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Use of Immobilised Lipase from *Candida antarctica* in Supercritical Fluid Extraction of Borage (*Borago officinalis* L.) Seed Oil

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Summary

This study aims at the investigation of the possibilities to use immobilised lipase from *Candida antarctica* in supercritical fluid extraction (SFE) of borage (*Borago officinalis* L.) seed oil. The first series of experiments was performed to measure the extract yields obtained with pure CO_2 and with the added entrainer (ethanol). The yield increased more than twice after increasing the extraction pressure from 15 to 25 MPa. Further increase to 35 MPa was less effective. The effect of the entrainer was not significant in most cases. Palmitic (13.1–16.1 %), oleic (13.4–23.8 %), linoleic (33.8–48.4 %) and linolenic (8.8–16.3 %) acids were dominant in all extracted oils. Further experiments involved the use of enzyme. In this case the first extractor was loaded with ground borage seeds, the second one was filled with the enzyme. The total yield obtained at 15, 25 and 35 MPa was (8.8±0.2), (23.6±0.2) and (28.9±1.1) %, respectively. Thin layer chromatography (TLC) of fatty acid ethyl esters showed that the content of esters was higher in the extract obtained in one extractor system at 15 MPa, compared to 35 MPa.

Key words: borage, *Candida antarctica*, supercritical CO_2 extraction, entrainer

Introduction

The excess fat intake can increase the risks of cardiovascular diseases, obesity, and certain types of cancer. Therefore, health-conscious individuals have consequently adjusted their dietary habits and consumed less fat. This trend has encouraged the food industry to develop structural modifications and substitutes of fats containing less energy and possessing the desirable functional properties. Structured lipids have gained attention as edible oil and for pharmaceutical purposes, and can be produced *via* enzymatic action (mostly *via* lipases), and/or chemical reactions (*1*). For instance, hydrolysis of oils using triacylglycerol lipases under mild conditions yields heat-labile polyunsaturated fatty acids, while esterification catalyzed by triacylglycerol lipases leads to products such as monoacylglycerols and a wide variety of esters (*2*).

Borage (*Borago officinalis* L.) seeds contain remarkable amount of γ-linolenic acid (GLA, 18:3ω6), which is not biosynthesised in human organism. Therefore, GLA isolation and enrichment are an important scientific and technological issue. Methods used for the isolation of GLA from natural sources include urea adduct formation (*3*), separation on Y-zeolite (*4*), solvent winterization (*5*) and lipase-catalysed reactions, such as selective hydrolysis of GLA-containing or selective esterification of GLA-containing fatty acid mixtures, derived from oils,

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with *n*-butanol (*6*,*7*). Selective hydrolysis of borage oil has been catalyzed by two lipase preparations of *Nigella sativa* L. seeds at 40 °C in a mixture of borage oil, water, and hexane; it has been shown that the relative inability of the applied lipase(s) to hydrolyze γ-linolenoyl moieties of TAG can be used for the enrichment of this acid in the unhydrolyzed acylglycerol fractions of GLA-containing oils (*8*). Oil containing 46 % GLA was produced by selective hydrolysis of borage oil (GLA content, 22 %) at 35 °C for 15 h in a mixture containing 50 % water and 20 U/g of the reaction mixture of *Candida rugosa* lipase (*9*). Finally, GLA was purified from borage oil by a two-step enzymatic method; first by hydrolysis of borage oil with lipase *Pseudomonas* sp. enzyme (GLA content, 22.5 %; recovery of GLA, 92.7 %); the second by selective esterification of borage free fatty (FFA) acids with lauryl alcohol by using *Rhizopus delemar* lipase (*10*).

To the best of our knowledge the use of lipase for enzymatic processing of borage seed oil has not been reported until now, while it has been recognised that the supercritical fluids (SF) offer few potential advantages for performing enzymatic reactions. In combination with an environmentally benign and safe medium, such as supercritical carbon dioxide (SC-CO_2), enzymatic catalysis makes supercritical fluids extremely attractive to the food industry (*11*). The main reason for the frequent use of lipase in SF is the increased solubility of hydrophobic lipid substrates in non-polar SF and the reversal of hydrolysis reactions in favour of synthesis such as esterification and transesterification. The separation and recovery of the enzyme catalysts after the reaction are possible due to their insolubility in nonpolar SF. Nonpolar substrates such as lipids are more soluble in dense gases than in aqueous solution. The following potential advantages of SF in performing enzymatic reactions should be mentioned: (*i*) flexible modification of solvent properties by adjusting pressure and/or temperature; (*ii*) the diffusivity of substrates is high and they can be removed from the reaction products by pressure and temperature reduction; (*iii*) critical temperature is sufficiently low for the processing of heat-labile materials and at the same time close to the preferable temperature for enzymatic reaction; (*iv*) the solvent can be readily recycled. SC-CO_2, being non-hazardous, inexpensive and possessing no waste-disposal problems, is an especially attractive solvent for enzyme-catalysed reactions (*12*).

Fats from oil-bearing seeds mainly consist of C16–C20 fatty acid triglycerides, which are fairly soluble in SC-CO_2 but far more so in short chain hydrocarbons, such as propane (*13*). A linear relationship between the logarithm of solubility and the logarithm of solvent density was obtained for *Echium*, borage, and *Lunaria* seed oils in compressed CO_2 (*14*). Adding a co-solvent to the SC-CO_2 can considerably enhance the solubility of lipids (*15*). For instance, the addition of caprylic acid methyl ester used as entrainer increased the yield of pure extract from borage seeds up to 47.8 times at 10 MPa and 2.4 times at 20 and 30 MPa (*16*). The esters are much more soluble in dense CO_2 as compared to free fatty acids (FFA) and therefore are more readily extracted (*17*).

This study is aimed at the investigation of the effect of the use of immobilised lipase from *Candida antarctica* in supercritical fluid extraction (SFE) of borage (*Borago officinalis* L.) seed oil. Some promising results with this enzyme were reported earlier in the SFE of cod liver oil (*18*). To our knowledge, the use of enzymes for the SFE of borage oils has not been previously reported.

Materials and Methods

Materials

The seeds of borage (*Borago officinalis* L.) were harvested from the experimental garden of Lithuanian Institute of Horticulture. They were dried at 30 °C in a ventilated drying oven and stored in paper bags at ambient temperature protected from light until further treatment. The samples were ground before extraction by Knifetec 1095 Sample Mill (Tecator AB, Höganäs, Sweden) for 20 s.

Immobilised non-specific lipase Novozym SP 435 L from *Candida antarctica*, supported on a macroporous acrylic resin, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The enzyme had a quoted activity of 5500 palm oil lipase units (PLU)/g. Other materials were carbon dioxide (99.99 %, Aga Gas, Sweden), hexane, diethyl ether, glacial acetic acid, ammonium sulphate, Silica Gel 60 plates (all from Merck, Darmstad, Germany), ethanol (99.5 %, Kemetyl, Sweden), 12 % boron trifluoride in methanol and 0.5 M sodium hydroxide in methanol (Acros, New Jersey, USA).

Apparatus and extraction

A Milroyal B-C pump (Dosapro Milton Roy, Pont-Saint-Pierre, France) was used in the extraction set (Fig. 1). The samples were covered with glass wool on the top and at the bottom of the vessel. The extraction was carried out at 40 °C and the pressure of 15, 25 and 35 MPa with or without the co-solvent.

In the first series of experiments 10 g of ground seeds were extracted in a 47-mL capacity vessel at CO_2 flow rate of 0.5 L/min (total amount of CO_2 was 100 L) without solvent circulation and the extracts were collected in the test tubes at –5 °C. In the second series 100 g of seeds were extracted in a 200-mL vessel (extractor 9 in Fig. 1) at the CO_2 flow rate of 0.02–0.025 kg/min (total amount was 10 kg) with solvent circulation. Ethanol was added into the system by the pump (16). Two 200-mL

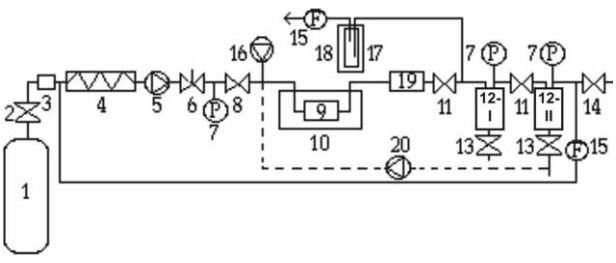

Fig. 1. Schematic drawing of the SFE equipment: 1 – gas container; 2 and 8 – shut-off valves; 3 – gas filter; 4 – ethanol bath (–22 °C); 5 – pump; 6 – safety valve; 7 – pressure meter; 9 – extractor; 10 – water bath; 11 – micrometering valve; 12 – separators (I on the left and II on the right); 13 – sampling valves; 14 – exhaust valve; 15 – flow meter; 16 – ethanol pump; 17 – test tube; 18 – cooling bath; 19 – enzyme container; 20 – extract pump

separators operating at 10 MPa and 40 °C (separator 12–I in Fig. 1) and 5 MPa and 40 °C (separator 12–II in Fig. 1) were used to separate the oil fraction from the entrainer. In the latter case the entrainer fraction from the second separator (12-II) was reused by the pump (20). The entrainer was removed from the extracts in a Büchi rotary vacuum evaporator (Büchi, Donau, Switzerland) and the remaining oil was weighed by the precision balances (Mettler AE 163, readability 0.01 mg, Mettler Instrumente AG, Greifensee, Switzerland). Two replications for each sample were performed and the results were expressed as a mean value.

Enzymatic treatment

The continuous lipase-catalysed reaction was performed as described elsewhere (*18*). The ratio of lipids and ethanol was 1 to 0.6. The enzymes were in the enzyme vessels (19) of 7 and 47 mL capacity, with 2-μm metal filters on the top and at the bottom, which were placed after the extraction vessels (9) of 47 and 200 mL capacity (Fig. 1). The amount of immobilised enzyme was 20 % of the seed mass.

Preparation of fatty acid methyl esters (FAME) and their analysis

The oil (150 μL) was first transesterified with boron trifluoride-methanol and 0.5 M methanolic sodium hydroxide, and then the fatty acid methyl esters (FAMEs) were extracted into hexane as described in AOAC method 969.33 as described by Staby and Mollerup (*17*).

FAMEs were analyzed on a Varian 3400 capillary gas chromatograph equipped with a fused silica capillary column, Supelcowax™ 10; 60 m, 0.32 mm i.d., 0.50 μm film thickness (Supelco, Bellefonte, PA, USA) and a flame ionization detector connected to a Vista 420 integrator (Varian Associates, Walnut Creek, CA, USA). The oven temperature was held at 180 °C for 8 min, then increased to 225 °C at 10 °C/min and held for 28 min. The temperature of the on-column injector was raised from 180 to 250 °C at 100 °C/min and kept at 250 °C for 30 min. The temperature of the detector was 250 °C. Helium was used as a carrier gas at a flow rate of 4 mL/min. FAMEs were identified by the comparison of their retention times with those of a reference solution analysed at identical GC conditions. Two replicate GC analyses were performed and the results were expressed in percentage of GC area as a mean value.

Thin layer chromatography (TLC) was used for the preliminary assessment of the content of various extracted lipid classes. The extracts, 20 mg of each, were dissolved in 1 mL of chloroform, and 5 μL of the obtained solution were applied on the silica gel plate. Solvent system consisting of hexane, diethyl ether and glacial acetic acid (80:20:1) was used to separate different lipid compounds, which were detected after the TLC separation by spraying with 20 % aqueous solution of ammonium sulphate and heating the plate at 180 °C for 20 min; the spots referring to different lipids were developed (*19*).

Results and Discussion

Effect of pressure and entrainer on the extract yield and fatty acid composition

The first series of experiments were carried out by extracting 10 g of borage seeds with 200 g (100 L at atmospheric pressure) of CO_2 without circulation in a 47-mL capacity extractor. The extracts were obtained at 15, 25 and 35 MPa pressure with pure CO_2 and by using ethanol as the entrainer. The main goal of these experiments was to obtain as high as possible extract yield with preferable ratio of lipids and ethanol (1 to 0.6) for the enzymatic reaction (*18*).

Table 1 provides the amounts of pure borage oil in the extracts after the removal of entrainer. It is evident that in all cases the yield of the extract increased more than twice after increasing the extraction pressure from 15 to 25 MPa. However, the increase was not so remarkable after further compression of CO_2 to 35 MPa (when 6 % of ethanol were added the yield even decreased). In our previously reported study the increase of CO_2 pressure from 10 to 35 MPa resulted in the increase in extract yield from 0.14 to 24.29 % (by mass) (*16*). It was also reported that the yields obtained with SC-CO_2 were very similar to those resulting from the conventional extraction process using hexane as a solvent (26.0 %). However, the quality of the oil extracted by supercritical fluid was higher: acidity 11.0 mg KOH/g oil, unsaponifiables 1.8 % (*20,21*).

Table 1. The yields of borage oil (%, by mass) obtained at different extraction conditions

Extraction conditions	Pressure/MPa		
	15	25	35
	Without enzymatic treatment		
CO_2	9.1±2.1	18.9±0.4	22.9±0.1
CO_2+3 % EtOH	11.0±2.6	26.4±2.9	25.5±0.8
CO_2+4 % EtOH	6.2±0.5	14.6±1.1	18.2±3.5
CO_2+5 % EtOH	8.0±0.1	16.8±1.3	20.5±2.9
CO_2+6 % EtOH	9.4±0.1	29.9±1.5	23.5±2.4
	With enzymatic treatment		
CO_2+4 % EtOH	8.8±0.2	–	–
CO_2+5 % EtOH	–	23.6±0.2	28.9±1.1

The effect of the entrainer in most cases was not significant and almost similar yields were obtained as compared to the extraction with pure CO_2. In general, the entrainer such as ethanol is used to increase the solubility of more polar compounds, which are usually present in plant material. Most likely, such compounds in borage seeds were in minor amounts and therefore ethanol effect on the total yield in this case was not significant. Taking into account the polar compounds, more detailed analysis of phenolics would possibly give some additional information of the effect of ethanol. As an exception, the use of 4 % of the entrainer at all pressures (the lowest yields) and 6 % of the entrainer at 25 MPa (the highest yield) should be mentioned. Most likely, the sol-

Table 2. Chemical composition of borage seed oil obtained at different extraction conditions, expressed in percentage of the total integrated area of GC peaks

Fatty acids	φ(added ethanol)/%														
	0			3			4			5			6		
	Pressure/MPa														
	15	25	35	15	25	35	15	25	35	15	25	35	15	25	35
16:0	14.9	13.1	13.3	14.1	13.3	13.2	16.0	16.1	13.3	15.4	14.0	13.5	15.0	14.2	13.5
18:0	4.5	6.8	2.8	5.4	4.2	4.4	4.9	5.7	5.4	4.2	4.3	5.0	4.5	4.7	3.2
18:1	23.1	23.4	23.8	17.4	16.5	16.0	18.8	20.5	16.0	18.6	19.9	17.5	17.5	15.4	13.4
18:2	35.3	33.8	36.8	37.0	38.4	40.3	35.2	38.9	37.6	40.1	39.4	37.4	38.8	40.6	48.4
18:3ω6	11.9	11.7	11.8	16.2	14.8	13.6	13.9	8.8	15.6	12.6	12.9	16.3	14.2	14.1	10.1
20:0	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.1
20:1	4.6	4.7	4.7	4.6	5.1	5.0	4.3	4.4	5.0	4.2	4.3	4.5	4.4	4.7	4.6
22:1	3.2	3.8	3.9	3.2	4.3	4.3	4.0	3.4	4.1	3.0	3.2	3.5	3.2	3.7	3.9
24:1	2.0	2.6	2.8	2.0	3.1	3.0	2.8	2.2	2.8	1.9	2.0	2.2	2.1	2.4	2.7

ubility of the borage extractives in two-solvent system at the above-mentioned conditions decreased; however, it is difficult to give a definite explanation on the basis of the results obtained within the scope of the present study. In our previous study with *Nigella damascena* seed we found significant increase of extract yield after adding 1 % of ethanol at all the extraction pressures applied (*22*).

The composition of fatty acids in borage seed oil obtained at different extraction conditions is presented in Table 2. The following fatty acids were dominant in all the oils: palmitic (13.1–16.1 %), oleic (13.4–23.8 %), linoleic (33.8–48.4 %), and linolenic (8.8–16.3 %). The content of longer chain unsaturated acids, C20:1, C22:1 and C24:1 constituted from 1.8 to 5.1 %. In previously published articles it had also been reported that the fatty acid composition of the borage seed oil extracted by SFE was rich in unsaturated fatty acids, especially γ-linolenic acid (21.7 %). For these reasons borage seed oil extracted with carbon dioxide could be competitive with the conventional process, since it simplifies considerably the oil refinement stages and completely eliminates solvent distillation stage, which is the most costly processing step in terms of energy consumption (*21*).

Some variations in the fatty acid composition at different pressures can be observed. The content of some individual fatty acids was possibly affected by the changes of the pressure and by the use of entrainer as well. However, some of these variations could also be caused by the insufficient separation of GC peaks, which in some cases was not good enough for the precise integration. For instance, the resolution of palmitic and stearic acid peaks was sufficient in all cases, so the variation in the content of these acids was not remarkable.

Extraction of borage seeds with the use of enzymes

It should be noted that immobilized lipase derived from *Candida antarctica* was successfully applied in some previous studies. For instance, it was reported that the activity of non-immobilized *Candida rugosa* lipase in SC-CO_2 decreased compared to atmospheric pressure, while the reaction rates of hydrolysis catalyzed by immobilized *Candida antarctica* lipase in SC-CO_2 were 1.5--fold higher than at atmospheric pressure (*23*). In another study, randomization of fats and oils was accomplished with an immobilized lipase in flowing SC-CO_2. Triglycerides adsorbed onto Celite were solubilized in CO_2 and carried over 1–10 g of immobilized lipase derived from *Candida antarctica*. The resultant randomized oil mixtures had properties such as solid fat index that make them potential candidates for incorporation into traditional margarine formulations (*11*).

The amounts of ethanol and lipids in the extracts were measured after the removal of carbon dioxide from the extracts. The ratio lipids/ethanol was calculated from the data obtained; the influence of the amount of entrainer in CO_2 on this ratio is presented in Table 3. The ratio was preferable for the enzymatic reactions (*18*) at the following extraction conditions: 4 % ethanol at 15 MPa; 5 % ethanol at 25 and 35 MPa.

Table 3. The influence of the amount of entrainer on the ratio lipids/ethanol in the extracts

Pressure / MPa	φ(added ethanol)/%			
	3	4	5	6
	ψ(lipids, ethanol)			
15	20.24	0.43	0.35	0.23
25	54.14	0.90	1.15	0.72
35	89.51	1.13	1.34	0.64

Other series of the experiments involved the use of enzymes. In this case the extractor (47 mL) was loaded with ground borage seeds, while the enzyme container (7 mL) was filled with enzyme (Fig. 1). Table 1 presents the amounts of the extract after enzymatic esterification, which were obtained only at the selected parameters, *i.e.* CO_2+4 % EtOH (15 MPa) and CO_2+5 % EtOH (25 and 35 MPa). The total yield obtained at 15, 25 and 35 MPa was (8.8±0.2), (23.6±0.2) and (28.9±1.1) % (by mass), respectively. All the yields were by 40 % higher than those obtained under the same conditions without the enzyme

treatment. This indicates that the lipase-catalysed esterification reaction took place during the applied process.

The composition of fatty acids in borage seed oil obtained by enzyme treatment and after methyl esterification is presented in Table 4. The amount of C16:0 fluctuated depending on the pressure from 13.4 to 17.2 %, while the amounts of C18:0, C18:1 and 18:3ω6 decreased from 5.5 to 4.3 %, from 41.2 to 36.9 % and from 18.0 to 9.7 % respectively, by increasing the pressure. The amount of polyunsaturated C18:2 increased from 10.0 to 22.9 % in the total fatty acid composition by increasing the pressure. The amount of some other compounds present at remarkably lower levels also increased by increasing the pressure.

Table 4. Composition of borage seed oil after enzymatic treatment, expressed in percentage of the total integrated area of GC peaks

Fatty acids	Pressure/MPa		
	15	25	35
16:0	15.0	17.2	13.4
18:0	5.5	5.5	4.3
18:1	41.2	38.2	36.9
18:2	10.0	18.8	22.9
18:3ω6	18.0	10.5	9.7
20:0	0.1	0.1	0.4
20:1	4.2	4.4	5.1
22:1	4.0	3.3	4.3
24:1	2.0	2.0	3.0

The presence of fatty acid ethyl esters (FAEE) in the extract was examined by thin layer chromatography (TLC) (Fig. 2). The largest spots of ethyl esters on the TLC plates were obtained from the extract isolated at 15 and 25 MPa, while at 35 MPa the same spot was smaller and less clear (sharp). However, the amount of extract obtained at the pressure of 15 MPa was not high. Therefore, the final experiment was carried out at the pressure of 25 MPa with 5 % of ethanol added as an entrainer. The extractions were performed in a 200-mL vessel containing 100 g of borage seeds (no. 9 in Fig. 1) and 47-mL vessel containing enzymes (no. 19 in Fig. 1). The amounts of extract after the extraction are presented in Table 5. The first extraction was accomplished without the use of enzyme in order to establish the influence of ethanol on

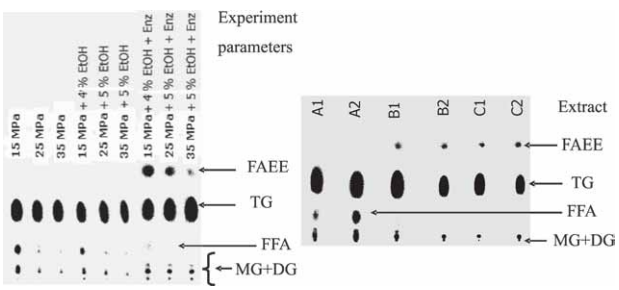

Fig. 2. TLC of borage seed extracts obtained at different extraction and separation parameters

Table 5. Influence of the extraction method on the yield of borage seeds oil (%, by mass)

Extraction method	Yield/%		
	Separator I	Separator II	Total
A – extraction without the use of enzyme	16.8±3.3	11.4±1.9	28.2±5.2
B – extraction with the use of enzyme	6.2±0.7	14.5±0.6	20.7±1.3
C – extraction with the use of enzyme and with recirculation of the obtained extract	13.1±0.5	16.4±0.6	29.6±1.1

the extract yield and distribution in the two separators (A-I – first separator, A-II – second separator). In this case higher amount of the extract was precipitated in the first separator (A-I). The amount of ethanol, measured after its removal in a vacuum drier, was 78.7 and 98.9 % in the first and second separator, respectively.

Next experiment was carried out under the same conditions but using enzymes (B-I – first separator, B-II – second separator). In this case the yield in the second separator (B-II) was 2.3 times higher compared to the first (B-I) one. However, the total yield was lower compared to the control extraction, without the use of enzyme. The content of ethanol in the extract before its drying was 95.0 and 94.3 % in the first and second separator, respectively.

The tendency of the yield to increase in the second separator (B-II) was used to calculate the output of the pump (20) in order to transfer the extract from the second separator back to the system to make it perform as an entrainer before the extractor (9) (Fig. 1). The pump (20) was disconnected from the second separator after passing 10 kg of CO_2; afterwards 2 kg of the solvent were additionally passed through the extraction vessel. The yield obtained (Table 5) from the first separator (C-I) by using this technique was (13.5±0.5) %, *i.e.* lower than in the case of extraction without the enzyme. However, the yield of dry extract collected in the second separator (C-II) was higher compared to the A-II. Consequently, the total extract yield obtained with enzymes in the recirculation system (C) was 29.6 %, *i.e.* higher by 5 % than in the extraction without the use of enzyme.

The composition of borage seed lipids (TLC) after using 200-mL vessel is presented in Fig. 2. The presence of free fatty acids in the extract from the second separator (A-II) obtained by using CO_2 with ethanol can be clearly observed. The extracts obtained from the first separator with the use of enzymes without extract circulation (B-I) contained some amount of ethyl esters, while remarkably lower amount of these esters, as it can be judged by the size of TLC spots, was present in the extract obtained by using re-circulation of ester fraction (C-I). The spots of ethyl esters in the second separator, both in the case of B-II and C-II, seem to be quite similar.

The chemical composition of extract fatty acids is presented in Table 6. The amounts of all fatty acids were almost similar in all extracts independently from their

Table 6. Influence of the extraction method on the composition of borage seed oil, expressed in percentage of the total integrated area of GC peaks

Fatty acids	A-I	A-II	B-I	B-II	C-I	C-II
16:0	12.9	13.8	11.5	14.0	12.8	13.6
18:0	4.9	4.2	4.9	5.0	4.0	3.4
18:1	16.6	8.8	11.1	10.6	10.1	19.0
18:2	37.3	49.2	49.1	41.5	50.2	40.9
18:3ω6	15.0	11.7	11.1	15.8	10.4	11.0
20:0	0.1	0.2	0.1	0.2	0.1	0.1
20:1	5.1	4.9	4.8	5.0	4.9	4.8
22:1	4.6	4.2	4.3	4.5	4.4	4.2
24:1	3.4	3.0	3.1	3.3	3.2	3.0

isolation method except for C18:1–18:3. The percentage of oleic acid (C18:1) was higher in the first separator (up to 16.6 % of the total area integrated by GC) than in the second one (8.8 %) when using CO_2 with ethanol. When the enzymes are used, the amount of oleic acid in the first separator decreased to 11.1 % in the extraction system without circulation and to 10.1 % in the system with recirculation of the extract collected in the second separator. The amount of C18:1 increased to 10.6 and to 19.0 %, respectively. It is interesting to note that in the case of using the enzyme the percentage of linoleic acid (C18:2) was higher in the first separator than in the second one. On the contrary, when the enzyme was not applied, the concentration of this acid was higher in the second separator. The opposite tendency was observed for linolenic acid (18:3ω6); its percentage was higher in the second separator for the system with the use of enzymes (B, C), while in the case of the extraction with CO_2 and ethanol the content of linolenic acid was higher in the first separator.

In fact, the extraction of borage seeds with SC-CO_2 and polar entrainer (ethanol) represents a dynamic system of three main substances, namely fluid carbon dioxide, ethanol and borage oil. The system also contains some minor components such as free fatty acids, mono-, diacylglycerols and other borage seed extractives. When the enzymatic reactor is included into the system, it becomes even more complex as a result of the formation of hydrolysis and esterification products. The effects of these factors were demonstrated by the redistribution of the extract components, particularly in terms of fatty acid composition between separators operating under supercritical and subcritical conditions. Further studies should be focused on measuring exact concentrations of different products obtained during enzymatic treatment under supercritical conditions to proceed with further process optimisation, *e.g.* with the aim to produce lipid compositions with specified structural and functional properties.

Conclusions

In general, the introduction of immobilised non-specific lipase from *Candida antarctica* into the extraction of borage seeds provides some possibility to increase the extract yield by the action of recirculating in the system the fatty acid ethyl esters produced in the course of the process. These esters may play a role of an additional extraction co-solvent. However, manipulation of process parameters and solvent composition as well as enzymatic treatment did not have remarkable effect on the distribution of fatty acids in the extracts obtained at different parameters.

Moreover, the TLC results suggest (Fig. 2) that the formation of ethyl esters was more intensive at the pressure of 15 MPa as compared to higher pressures. Consequently, it can be expected that the increase in oil yield should be more considerable in the extraction carried out at 15 MPa; however, the efficiency of the extraction of oil increased more remarkably with the increase of pressure (Table 1). As a possible explanation the effect of the two main factors, namely the presence of esters and the extraction pressure, as well as of some other factors should be considered. Most likely, the effect of the pressure was prevalent compared to the effect of the produced esters. Also, the formation of a multicomponent system, carbon dioxide/seed extractives/ethanol, should be taken into account; it cannot be excluded that even slight changes in the system composition can exert some effect on its solvation properties, and the effect of the entrainer (in this case produced esters) is not necessarily linear. Therefore, it can be suggested that at significantly higher pressures even smaller concentrations of the entrainer can possess more remarkable effect on the extract yield. Further research involving a number of model systems is required to prove or disprove this hypothesis.

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