Rapid screening of antioxidant activity of sage (*Salvia officinalis* L.) extracts obtained by supercritical carbon dioxide at different extraction conditions

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Sage herb (*Salvia officinalis* L.) was extracted at supercritical fluid extraction (SFE) conditions with carbon dioxide at different parameters and the extracts tested on their antioxidant activity (AA). SFE of sage herb at 35 MPa pressure was found to be an effective method to obtain pure extracts. The yields of the extracts were substantially increased by using 1% of entrainer solvent ethanol. The fractionation of sage extract was a complex procedure in terms of extract distribution between separators operating at various pressure and temperature conditions. It was also proved by testing the AA of the extracts in rapeseed oil. The effect of the extracts on the rapeseed oil weight gain varied in a wide range (from 'very low' to 'high') depending on the fractionation conditions. Preliminary results showed that to obtain more effective antioxidant fractions separation steps should be started at 10 MPa lower pressure than that used for the extraction.

1 Introduction

Since the prehistoric era, spices and herbs have been used not only for the flavouring of food but also for their antiseptic or other medicinal properties [1]. Although the presence of antioxidative substances has been reported in many Labiatae species the sage and rosemary are the most comprehensively investigated plants as a source for natural antioxidants [2–6]. Such antioxidatively active constituents as carnosic acid, carnosol, rozmarinic acid, rosmanol, epirosmanol and isorosmanol are the main compounds identified in sage and rosemary [6–9]. Some important phenolic glycosides have also been reported [10].

Natural antioxidants from rosemary and sage are usually obtained by conventional extraction with various solvents [11–14]. Further processes of purification and fractionation of the crude extracts are necessary to obtain colourless, odourless and tasteless products, which can be used as natural additives in food, cosmetics, drug and other applications [15].

Supercritical CO_2 extraction is considered as an alternative way to obtain antioxidants from plant material. This sophisticated technology provides solvent free extracts; the selectivity of the supercritical CO_2 can be changed to obtain the fractions consisting of desirable compound and/or their mixtures. However, only a few reports were published on the use of supercritical CO_2 to obtain natural antioxidants. For instance, *Lopez-Sebastian* et al. [16] applied SFE techniques for the deodorization of rosemary extracts; *Djarmati* et al. [15] reextracted antioxidants from ethanol extract of sage.

It has been reported that the solubility of synthetic and natural antioxidants in CO_2 is very low. For instance, one of the strongest antioxidant compound in various Labiatae family plants, carnosolic acid was found to be almost insoluble in supercritical CO_2 below 30 MPa [17], the solubility of synthetic gallates, ascorbic acid and ascorbyl palmitate was lower than 10^{-4} mol/fract at the pressure up to 25 MPa [18]. On the contrary, the essential oils, sage oil particularly, usually are soluble at pressures lower than 10 MPa at 40–55 °C; at 20 MPa larger quantities of higher molecular weight compounds are extracted [19, 20]. Thus, the fractional extraction at high pressures is very effective in obtaining carnosolic acid with a low content of undesirable compounds [21]. The US-patent [22] describes the possibility to fractionate spice and herb extracts into several fractions by the change of extraction pressure and temperature. Similar method describing isolation of antioxidants by SFE was also patented in USA in 1991 [23]. The extracts obtained in such way are physiologically safe, effective at low concentrations, oil-soluble, stable during processing, immediately available, they possess favourable price/performance ratio [24].

The goal of the present study was to prepare sage extracts with supercritical carbon dioxide at various pressure parameters (with and without entrainer solvent ethanol) and to determine the AA of the extracts and their fractions by the method of weight gain in rapeseed oil during storage.

2 Materials and methods

2.1 Materials

Sage herb (*Salvia officinalis* L.) was grown in 1998 in the collection of aromatic and medicinal plants of Kaunas Botanical Garden at Vytautas Magnus University (Lithuania). The plants were harvested during flowering period, dried at 30 °C in a ventilated drying oven ("Vasara", Utena, Lithuania) and stored in paper bags at ambient temperature protected against direct light until further analysis. The samples were ground in a hammer mill equipped with 0.8 mm sieve before extraction.

Carbon dioxide (99.99%) was purchased from AGA (Sweden), ethanol from Merck (Darmstadt, Germany), synthetic antioxidant 2,6-*di*-*tert*-butyl-4-methylphenol (BHT) from Aldrich-Chemie (Steinheim, Germany). Fresh, refined and deodorized rapeseed oil without any additives was kindly donated by the Joint Stock Company "Obelių Aliejus" (Lithuania). Some quality characteristics of rapeseed oil were as follows: peroxide value (PV) 2.88 meq/kg, erucic acid content 0.56%, linolenic acid content 9.8% and total content of natural tocopherols 769 mg/kg.

2.2 Extraction

A schematic diagram of experimental apparatus used in this study is shown in Figure 1. A Dosapro Milton Roy (France) pump Milroyal B-C was used for the extraction. For supercritical extraction 500 g of ground

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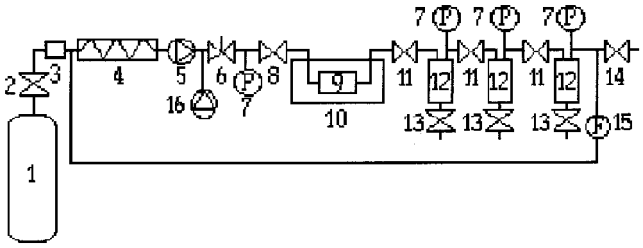

Figure 1. Schematic drawing of the supercritical extraction equipment: 1. Gas tube; 2. Shut-off valve; 3. Gas filter; 4. Ethanol bath –22 °C; 5. Pump; 6. Safe valve; 7. Pressure meters; 8. Relief valve; 9. Extractor; 10. Water bath; 11. Micro metering valve; 12. Separators; 13. Taken out valves; 14. Extra valve; 15. Flow meter; 16. Entrainer pump.

Table 1. Extraction conditions (the pressure in the second separator was 10 MPa, in the third one 5 MPa; temperature was 40 °C).

No.	Pressure [MPa]		Content of entrainer (ethanol) [%]
	Extraction	Separator 1	
1	35	30	–
2	35	25	–
3	35	30	1
4	35	25	1
5	35	20	1
6	35	25	2
7	25	20	1

herb were extracted in 1000 ml capacity vessel (covered by glass wool on the bottom and on the top) by CO_2 flow at the rate of 0.05 kg/min. The extraction was performed at 100 °C temperature and at 25 and 35 MPa pressure with or without adding 1% or 2% of ethanol as an extraction entrainer. The extracts were collected into the three separators, 200 ml capacity each. Different densities and temperatures were kept in the separators to obtain three extract fractions: 20, 25, 30 MPa pressure and 40 °C temperature in the first separator, 10 MPa pressure and 40 °C temperature in the second separator and 5 MPa pressure at 40 °C temperature in the third separator. Extraction and separation conditions are summarized in Table 1. The extraction was terminated after passing 10 kg of CO_2. The entrainer was removed at 40 °C in a Büchi rotavapor (Büchi, Donau, Switzerland) and the precision balances Mettler AE 163 weighed the remaining extract with a readability of 0.01 mg (Mettler Instrumente AG, Switzerland). Three replicates were extracted for each parameter set.

2.3 Assessment of antioxidant activity (AA)

AA was assessed by the rapeseed oil weight gain during storage. For this purpose the weight of 150 ml open beakers containing 25.00 g of oil was measured at timed periods during 9 days [25]. The extracts, 0.1% w/w each were dissolved in tested oils; blank sample was left without any additives. The sample with 0.02% of BHT was also tested for the comparison reasons. The extracts were incorporated into the oil by sonicator cell disruptor W-375 (Heat system, Ultrasonics, inc.) for 1 min. The samples were placed in KC-65 (PREMED, Poland) thermostat at 80 °C temperature protected against light [26–28]. The amount of oxygen consumed for oil oxidation reaction was calculated by the formulae:

$$Y\% = [M_2 - (M_1 - M_0)] / M_0 \times 100;$$

where: M_0 – weight of oil,
M_1 – weight of glass with fresh oil,
M_2 – weight of glass with stored oil.

The oxidation induction period was considered after the weight gain by 0.03% [29]. The protection factor (PF) was calculated by the formulae:

$$PF = IP_X / IP_K$$

where: IP_X – induction period of sample with additive [h];
IP_K – induction period of sample without additive [h].

Two replicates of each sample were analysed and the standard deviation was from 3 to 15%.

3 Results and discussion

3.1 The effect of extraction conditions on the extract yield

It has been reported that compounds with antioxidant properties are soluble in CO_2 at the pressures higher than 30 MPa at 100 °C, particularly when ethyl alcohol is used as a co-solvent [21, 23]. Therefore, the parameters defined in the above mentioned sources were considered as a target ones in our study. It was also assumed that the pressure in the first separator could not be lower than 20–25 MPa to prevent collecting of chlorophyll [30, 31]. The use of ethanol as an entrainer causes additional problems as regards the transferring of too high amount of chlorophyll into the extracts. The temperature of 40 °C in the separators was selected due to a good solubility of waxes and essential oil in CO_2 at the pressures higher than 20 MPa. Thus, by decreasing the pressure in the second separator to 10 MPa it was expected to separate chlorophyll and waxes, while further reduction in the third separator to 50 bar was applied to precipitate essential oil from CO_2 [31].

The results of sage extraction are present in Table 2. First (No. 1) and second (No. 2) experiments were performed at 35 MPa pressure without using co-solvent. The total yield of the extract in both experiments was close; however, redistribution of the fractions between separators was different depending on the pressure in the first separator. When the pressure in the first separator was reduced from 30 to 25 MPa the amount of the precipitate in it increased 6 times. It is interesting that the reduction of the pressure in the first separator completely changed the yields of the extracts in the second and third separators. Thus almost all extract (70%) was moved to the third separator when 30 MPa were maintained in the first separator, whereas approximately halve of it (45%) remained in the second separator, when the pressure in the first separator was reduced to 25 MPa.

The total yield of the extract was considerably increased after adding to CO_2 1% of a co-solvent (also called entrainer) ethanol (No. 3, 4 and 5). Further increase of the amount of the

Table 2. Effect of extraction and separation conditions by supercritical CO_2 on the yield of sage extract [g/100 g]; n = 3 (for extraction conditions see Table 1).

No	Separator 1	Separator 2	Separator 3	Total
1	0.31 ± 0.01	3.31 ± 0.25	8.53 ± 0.04	12.15 ± 0.10
2	1.93 ± 0.28	5.83 ± 0.62	4.98 ± 0.33	12.74 ± 1.23
3	0.24 ± 0.06	27.50 ± 2.96	18.52 ± 5.46	46.26 ± 8.48
4	20.35 ± 4.38	12.64 ± 3.08	11.01 ± 2.61	43.99 ± 10.07
5	28.74 ± 3.51	12.06 ± 0.10	3.35 ± 1.02	44.15 ± 4.63
6	25.43 ± 0.17	9.96 ± 2.82	3.99 ± 0.21	39.38 ± 3.20
7	16.01 ± 4.45	2.05 ± 0.56	2.75 ± 0.81	21.81 ± 5.82

ethanol to 2% was not efficient (No. 6), however high pressure in the extraction was crucial parameter. At the extraction pressure of 25 MPa (1% of the entrainer) the yield dropped approximately two times (No. 7). Three different pressures were maintained in the first separator in case of using 1% of the entrainer. It is reasonable that every pressure reduction step in the first separator resulted in higher extract yields in it. Again, very small amounts of the sage extractives precipitated at 30 MPa. The main part of the extract at these conditions was found in the second separator operating at 10 MPa. The decrease of the pressure in the first separator also caused in the reduction of the extract yield in the third separator. It is likely that distribution of the extracts between separators at different conditions also depends on the composition of the substances occurring in the extracts. The composition of the extracts carried to the second and third separator changes, because a higher amount of the initially extracted substances is precipitated in the first separator after decreasing of pressure.

The results clearly show that a great part of sage substances is soluble at 30 MPa and higher pressures. The pressure between 25 and 30 MPa can be considered as a critical one in terms of solubility of approximately 50% of sage extractives isolated at 35 MPa with CO_2 enriched by 1% of ethanol.

3.2 The effect of the extracts on the rapeseed oil oxidation

The sage extracts obtained from supercritical CO_2 extraction were examined in rapeseed oil to establish their antioxidant effect. Synthetic antioxidant BHT was used for comparison purposes. The experiment was carried out at 80 °C in an oven with active air circulation. The rate of hydroperoxide formation in lipids can be related to the amount of oxygen used in oxidation reaction and consequently to the oil weight gain at accelerated oxidation conditions. It should be said that BHT at the used experimental conditions was completely ineffective in terms of rapeseed oil oxidation induction period, which was almost the same in the oil with BHT and in the oil without additives. The activity of the extracts was ranked depending on PF into the following groups: 'very low' (PF = 1–1.5), 'low' (PF = 1.5–2), 'medium' (PF = 2–2.5), 'high' (PF = 2.5–3) and 'very high' (PF > 3) [32]. It was demonstrated that the antioxidant activity of the extracts produced by supercritical CO_2 depends on the extraction and separation conditions (Table 3). The pressure in the first separator was very important on the antioxidant activity of all sage extracts fractions. When this pressure was kept at 25 MPa the protector factors were highest for all sage extract fractions (antioxidant activity 'medium' or 'high'). The activity of the fractions obtained at 30 MPa in the first separator was 'very low' or 'low'. This, from the first sight, rather strange result can be preliminary explained by the following presumption. Sage extracts consist of a great number of substances, both possessing antioxidant and prooxidant activities. Also the compounds which do not interfere with lipid oxidation processes are isolated during SFE. All these constituents can interact between themselves thus changing the mechanisms of their effect on the oxidation of unsaturated fatty acids present in rapeseed oil. It is likely that pressure changes in the first separator can significantly change the distribution of sage extractives between all separators used and consequently to alter antioxidant activity of the chemical complex precipitated in one or another separator. The answer to this question can give comprehensive analysis if the composition of the fractions obtained at different conditions.

Table 3. Antioxidant activity of sage extracts obtained at different extraction conditions (n = 2) (for extraction conditions see Table 1).

No	Separator 1		Separator 2		Separator 3	
	IP [h]	PF	IP [h]	PF	IP [h]	PF
1	86	1.26	110	1.62	78	1.15
2	144	2.12	196	2.88	189	2.78
3	77	1.13	108	1.59	117	1.72
4	167	2.46	173	2.54	178	2.62
5	102	1.50	97	1.43	111	1.63
6	131	1.93	161	2.37	159	2.34
7	140	2.06	145	2.13	188	2.76

IP – Induction period; PF – Protector factor; IP of blank sample was 68 (PF = 1), IP of the sample with BHT was 67 (PF = 0.98).

The AA of the extracts obtained with 1% of the entrainer at 20 MPa in the first separator also was low, whereas the effectiveness of the extracts isolated at lower extraction pressure (25 MPa, 1% of ethanol) and at the same separation conditions were higher. For instance, the activity of the fraction precipitated in the third separator was 'high'. Comparing the activity of the extracts obtained in different separators it can be observed that in many cases more effective fractions were obtained in the second and in the third separators.

It is difficult to compare our results with previously reported [23] due to the differences in extraction, separation and testing conditions, however, it can be stated that the pressures above 30 MPa or use of a polar co-solvent are very important for the effective isolation of antioxidant substances.

4 Conclusion

SFE extraction of sage herb at 35 MPa pressure is an effective method to obtain pure extracts. The yields of the extracts can be substantially increased by using 1% of entrainer solvent ethanol. However, the fractionation of sage extract is a complex procedure in terms of extract distribution between separators operating at various pressure and temperature conditions. The testing of AA of the extracts in rapeseed oil also proved it. The effect of the extracts varied in a wide range (from 'very low' to 'high') depending on the fractionation conditions. Preliminary results show that to obtain more effective antioxidant fractions separation steps should be started at 10 MPa lower pressure than that used for the extraction.

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