THE ULTRASOUND EXTRACTION AND SUPERCRITICAL CO_2 RE-EXTRACTION OF OBTAINED EXTRACT OF SAGE (*Salvia officinalis* L.)

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Abstract (na karaju rada moze te pronaci abstract koji je poslat prethodno)

Knowing that supercritical fluid extraction (SFE) of sage (Salvia officinalis L.) allow isolation of wide spectrum of phytochemicals in comparison to the other extraction methods which are limited to either volatiles isolation (hydrodistillation) or higher molecular compounds isolation (Soxhlet extraction) the combination of ultrasound assisted extraction followed by re-extraction of obtained extract with supercritical CO_2 was performed in this study. The goal of performed investigation was to concentrate diterpenes present in sage extract which are generally considered to be responsible for antioxidant activity of sage.

The ultrasound assisted extraction was performed at 40 °C using ethanol – water mixture (70:30) and 18 mass% yield of extract after 45 min of treatment was detected. Obtained extract was separated into four fractions (F1-F4) through re-extraction with supercritical CO_2 at 50 °C and 15 MPa. Namely, the sage re-extract had a higher content of diterpenes (according to GC analysis, about 45 mass%) in comparison to the extract obtained by hydrodistillation (1.5 mass%), Soxhlet extraction (about 30 mass%) or SFE extraction (50 °C and 15 MPa; 30 mass%) and lower content of aromatic and volatile compounds compared to extract or fractions obtained by other extraction technique. The combined procedure, the supercritical CO_2 extraction of the previously treated plant material (distilled water extraction assisted with ultrasound) could give the two valuable products (water extract and diterpenes' concentrate extract).

Keywords: *Salvia officinalis* L; Supercritical; Ultrasonic; Diterpenes.

1. Introduction

Salvia officinalis L. (Lamiaceae) extracts, among its most possible applications (antibacterial, hypoglycemic, anti-inflammatory, fungistatic, virustatic, astringent, eupeptic, anti-hydrotic and antidiabetic properties [1-10]), it is well known as a significant antioxidant [1-6] which could be used in food preservation or for medical/pharmaceutical care.

There are so many different extraction techniques described in literature for the sage extraction as hydrodistillation, the conventional organic solvent extraction, ultrasound assisted solvent extraction and subcritical or supercritical extraction using CO_2 (SFE or $SCCO_2$ extraction). All of them had an advantages and disadvantages with respect to operating cost, capital cost, yield, and quality of obtained extracts and only the SFE possesses environmental friendly and human health safety attributes. None of the above mentioned conventional techniques could be used for the concentration of different groups of compound in the sage's extract. Most of these techniques could isolate only light fractions or sage's essential oil or total/crude extract (with heavy components as a waxes, oleoresins,

polysaccharides, chlorophyll etc.) but most valuable components in sage extract poses antioxidative characteristic like diterpenes [12].

Using the SFE and CO_2 as a solvent it could concentrate diterpenes by varying the temperature and pressure (or density) and differentially collected fractions as was previously published [1]. Regarding the total extract composition, the best operating conditions for the best selectivity to diterpenes are 150 bar and 50 °C [1].

In Eastern Europe herbal tinctures are often employed in medical treatment. Their production from conventional processes is not, however, very effective. A comparative study of the composition of the ethanol extract obtained from *Salvia officinalis* by conventional and ultrasound-assisted extraction indicates more efficient extraction using the latter method [12-14]. The use of ultrasound resulted in increased component extraction in a shorter time and at lower temperatures [11]. The mechanical effect of ultrasound is able to accelerate the extraction of organic compounds, contained within the body of plants, due to disruption of the cell walls and enhanced mass transfer of cell contents.

Comparative analysis of combined extraction techniques (e.g. the ultrasound assisted solvent extraction followed by supercritical extraction of obtained ultrasound extract), which might fulfil the main task to obtain the extract with desired concentration of phenolic diterpenes is the main task of this study.

The objectives of this study were focused on concentration of diterpenes by using combination of several extraction techniques: (a) the fractionation using the $SCCO_2$; (b) ultrasound assisted solvent extraction and re-extraction of obtained extract; (c) ultrasound assisted solvent extraction and extraction of the plant material obtained after solvent extraction.

2. Material and methods

2.1. Materials

Dalmatian sage (Salvia officinalis L.) was collected in September/October 2008 (late flowering stage) from the region Konavle (the very southern tip of Croatia) and the voucher specimen (no. 16282) was deposited at Herbarium of Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade (Belgrade, Serbia). Aerial parts of sage (leaves to plant ratio=70:30) were dried in the air protected against direct sunlight. Leaves were milled in a blender for 60 seconds and immediately subjected to supercritical CO_2 extraction, or ultrasound assisted extraction. The average particle size of milled sage was 0.55 mm. The moisture content of the air-dried plant material determined by Karl Fischer volumetric titration was 13.6 mass%.

Commercial carbon dioxide (99% purity) was supplied by Tehnogas (Messer-Tehnogas, Serbia), 95% ethanol (Zorka Pharma, Serbia) for Soxhlet extraction and chloroform (GC purity, Sigma-Aldrich, Germany). The standard compounds used for the chemical analyses were carnosic acid and carnosol (Sigma Chemical Co. (St. Louis, USA)). These chemicals were of analytical reagent grade.

2.2. Equipment and methods

2.2.1. Supercritical carbon dioxide extraction

Extraction with supercritical CO_2 was carried out in a semi-batch Autoclave Engineers Screening System previously described in details [1,15]. All extraction was performed isothermally at 50 ^{o}C and at 150 bars as an experimental condition chosen as optimal one for the diterpenes concentration based on results of our previous work [1]. The mass of 30 g of

the plant material was used for the extraction while the flow rate of CO_2 was 0.4 kg h^{-1} in all performed runs. Differential quantities of extract (fractions) were collected during the extraction with main goal to monitor the changes of the chemical composition of the extract with respect to CO_2 consumption and pressure. The standard deviation of obtained yield (triplicate procedure) is 2.3% for all extraction conditions. All yields and composition calculations were made on a moisture basis of sage (16.3 mass%).

2.2.2. Ultrasound assisted extraction with different solvents

The milled plant material (50 g) was extracted with different solvents (distilled water and mixture of distilled water and ethanol (30:70)) and different solvents ratio (1:12 or 1:6 mass/volume of plant material to solvents) for 45 min at 40 °C using ultrasound bath. At the end of extraction the liquid extract was evaporated to the dryness by rotary vacuum evaporator up to 50 °C. The obtained extracts were kept in a sealed vial at 4 °C. All extractions procedure was performed in triplicate with the standard deviation of obtained 0.7 %. The ultrasonic bath Sonorex RK 52 (BANDELIN electronic GmbH & Co. KG, Munich, Germany) operating at a frequency of 35 kHz was used with HF power of 60 W and ultrasonic peak output 240 W.

2.3. Analytical Procedures

2.3.1. Analytical gas chromatography (GC/FID)

Gas chromatography analysis of the extracts was carried out on a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with split-splitless injector and automatic liquid sampler, attached to HP-5 column (25 m x 0.32 mm, 0.52 μm film thickness) and fitted to flame ionisation detector (FID). Carrier gas flow rate (H2) was 1 ml/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40-260 °C (at rate of 4 °C/min), and then kept isothermally at 260 °C for further 10 minutes. Solutions of samples in ethanol (or mixture of ethanol and chloroform, 50:50) (~1 mass%) were consecutively injected in amount of 1 μl. Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification analysis.

2.3.2. Gas chromatography/mass spectrometry (GC/MS)

The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with column HP-5MS (30 m x 0.25 mm, 0.25 μm film thickness), using HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Helium was used as carrier gas. Transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV); in m/z range 40-450. The amount of 0.2 μl of sample solution in ethanol (or mixture of ethanol and chloroform, 50:50) (~1 mass%) was injected.

The components of the oil were identified by comparison of their mass spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.) [16], compared to those from available literature (Adams, 2007) [17], and used as additional tool to approve MS findings. Percentage of each volatile compound and fatty acids methyl esters was the mean of three replicates (± standard deviation, SD).

3. Results

Major compounds in sage extract and the content of different compound families such as monoterpenes (M), oxygenated monoterpenes (OM), sesquiterpenes (S), oxygenated sesquiterpenes (OS), diterpenes (D), triterpenes (T), esters (E) and waxes (W) identified by means of GC-FID and GC-MS methods were listed in **Table 1** (average result of cumulative extract collected at specific condition used for extraction).

Table 1. The major compounds identified in different sage extracts

Components	**KI**	**RT/MS**	**SD**	**US EtOH+ water**	**NKE 300 bar**	**NKE 150 bar**	**Total in plant material**
				g/100g of plant material			
Monoterpenes				**0.06**	**0.27**	**0.24**	**0.27**
α-pinene	934	7.40	0.01	0.02	0.10	0.09	0.10
camphene	949	7.84	0.03	0.02	0.07	0.06	0.07
β-pinene	977	8.73	0.01	0.00	0.02	0.02	0.02
myrcene	995	9.30	0.15	0.00	0.01	0.01	0.01
p-cymene	1027	10.37	0.21	0.00	0.01	0.01	0.01
limonene	1031	10.50	0.02	0.01	0.02	0.01	0.02
γ-terpinene	1061	11.56	0.26	0.00	0.04	0.03	0.04
trans-sabinene hydrate	1101	12.93	0.01	0.00	0.01	0.01	0.01
Oxygenated monoterpenes				**1.01**	**2.09**	**1.89**	**2.09**
1,8-cineole	1033	10.57	0.02	0.02	0.23	0.17	0.23
β-thujone	1109	13.21	0.02	0.17	0.60	0.62	0.60
α-thujone	1119	13.57	0.02	0.05	0.39	0.29	0.39
iso-3-thujanol	1138	14.22	0.02	0.00	0.01	0.01	0.01
trans-pinocarveol	1140	14.32	0.02	0.00	0.01	0.01	0.01
camphor	1146	14.52	0.01	0.18	0.51	0.48	0.51
neo-3-thujanol	1155	14.80	0.01	0.00	0.11	0.09	0.11
borneol	1169	15.30	0.01	0.14	0.02	0.01	0.02
terpinen-4-ol	1181	15.74	0.05	0.00	0.00	0.00	0.00
α-terpineol	1198	16.31	0.04	0.00	0.01	0.01	0.01
myrtenol	1202	16.46	0.03	0.06	0.03	0.03	0.03
bornyl acetate	1289	19.41	0.02	0.04	0.04	0.03	0.04
α-campholenic acid	1346	21.28	0.02	0.29	0.10	0.09	0.10
acetophloroglucine*	1354	21.52	0.02	0.06	0.03	0.03	0.03
Sesquiterpenes				**0.71**	**0.18**	**0.16**	**0.71**
cis-α-bergamotene	1418	23.55	0.02	0.38	0.01	0.01	0.38
trans-caryophyllene	1422	23.68	0.02	0.16	0.07	0.05	0.16
α-humulene	1456	24.74	0.01	0.16	0.09	0.10	0.16
Oxygenated sesquiterpenes				**0.98**	**0.33**	**0.35**	**0.98**
caryophyllene oxide	1585	28.61	0.31	0.09	0.03	0.03	0.09
viridiflorol	1594	28.88	0.17	0.61	0.20	0.22	0.61
humulene epoxide I	1599	29.02	0.03	0.02	0.01	0.01	0.02
humulene epoxide II	1611	29.36	0.03	0.19	0.06	0.06	0.19
muurola-4.10(14)-dien-1-β-ol	1634	29.98	0.02	0.06	0.02	0.03	0.06
14-hydroxy-*cis*-caryophyllene	1660	30.69	0.02	0.02	0.01	0.01	0.02
Diterpenes				**4.25**	**1.33**	**0.94**	**4.25**
manool	2059	40.68	0.15	1.87	0.81	0.70	1.87
carnosol derivative*			0.15	2.17	0.45	0.24	2.17
trans-ferruginol	2330	46.56	0.15	0.21	0.07	0.00	0.21
Ester derivatives				**0.30**	**0.11**	**0.09**	**0.30**
methyl hexadecanoate	2096	41.53	0.01	0.15	0.04	0.03	0.15
methyl undecanoate	2103	41.68	0.01	0.02	0.01	0.01	0.02
methyl oleate	2130	42.28	0.01	0.12	0.05	0.04	0.12

methyl octadecanoate	2150	43.57	0.01	0.01	0.01	0.00	0.01
Waxes				**2.05**	**0.18**	**0.15**	**2.05**
heptacosane	2731	54.45	0.01	0.16	0.04	0.02	0.16
octacosane	2879	57.43	0.01	0.00	0.07	0.06	0.00
nonacosane	3031	60.40	0.03	0.88	0.03	0.01	0.88
untriacontane	3115	62.13	0.02	1.00	0.05	0.05	1.00
Triterpenes				**4.85**	**0.17**	**0.10**	**4.85**
t-sitosterol*	2812	56.10	0.01	0.18	0.02	0.01	0.18
olean-18-ene	2798	55.82	0.01	3.53	0.11	0.07	3.53
lupeol	2900	57.85	0.01	1.14	0.04	0.03	1.14
Total, g/100g of plant material				**14.21**	**4.65**	**3.92**	**15.50**

KI – Kovats index; RT/MS - retention time of corresponding constituent obtained by GC/MS; SD- standard deviation; * tentative identification.

Sage's extract mainly contain: monoterpenes (2.27-5.82%), oxygenated monoterpenes (29.06-45.08%), sesquiterpenes (3.67-5.48%), oxygenated sesquiterpenes (6.79-19.08%), diterpenes (25.19-29.85%), esters (2.23-5.36%), triterpenes (2.77-4.94%) and waxes (2.36-4.60%)(**Table 1**). The oxygenated monoterpenes were the most abundant in the SFE extract along with diterpenes and oxygenated sesquiterpenes, and the major compounds were manool (15.41-21.57%), β-thujone (7.95-16.56%), carnosol derivatives (6.25-13.09%), camphor (7.95-10.28 %) and viridoflorol (9.58-4.14%). In this study carnosol derivatives were identified instead of carnosic acid and carnosol which are commonly found in sage extract due to their thermal conversion during gas-chromatography analysis [18].

Based on these compositions and the values of the obtained extract yield, it was calculated and proposed the hypothetical composition of the plant material (**Table 2**).

Table 2. Composition of sage plant material

Components	**Composition in sage plant material** (mass% per dried plant)	
Other plant material (cellulose)		**53.1**
Humidity		**13.6**
Sugars		**26.0**
Chlorophyll		**2.4**
Extract (containing 0.7 mass% of essential oil)		**4.9**
	g in 100g of plant material	mass% in extract
Monoterpenes	0.09	1.74
Oxygenated monoterpenes	0.22	13.48
Sesquiterpenes	0.31	4.58
Oxygenated sesquiterpenes	0.66	6.32
Diterpenes	1.34	27.42
Ester derivatives	0.09	1.94
Waxes	0.65	13.23
Triterpenes	1.53	31.29
Total of terpeness	***4.90***	***100.00***
Total		**100**

3.1. Fractionation with supercritical CO_2 – experiment 1

Fractional extraction of sage enable concentration of lower molecular compounds (monoterpenes and oxygenated monoterpenes) followed by minimal co-extraction of higher molecular compounds. Supercritical extraction performed at 15 MPa followed by higher CO_2

consumption (>37 $kg_{CO2}/_{kgplant\ material}$) is favourable for concentrating the higher molecular compounds in extract. The average selectivity of diterpenes compared to heavier compounds (triterpenes, esters and waxes) was the highest at 15 MPa due to lower solubility of triterpenes, esters and waxes in CO_2 at that pressure.

The differential selectivity of diterpenes compared to monoterpenes and sesquiterpenes, as well as differential selectivity of diterpenes compared to heavier compounds (esters, triterpenes and waxes) was the highest at 15 MPa. It is result of a very small amount of other heavier compounds present in extract isolated using carbon dioxide at 15 MPa. Presence of the carnosol derivatives even in the first fractions collected at 15 MPa (**Figure 1**) can be explained by carnosic acid solubility in CO_2 even at lower pressure (lower CO_2 density) [1]. Namely, it was found that up to 30 MPa only carnosic acid is soluble in CO_2, while above 30 MPa solubility of carnosol starts to increase significantly as well.

This study indicated that lower CO_2 consumption (3-10 $kg_{CO2}/kg_{plant\ material}$) were favorable for the isolation of low molecular compounds (monoterpenes and oxygenated monoterpenes). A longer extraction time (a higher CO_2 consumption) seemed to be favorable for extraction of higher molecular compounds. This is a result of increased solubility of different compounds present in sage in supercritical CO_2 mainly at higher pressure and at the same time it is followed by decrease of CO_2 selectivity of diterpenes. However, the resistance for diffusion of heavier compound throughout the structure of plant material must be also taken into the account for detailed analysis of diterpenes extraction selectivity.

Using SFE fractionation it could be obtained the fraction of up to 62 mass% of diterpenes but the amount of obtained fraction is very small comparing to total supercritical extract (up to 2 mass%). That fraction could be obtained only at the end of the performed fractionation, after the pressure in extractor increase to 30 MPa and carnosol start to extracted (**Figure 1**, fraction 8).

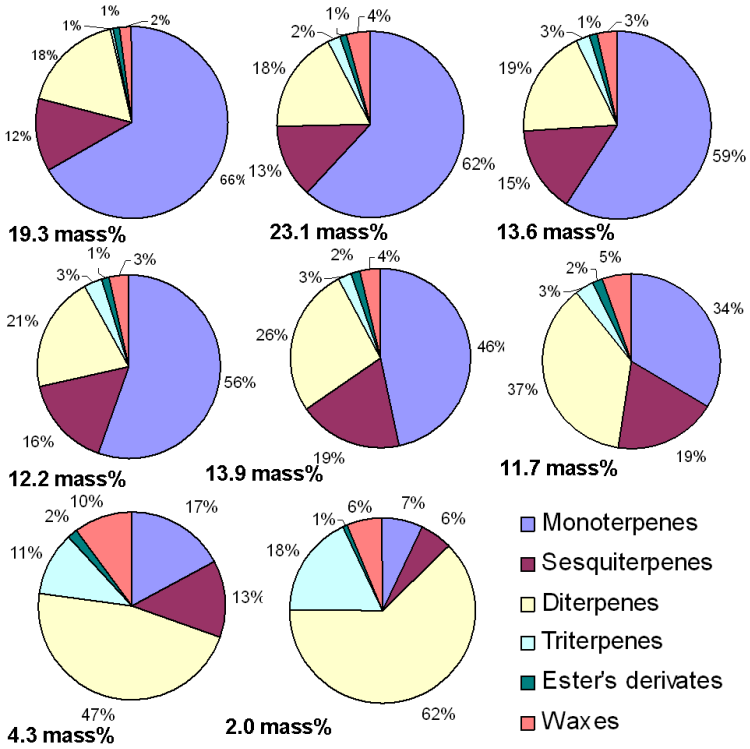

Figure 1. The chemical composition of obtained fraction during fractionation with CO_2 at 15 MPa and 50 °C

First four fractions (representing the 68.2 mass% of total extract) have the same composition and they contain mainly monoterpenes. Such composition profile was expected due to similar solubility of the components in supercritical CO_2. The fractionation with supercritical CO_2 could be used to concentrate diterpenes in comparison to diterpenes present in total extract as well as to the amount present in the plant material (**Table 1** and **2**). However, the mass of all collected fractions is only a smaller part (16 mass%) of the total extract.

3.2. Combined the ultrasound assisted solvent extraction and $SCCO_2$ fractionation

3.2.1. The re-extraction from the extract obtained by ethanol-water mixture and ultrasound – experiment 2

The ultrasound assisted extraction was performed at 40 °C using ethanol – water mixture (70:30) and 18 mass% yield of extract after 45 min of treatment was detected. Obtained extract was separated into four fractions (F1-F4) through re-extraction with supercritical CO_2 at 50 °C and 150 bars. The yield of deodorized sage re-extract was 6.0 mass% (with respect to mass of ultrasonically obtained extract) or 1.08 mass% with respect to initial mass of plant material used for combined process of ultrasound extraction - $SCCO_2$ re-extraction of extract. The experimental procedure was shown in **Fig.2**.

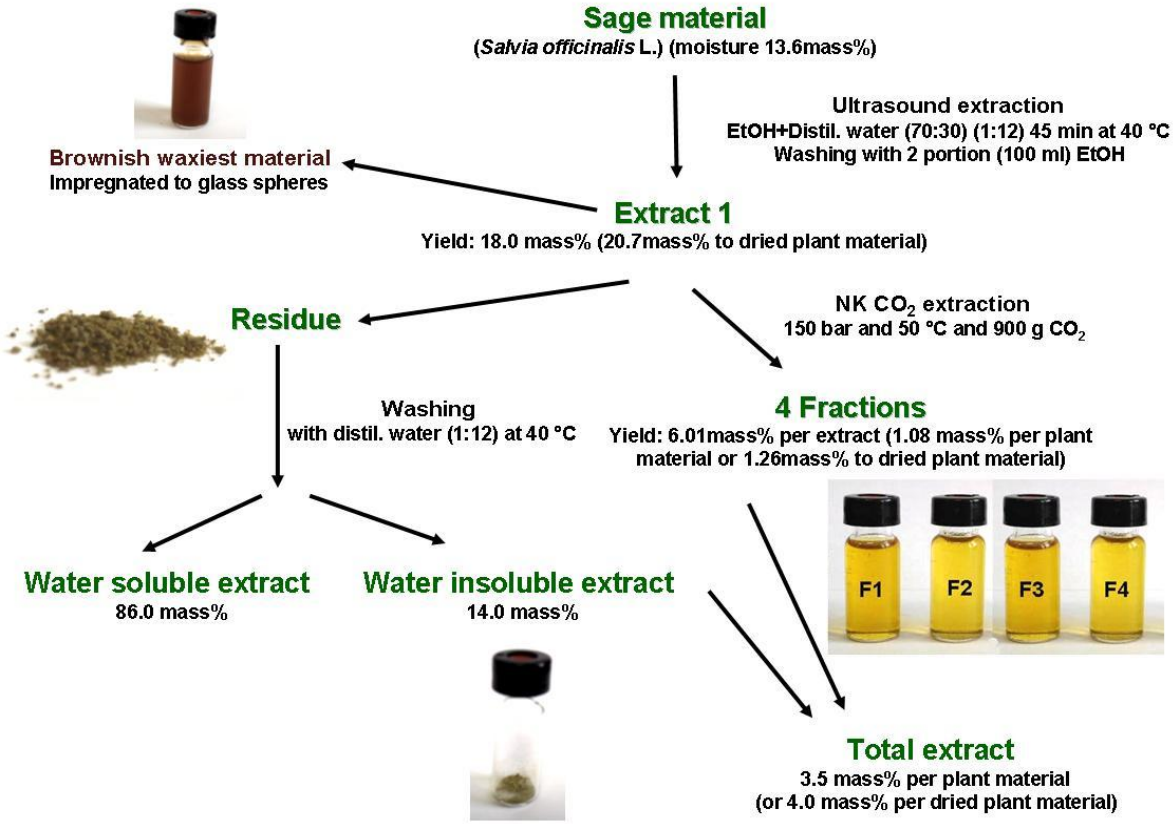

Figure 2. The schematic presentation of the experiment 2: ultrasound assisted water-ethanol extraction and re-extraction of the obtained extract at 15 MPa and 50 °C

The investigation of composition of F1-F4 fractions of sage re-extract was obtained by GC/FID and GC/MS analysis and the results are presented in **Table 2**.

Table 2. The chemical composition of the fractions, starting and end extract obtained in experiment 2

Components	**Composition,** mass%						
	Extract	**residue in extractor**	**total in fractions**	**Fractions**			
				F1	**F2**	**F3**	**F4**
Monoterpenes	0.25	0.25	0.96	0.60	0.55	0.50	0.25
Oxygenated monoterpenes	4.24	4.21	8.65	9.59	8.11	7.13	4.24
Sesquiterpenes	2.97	3.19	1.92	2.58	2.21	2.02	2.97
Oxygenated sesquiterpenes	6.90	6.48	20.19	21.13	18.42	16.95	6.9
Diterpenes	29.85	30.45	48.08	48.52	46.70	48.94	29.85
Ester derivates	2.02	1.48	12.50	9.42	13.20	14.28	2.02
Waxes	14.36	15.78	5.77	4.10	6.94	4.57	14.36
Triterpenes	34.02	34.02	1.92	0.00	0.21	2.56	34.02
Total, mass%	**94.61**	**94.61**	**100**	**95.93**	**96.34**	**96.94**	**94.61**
Total mass, g	**17.03**	**15.99**	**1.04**	**0.38**	**0.36**	**0.22**	**0.08**

Difference in composition profiles of F1-F4 fractions i.e. in total re-extract and extract obtained from sage (*Salvia officinalis* L.) performing only by supercritical CO_2 extraction at 50 °C and 150 bars seemed that combined technique is effective for diterpenes isolation. Namely, the sage re-extract had a higher content of diterpenes (about 61 mass%) in comparison to the extract obtained by hydrodistillation (1.5 mass%), Soxhlet extraction (about 30 mass%) or SFE extraction (30 mass%) and lower content of aromatic and volatile compounds compared to extract or fractions obtained by other extraction technique. The obtained small amount of extract was probably due to incorporation of extract to sugar crystals.

3.2.2. The re-extraction from the extract obtained by water and ultrasound extraction– experiment 3

Based on the previous results, the next experiment was performed with the aim firstly to remove the sugars from the extract. So, in the first step, the ultrasound assisted extraction was performed at 40 °C using distilled water giving 22.5 mass% of extract after 45 min of treatment. The treated plant material was further treated by ultrasound at 40 °C using ethanol – water mixture (70:30) and 6.3 mass% of extract after 45 min of treatment was obtained. After the evaporation in vacuum, this amount was re-extracted with supercritical CO_2 at 50 °C and 150 bars and three fractions was collected. The yield of deodorized sage re-extract was 19.9 mass% (with respect to mass of ultrasonically obtained extract, extract 2 from the **Fig.3**) or 1.25 mass% with respect to initial mass of plant material used for combined process of ultrasound extraction - $SCCO_2$ re-extraction. The detailed experimental procedure performed in this experiment was shown on **Fig.3**.

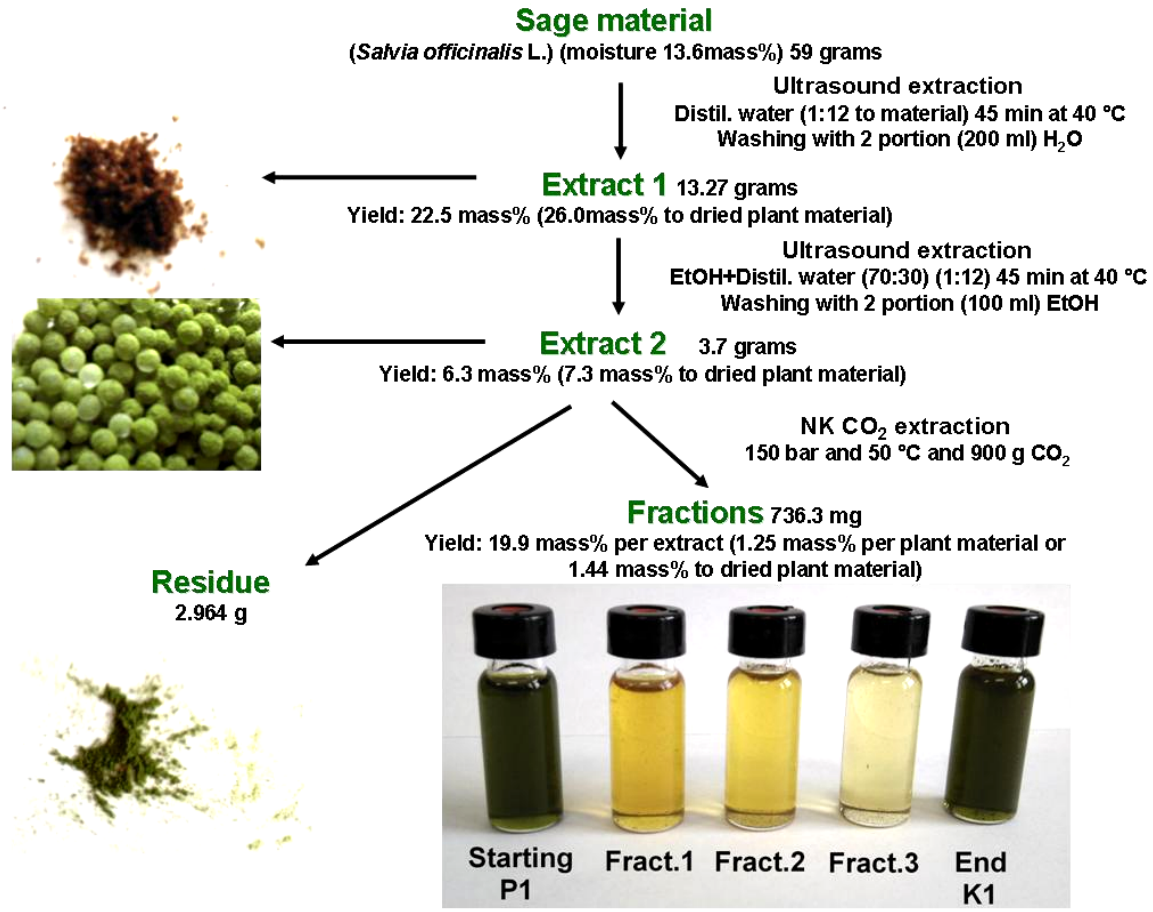

Figure 3. The schematic presentation of the experiment 3: ultrasound assisted water and water-ethanol extraction and re-extraction of the obtained extract at 15 MPa bar and 50 °C

The obtained results indicated that the removal of polysaccharides did not solve the problem, namely, still the almost complete extract remain in the residue and could not be re-extracted (only 33.3 mass% of total amount could be extracted using $SCCO_2$). These results are quit similar to the previous one (experiment 2); removed amount of polysaccharides increases the mass of supercritical extract for 15% but still the co-extracted material during ultrasound assisted treatment (probably chlorophyll, based on the extract color) unable a larger extraction of diterpenes. The composition of the obtained fraction are quite similar to the previous one and the concentration of the diterpenes could not exceed the 50 mass% in fractions.

3.2.3. The $SCCO_2$ extraction from the plant material previously treated with distilled water and ultrasound – experiment 4

The plant material was prepared before the $SCCO_2$ extraction according to the following procedure: the ultrasound assisted extraction was performed at 40 °C using distilled water with the aim to remove the water soluble components, mainly polysaccharides, from the plant material. The obtained yield of extract was 22.4 mass% after 45 min of ultrasound treatment. The insoluble components, i.e. valuable terpeness' extract remain in the plant material. The treated plant material was dried at the room temperature for the 12 hours and

remain humidity in the plant material was 41.3 mass%. Prepared plant material was extracted with supercritical CO_2 at 50 °C and 150 bars and the yield of sage extract was 2.11mass% calculated to the plant material or 3.59 mass% calculated to mass of dried plant material.

4. Discussion

The combination of extraction techniques is performed with the aim to enhance the yield of extract, as well as, to try to concentrate the diterpenes in the extract. Namely, it could be concluded that the $SCCO_2$ performed at 30 MPa and 50 °C yielded the maximum of the valuable extract from plant material [1]. The other techniques could extract light fractions only (yield 0.7 mass% of essential oil, hydrodistillation) or total/crude extract (yield 22 mass%, containing heavy components as a waxes, oleoresins, sugars, chlorophyll etc., Soxlet, ultrasonic assisted solvent extraction). Furthermore, detailed analyses of the chemical composition of some of extracts or their fractions shows that the complete valuable extract (rich in terpeness, oleoresins and esters) could be obtained only with $SCCO_2$ extraction.

The best operating conditions for obtaining the highest selectivity to diterpenes in extract is 15 MPa and 50 °C, yielding the 16% lower yield comparing to SFE at 30 MPa and 50 °C but with the concentration of the diterpenes of only 25 mass%.

The obtained extraction curves from the all performed experiments (1-4) in this study are presented at the **Fig.4.**.

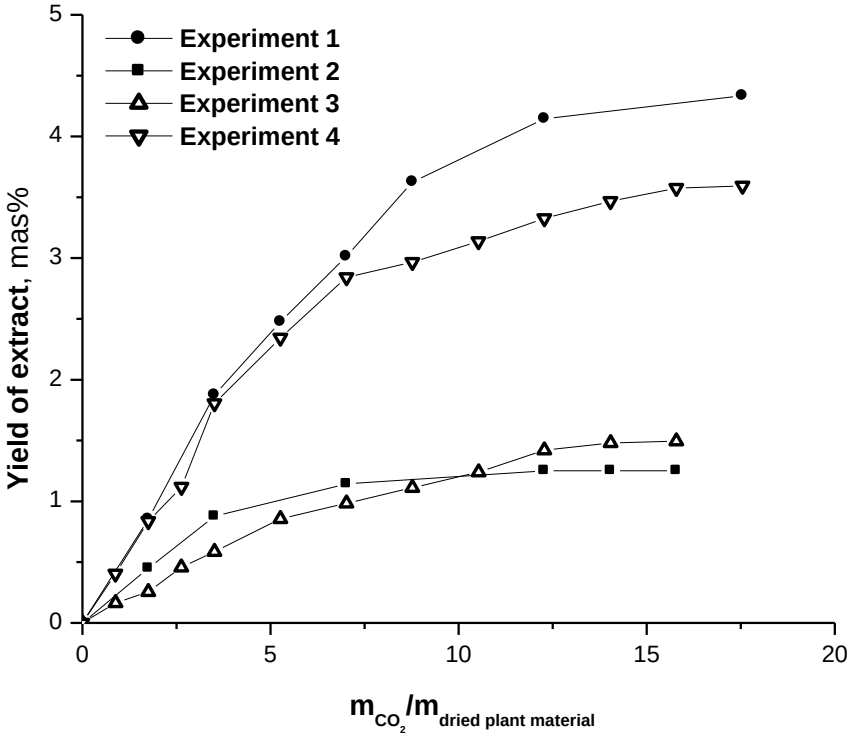

Figure 4. The extraction curves for all experiments (yield of extract was calculated to the mass of the dried plant material)

The fractionation with supercritical CO_2 (15 MPa and 50 °C) could concentrate diterpenes (up to 39.7 mass%) in fraction in comparison to 25 mass% to the total extract as well as to total composition in the plant material (**Table 1** and **2**), but the mass of fraction takes only 16 mass% of the total extract.

Using the combination techniques (experiment 2 and 3) it could be concentrate the diterpenes up to 50 mass% but the total mass of obtained fraction yielded maximum of the 33.3 mass% of the total possible extract (total extract obtained at 15 MPa and 50 °C). As could be seen, the re-extraction from the extract obtained with different solvents (ethanol-water (experiment 2), or water and further ethanol-water ultrasound extraction (experiment 3) are quite similar giving the similar composition of the obtained fractions. The $SCCO_2$ extraction from the plant material previously treated with distilled water and ultrasound (experiment 4) shows the extraction curve similar to extraction of non treated plant material in the first stage of extraction (up to consumption of 7 $g_{CO2}/g_{plant\ material}$). The further extraction yielded the 17.1% a lesser amount of extract. The explanation of such results could be probably attributed to the water extraction of manool (representing the 74.5mass% of total diterpenes, and 17.9 mass% to total extract). The further chemical composition analyzes of obtained fraction should confirm this conclusion.

Summarizing all obtained results and derived conclusions, it could be proposed the new extraction procedure of pretreatment of plant material with distilled water and further supercritical CO_2 extraction of plant material. Such procedure could give two valuable products.: a) water extract – rich in polysaccharides which possess the immunomodulatory activity [19], and b) supercritical extract – rich in diterpenes (up to 50 mass%).

4. Conclusion

The combination of different extraction techniques are performed in this study with the aim to concentrate the diterpenes in obtained extract. The fractionation using the $SCCO_2$, ultrasound assisted solvent extraction and re-extraction of obtained extract as well as, the ultrasound assisted solvent extraction and extraction of the plant material obtained after solvent extraction was analyzed and comparison are done. It could be concluded that combination of the pretreatment of plant material with distilled water and further extraction of plant material using the supercritical CO_2 could give two valuable products.

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References

[1] S. Glisiç, J. Ivanovic, M. Ristic, D. Skala, Extraction of Sage (Salvia officinalis L.) by Supercritical CO2: Kinetic Data, Chemical Composition and Selectivity of Diterpenes, The Journal of Supercritical Fluids, 52 (2010) 62–70.

[2] K. Miura, H. Kikuzaki, N. Nakatani, Antioxidant Activity of Chemical Components from Sage (*Salvia officinalis* L.) and Thyme (*Thymus vulgaris* L.) Measured by the Oil Stability Index Method, J. Agricultural Food Chemistry. 50 (2002) 1845-1851.

[3] A. Dapkevicius, R. Venskutonis, T. A van Beek, J. P H. Linssen, Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania, J. of The Science of Food and Agriculture. 77 (1998) 140-146.

[4] Z. Đarmati, R. M. Jankov, E. Schwirtlic, B. Đulinac, A. Đorđević, High Antioxidant Activity of Extracts Obtained from Sage by Supercritical CO_2 Extraction, J. the American Oil Chemists' Society. 68 (1991) 731-734.

[5] B. Bozin, N. Mimica-Dukic, I. Samojlik, A. Jovin, Antimicrobial and Antioxidant Properties of Rosemary and Sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L.. Lamiaceae) Essential Oils, J. Agricultural Food Chemistry. 55 (2007) 7879–7885.
[6] S. Fellah, P. N. Diouf, M. Petrissans, D. Barth, M. Romdhane, D. Perrin, M. Abderrabba, Supercritical CO_2, hydrodistillation extractions of *Salvia officinalis* L. Influence of extraction process on antioxidant properties, 10th European Meeting on Supercritical Fluids. Reactions. Materials and Natural Products. Strasbourg/Colmar (France), 12-14 December. 2005. Natural Products Processing N17
[7] El A. Hayouni, I. Chraief, M. Abedrabba, M. Bouix, J. Y. Leveau, H. Mohammed, M. Hamdi, Tunisian *Salvia officinalis* L. and *Schinus molle* L. essential oils: Their chemical compositions and their preservative effects against Salmonella inoculated in minced beef meat**,** Int. J. Food Microbiology 125 (2008) 242–251.
[8] S. Weckesser, K. Engel, B. Simon-Haarhaus, A. Wittmer, K. Pelz, C. M. Schemppa, Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance, Phytomedicine 14 (2007) 508–516.
[9] A. Menaker, M. Kravets, M. Koel, A. Orav, Identification and characterization of supercritical fluid extracts from herbs, Comptes Rendus Chimie 7 (2004) 629–633.
[10] H.J.D. Dorman, A. Peltoketo, R. Hiltunena, M.J. Tikkanen, Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs, Food Chemistry 83 (2003) 255–262.
[11] M. Ollanketo, A. Peltoketo, K. Hartonen, R. Hiltunen, M. L. Riekkola, Extraction of sage (*Salvia officinalis* L.) by pressurized hot water and conventional methods: antioxidant activity of the extracts, European Food Research and Technology 215 (2002) 158-163.
[12] D.T. Veličković, D.M. Milenović, M. S. Ristić, V. B. Veljković, Kinetics of ultrasonic extraction of extractive substances from garden (*Salvia officinalis* L.) and glutinous (*Salvia glutinosa* L.) sage, Ultrasonics Sonochemistry 13 (2006) 150–156.
[13] M. Sališova, Š. Toma, T. J. Mason, Comparison of conventional and ultrasonically assisted extractions of pharmaceutically active compounds from *Salvia officinalis*, Ultrasonics Sonochemistry 4 (1997) 131-134
[14] N. E. Durling, O. J. Catchpole, J. B. Grey, R. F. Webby, K. A Mitchell, L.Yeap Foo, N. B. Perry, Extraction of phenolics and essential oil from dried sage (*Salvia officinalis*) using ethanol–water mixtures, Food Chemistry 101 (2007) 1417–1424.
[15] S. Glisic, A. Smelcerovic, S. Zuehlke, M. Spiteller, D. Skala, Extraction of hyperforin and adhyperforin from St. John's Wort (Hypericum perforatum L.) by supercritical carbon dioxide, J. Supercritical Fluids 45 (2008) 332-337.
[16] Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.), National Institute of Standards and Technology (NIST), Standard Reference Data Program, Gaithersburg, MD (USA)
[17] R.P. Adams (2007): Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry, 4th Ed., Allured Publishing Corporation, Carol Stream, Illinois, USA.
[18] M.A. Thorsen and K.S. Hildebrandt, Quantitative determination of phenolic diterpenes in rosemary extracts – aspects of accurate quantification, J. Chromatography A 995 (2003) 119–125.
[19] P. Capek and V. Hribalova, Water-soluble polysaccharides fro *Salvia officinalis* L. possessing immunomodulatory activity, Phytochemistry 65 (2004) 1983-1992.