, D.A., U. S. Pat. 5,017,397, **1991.**

[5] LOPEZ-SEBASTIAN, S., RAMOS, E., IBAÑEZ, E., BUENO, J.M., BALLESTER, L., TABERA, J., REGLERO, G., J. Agric. Food Chem., **1998**, Vol. 46, p. 13.

[6] DJARMATI, Z., JANKOV, R.M., SCHWIRTLICH, E., DJULINAC, B., DJORDJEVIC, A., J. Amer. Oil Chem. Soc., **1991,** Vol. 68, p. 731.

[7] VAGI, E., RAPAVI, E., HADOLIN, M., PEREDI, K.V., BALAZS, A., BLAZOVICS, A., SIMANDI, B., J. Agric. Food Chem., **2005**, Vol. 53, p. 17.

[8] MARONGIU, B., PORCEDDA, S., PIRAS, A., ROSA, A., DEIANA, M., DESSI, M.A., Phytother. Res., **2004**, Vol. 18, p. 789.

[9] ZIAKOVA, A., BRANDSTETEROVA, E., J. Liquid Chromatogr. & Related Technol., **2002**, Vol. 25, p. 3017.

[10] RIBEIRO, M.A., BERNARDO-GIL, M.G., ESQUIVEL, M.M., J. Supercrit. Fluids, **2001**, Vol. 21, p. 51.

[11] ROZZI, N.L., PHIPPEN, W., SIMON, J.E., SINGH, R.K., Lebensm.-Wiss.-Technol., **2002**, Vol. 35, p. 319.