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HPLC in reversed phase mode: Tool for investigation of kinetics of blackcurrant seed oil lipolysis in supercritical carbon dioxide☆

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Abstract

Blackcurrant (*Ribes nigrum*) seed oil is rich in α- and γ-linolenic acids, the latter in particular being of potential use in medicine. The enzymatic hydrolysis of the oil was carried out in supercritical carbon dioxide using lipase Lipozyme as catalyst and changes in the composition of acylglycerols were recorded. Mono-, di-, and triacylglycerols and free fatty acids were separated by non-aqueous high-performance liquid chromatography in reversed phase mode and detected by UV diode array and ^{1}H NMR detectors. Lipozyme was found to exert low specificity to individual fatty acids in the hydrolysed oil.

Keywords: RP-HPLC; NMR; Oil hydrolysis; Supercritical carbon dioxide; Enzyme

1. Introduction

Blackcurrant seed oil (BCO) is rich in essential fatty acids α- and γ-linolenic acids, the compounds that play an important role in human metabolism. The acids are present in the oil in approximately equal amounts. They belong to different fatty acid groups that are not interconvertible in humans, display different physiological functions and act in concert with one another to regulate biological process. One of the possibilities to concentrate them in the mixture of fatty acids is via the oil reaction, like hydrolysis or ethanolysis, catalysed by an enzyme that is specific to some of the acids in the mixture [1]. Supercritical carbon dioxide is a suitable reaction medium, as it allows to combine the advantages of biocatalysts and supercritical fluids, namely high mass-transfer rate, the easy separation of reaction products from the solvent. The product of these reactions is a mixture of triacylglycerols (TAGs), diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FAs), glycerol, and, in the case of ethanolysis, fatty acid ethyl esters.

Besides gas chromatography, different types of high performance liquid chromatography (HPLC) were applied convenient as analytical and separation technique. Among different methods of HPLC in normal phase mode, argentation chromatography [2] was found to be efficient isomers separation technique. It separates TAGs according to the degree of their unsaturation, unlike reversed-phase HPLC (RP-HPLC) where the separation is the more complex, depending on the chain length of the constituent FAs and the degree of unsaturation. The separation of acylglycerols was achieved with the use of non-aqueous RP-HPLC with methanol–2-propanol–hexane [3], dichloromethane–acetonitrile [4,5], dichloromethane–acetonitrile–propionitrile [6,7], pure propionitrile [8], acetone–acetonitrile, chloroform–acetonitrile [9], or methanol–2-propanol [10].

Successful resolution of free fatty acids and acylglycerols was effected by an aqueous gradient elution system acetonitrile–water combined with non-aqueous 2-propanol–hexane mobile phase [3] and gradient elution system acetonitrile–water combined with 2-propanol–acetonitrile [11].

It is well documented [12,13] that the RP-HPLC separated TAGs in descending order of the combined number of acylcarbons (CN), and each of the double bonds (DB) in the acyl chains reduces the retention of the compound. The equivalent carbon

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numbers (ECN) help to identify TAGs peaks separated:

$$ECN = CN - 2DB$$

TAGs in *Ribes nigrum* were separated by RP-HPLC with particular respect to separation and identification of the TAGs containing γ- and α-linolenic acids [12,14].

In this study, changes in the composition of acylglycerols in the hydrolysed blackcurrant seed oil were investigated by non-aqueous HPLC in reversed phase mode (RP-HPLC) using analytical column with triacontylsilyl silica phase. The enzymatic hydrolysis was carried out in supercritical carbon dioxide (SC-CO_2) medium with lipase Lipozyme as biocatalyst.

2. Experimental

2.1. Chemicals and materials

Methanol Super Gradient HPLC, Anasolv™ (Analytika s.r.o., CR); *tert*-butyl methyl ether 99.8% HPLC grade, Sigma–Aldrich (Steinheim, Germany); chloroform-*d*, Eurisotop (France); acetonitrile HPLC grade, Fluka Riedel-deHaën (Schweiz).

Standards: glyceryl trilinoleate, glyceryl trilinolenate, glyceryl trioleate, 1,2-dilinoleoyl-3-oleoyl-rac-glycerol, 1,3-dilinoleoyl-rac-glycerol, 1-monolinoleoyl-rac-glycerol, γ-linolenic acid, α-linolenic acid, linoleic acid, oleic acid, palmitic acid, stearic acid; all standards were purchased from Sigma (St. Louis, MO, USA).

Enzyme Lipozyme® from *Mucor miehei*, immobilised, supplied by Sigma–Aldrich (Steinheim, Germany).

Black currant (*R. nigrum*) seeds were supplied by a cooperative farm at Chelcice (Czech Republic). The oil was extracted from the ground seeds with supercritical carbon dioxide at 28 MPa and 45 °C, dissolved in light petroleum/acetone mixture and subjected to column chromatography on silica gel; the main fraction consisting of triacylglycerols was used as a substrate in enzymatic hydrolysis. Table 1 presents the fatty acid composition in the main fraction [1].

2.2. Instrumentation

The HPLC system consists of the HP 1090 M chromatograph, the ultraviolet diode array detector and refractometer (Hewlett-Packard, Waldbron, Germany). Separations were achieved using the analytical column 250 mm × 4 mm I.D., spherical 5 μm particles with triacontylsilyl silica phase (C_{30}) Develosil (Nomura Chemical Co. Ltd., Japan) and security guard 3 mm × 4 mm I.D., C_{30} Develosil.

The LC-NMR set consists of the NMR Spectrometer (Varian Inova 500, USA) and the Varian LC Star module controlled by Star Chromatography Work station 2. Two analytical columns are used in series, namely 150 mm × 4.6 mm I.D., C_8 Luna 100 5 μm and 250 mm × 4.6 mm I.D., C_{18} Luna 100 5 μm (both Phenomenex USA).

Table 1
Fatty acid composition of blackcurrant seed oil

CN:DB	Fatty acid	Abbreviation	Composition (wt.%)
16:0	Palmitic acid	P	5.66
18:0	Stearic acid	S	1.38
18:1n-9	Oleic acid	O	10.75
18:2n-6	Linoleic acid	L	45.81
18:3n-3	α-Linolenic acid	Ln	13.87
18:3n-6	γ-Linolenic acid	Ln	14.30
18:4n-3	Stearidonic acid	Sn	2.86
	Others		5.37
Sum			100.00

Table 2
Variable reaction conditions: amount of enzyme in the reactor (N_e) and flow rate of CO_2 containing substrates (Q)

Experiment	Ne (mg)	Q (l/min)
HL-1	27.8	0.3
HL-2	54.3	0.6
HL-3	54.3	0.5
HL-4	54.3	0.7
HL-6	75.0	0.3

2.3. Sample preparation

The equipment for enzymatic hydrolysis consists of a high-pressure syringe pump for CO_2 (ISCO 260, USA), three HPLC columns of 8 mm I.D. and volume of 4–12 ml (Tessek Ltd., Czech Republic), a home-made heated micrometer valve, gas meter and a water bath. Carbon dioxide was pumped under constant pressure into the first 12-ml column filled with inert glass beads and water where it was saturated with water, to the second 12-ml column filled with glass beads and the blackcurrant oil where it was saturated with oil, and finally the third, 4-ml column, filled with enzyme and glass beads, which serves as the reactor. Both the second and the third column were immersed in the water bath heated to the reaction temperature. The solution flow rate was controlled by the micrometer valve at the reactor outlet where the solution expanded to ambient pressure. The precipitating reaction mixture of TAGs, DAGs, MAGs, and FAs was collected in a vial and the gaseous CO_2 flowed through the gas meter and was vented. The vials with obtained hydrolysates were stored in the refrigerator for further analysis.

The lipase Lipozyme was selected as immobilized catalyst because we observed in previous study [1] that this enzyme is specific towards linolenic acids, the components of interest. The reaction pressure was 15 MPa and the temperature was 40 °C throughout the experiments; the varied reaction conditions were the solvent flow rate and the amount of enzyme in the reactor. Their values are listed in Table 2; the CO_2 flow rate was measured at ambient conditions.

2.4. Methods

2.4.1. Method 1

The separation of TAGs, DAGs and MAGs was performed in a non-aqueous HPLC system in reversed phase mode on the

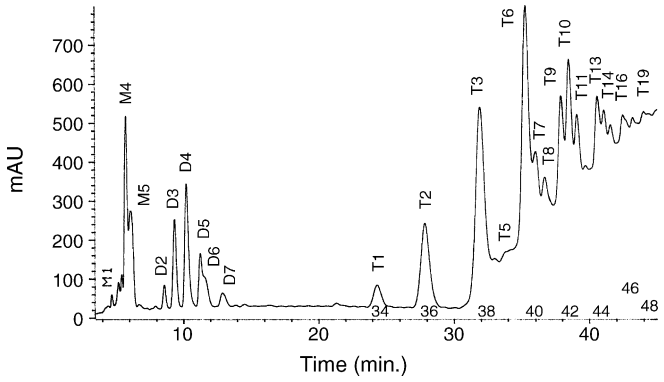

Fig. 1. RP-HPLC separation of blackcurrant seed oil acylglycerols after enzymatic hydrolysis. Column 250 mm × 4 mm I.D. C30 Develosil 100 5 μm, security guard 3 mm × 4 mm I.D. C30 Develosil. Mobile phase methyl-*tert*-butylether–methanol in a gradient mode (see Section 2); flow-rate of 0.5 ml/min; UV detection at 210 nm. Peaks: Mn, monoacylglycerols; Dn, diacylglycerols and Tn, triacylglycerols. Peak identification in Table 3. Numbers under the chromatogram line correspond to equivalent carbon numbers (ECN).

HPLC system with multi-stage non-linear gradient elution. The chromatographic conditions were as follows: ambient temperature about 20 °C, flow rate of the mobile phase 0.5 ml/min and UV detection at λ = 210 nm. The external standard method was applied for identification and quantification of the most important triacylglycerols (peaks Tn in chromatogram), diacylglycerols (peaks Dn in chromatogram) and monoacylglycerols (peaks Mn in chromatogram) (Fig. 1). Calibration graphs were obtained by plotting the chromatographic peak areas (or peak heights, if necessary) against the concentrations of 1,3-dilinoleoyl-rac-glycerol, 1,2-dilinoleoyl-3-oleoyl-rac-glycerol, glyceryl trilinoleate and glyceryl trilinolenate.

Standards and samples of the hydrolysates were dissolved in the mixture of components of mobile phase methanol–*tert*-butyl methyl ether 1:1 (v/v) at a concentration of 10–60 mg/ml. These solutions in amount of 5 μl were applied on the HPLC column.

The gradient elution was used. Solvent A: methanol, solvent B: *tert*-butyl methyl ether, A–B: 25 min 75:25 (v/v) isocratic, gradient 15 min to 50:50 (v/v), 5 min 50:50 (v/v) isocratic.

The separation of MAGs, DAGs and TAGs was finished after 45 min and then the column was equilibrated under the start conditions for 10 min.

A good resolution for trilinolenin and the next TAG peak was achieved: $R_s = 3.68$.

2.4.2. Method 2

The chromatographic conditions for the separation of free fatty acids (FAs) were as follows: two analytical columns C_8 and C_{18} in tandem mode, the mobile phase consisting of acetonitrile–chloroform-*d*-mixture 90:10 (v/v), and the mobile phase flow rate 0.5 ml/min. The quantitative analysis was performed via on-flow ^{1}H NMR differentiating between their individual signals of CH_3 groups. The external standard method was applied for the quantification of the free FAs and the analysis was finished after 25 min. Standards of γ-linolenic acid, α-linolenic acid, linoleic acid, oleic acid, palmitic acid, and stearic acid were used in the calibration.

Table 3
Peak identification of acylglycerols analysed by non-aqueous RP-HPLC

Pn	CN:DB	Acylglycerol
D5		L L
T1	54:10	Ln Ln Sn(?)
T2	54:9	Ln Ln Ln
T3	54:8	L Ln Ln
T6	54:7	L L Ln
T7	54:7, 52:6	O Ln Ln + P Ln Ln(?)
T9	54:6	L L L
T10	54:6	O L Ln
T13	54:5	O L L
T16	54:4	O O L
T19	54:3	O O O + O L S(?)

Peak numbers (Pn) refer to those presented in the chromatogram in Fig. 1.

3. Results and discussion

3.1. HPLC

HPLC analysis was performed with the use of the C_{30} column, recommended by the manufacturer for the separation of compounds with long aliphatic chains, and of the recommended non-aqueous mobile phase, methanol–*tert*-butyl methyl ether. The separation of MAGs, DAGs and TAGs was better than the separation with stationary phase C_{18} and with conventional HPLC methods. The separation of LnLnLn containing α- and γ-linolenic acids was much better (peak T2 in Fig. 1). Five peaks of MAGs, seven peaks of DAGs and 22 peaks of TAGs were obtained. Most of the peaks were identified using available standards of TAGs and DAG, ECN numbers, and data on fatty oil composition in blackcurrant seed oil (Table 3).

The resolution of DAGs and important TAGs listed in Table 4 was calculated according to equation [15]

$$R_{i,j} = \frac{\sqrt{n}}{4}\left(\frac{r_{j,i}-1}{r_{j,i}+1}\right)\left(\frac{k}{k+1}\right)$$

where the capacity factor, k, and the number of theoretical plates, n, are arithmetical means of the values calculated separately for the component (peak) i next to the component j in the chromatogram, and $r_{j,i} = k_j/k_i$ is the retention ratio.

Table 5 shows the content of diacylglycerol D5 and triacylglycerol T13 in the hydrolysates and of T13 in the oil. It is

Table 4
Resolution ($R_{i,j}$) of diacylglycerols D1–D7 and triacylglycerols T2, T3, T5–T7, the peak of compound i next to the peak of compound j in the chromatogram, and number of theoretical plates of the column (n)

Compounds separated i, j	$R_{i,j}$	n
D1, D2	1.24	5132
D2, D3	1.43	6648
D3, D4	1.51	6328
D4, D5	1.48	5523
D5, D6	0.51	8591
D6, D7	2.31	9820
T2, T3	2.18	15369
T5, T6	1.46	18673
T6, T7	0.71	53998

Table 5
Content of dilinoleoyl glycerol (D5) and triacylglycerol OLL (T13) in the samples of hydrolysates and oil

Experiment	D5 (mg/g)	T13 (mg/g)
HL-1	28.4	82.8
HL-2	29.7	107.6
HL-3	22.2	104.2
HL-4	16.0	104.9
BCO	–	133.7

evident that the maximum conversion was achieved in the first and likely also the second run as judged from the concentration of D5. In the next two runs, the reaction rate decreased regardless of the residence time of the solution in the reactor, which was inversely proportional to the flow rate Q.

When, however, the enzyme activity after the first four runs was mesured with the use of standard method, outside the reactor at ambient pressure, it was even higher than the activity of fresh enzyme. Glycerol is in SC-CO_2 by order of magnitude less soluble than other reaction mixture components; it thus dissolves in SC-CO_2 only partially and accumulates in the reactor. Therefore, the most probable explanation for the decrease in reaction rate in SC-CO_2 medium is the gradual blocking of the immobilized enzyme by glycerol produced by hydrolysis.

3.2. LC-NMR

Using the HPLC method 1, the free FAs co-eluted with the smallest MAG (C11). Therefore, the content of the free FAs (α- and γ-Ln, L, O, P, and S) in the samples of the BSO hydrolysate was determined by the LC-NMR method, using the standards of six major fatty acids in the oil (Fig. 2). Table 6 shows the results of the analysis for experimental run HL-6 where the degree of conversion to free fatty acids was 79.7 wt.%. A small change is evident in the α-linolenic-to-γ-linolenic acid ratio, compared to the blackcurrant seed oil. It shows that the enzyme Lipozyme was slightly specific to the linolenic acids as the ester bond connecting γ-linolenic acid with the glycerol skeleton was hydrolysed more readily than the bond of α-linolenic acid.

Table 6
Content of the six major free fatty acids in hydrolysate HL-6 determined by LC-NMR (T_R: retention time)

T_R (min)	Fatty acid	Composition (wt.%)
16.1	Linoleic acid	51.0
20.1	Palmitic acid	6.5
20.1	Oleic acid	11.8
14.2	α-Linolenic acid	15.8
14.3	γ-Linolenic acid	13.3
24.7	Stearic acid	1.6
Sum		100.00

3.3. Kinetics of hydrolysis

Before the chromatographic methods were at disposal, our study of reaction kinetics was based on the results of colorimetric determination of the content of free fatty acids in the reaction mixture [16]. A simple model comprising the effects of reaction

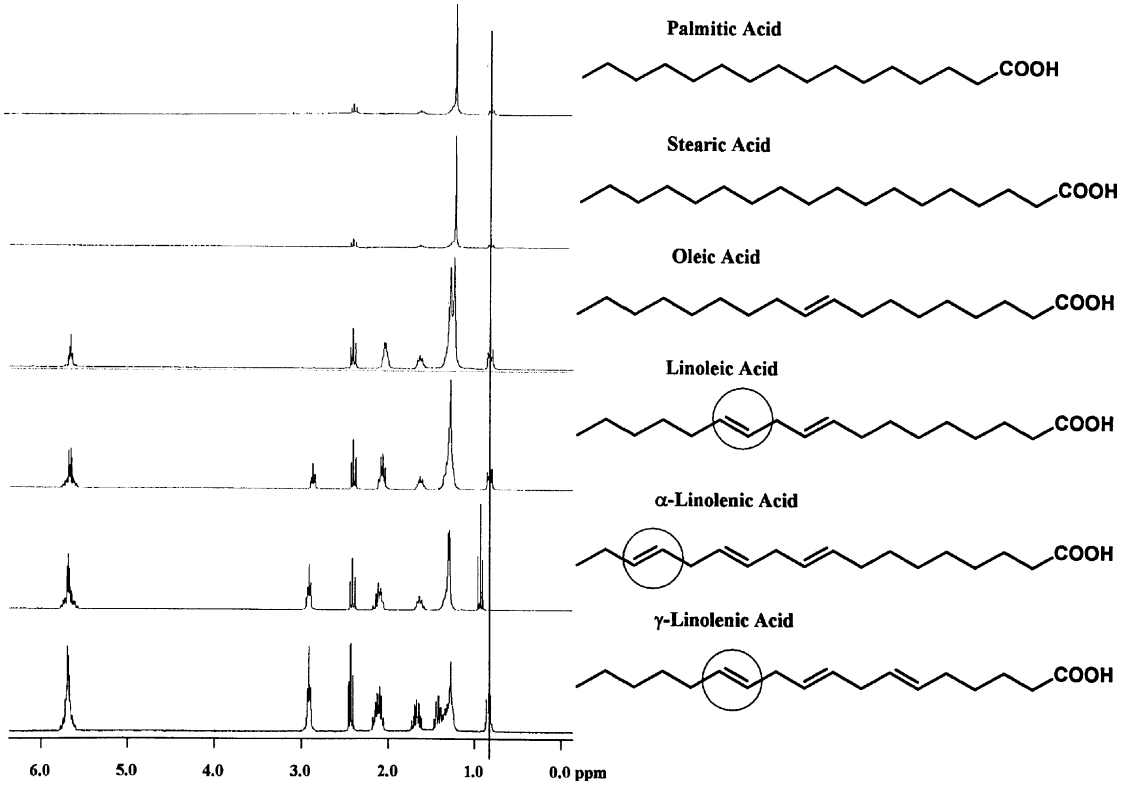

Fig. 2. ^{1}H NMR spectra of the free fatty acids standards.

kinetics and mass transfer rate in one parameter k_r was developed and will be described elsewhere. Its main features are the first-order kinetics (the amount of water in the reaction mixture is much higher than reaction stoichiometry requires), the reaction time t_h proportional to the product $t_d N_e$, where t_d is the residence time and N_e is the amount of enzyme in the reactor, and the rate constants proportional to the number of fatty acids bound on the glycerol backbone. Thus, the set of equations is

$$\frac{dc_1}{dt} = -r_1 c_1, \qquad \frac{dc_2}{dt} = r_1 c_1 - r_2 c_2,$$

$$\frac{dc_3}{dt} = r_2 c_2 - r_3 c_3, \qquad \frac{dc_4}{dt} = r_1 c_1 + r_2 c_2 + r_3 c_3,$$

$$\frac{dc_5}{dt} = r_3 c_3,$$

where c_1, c_2, c_3, c_4, and c_5 are the concentrations of TAGs, DAGs, MAGs, FAs, and glycerol, respectively, r_1, r_2, and r_3 ($r_1 = 3r_3$, $r_2 = 2r_3$) are the effective rate constants for the hydrolysis of TAGs, DAGs, and MAGs, respectively, and t is the time. To simulate the steady state in a continuous-flow reactor with plug flow pattern, the equations were integrated with initial condition $c_1 = c_{10}$, $c_2 = c_3 = c_4 = c_5 = 0$ for $t = 0$. The resulting concentrations of TAGs, DAGs, MAGs, FAs and glycerol are functions of $r_3 t_h$. As the reaction pressure and temperature were constant, the residence time was dependent only on the CO_2 flow rate Q and the term $r_3 t_h$ can be expressed as $k_r N_e/Q$, where parameter k_r is directly proportional to the effective rate constant r_3. For example, the concentration of TAGs is

$$c_1 = c_{10} \exp\left(\frac{-3k_r N_e}{Q}\right).$$

With N_e expressed in grams and Q in ml/min (measured at ambient conditions), parameter k_r was in the range 0.012–0.016 ml/g min for fresh enzyme, after a longer use of enzyme it decreased, and finally it oscillated between 0.002 and 0.005 ml/g min. The evaluation of model parameters will continue using the more informative data from chromatographic analysis, enabling us to determine more precisely the relations between rate constants r_1, r_2, and r_3.

4. Conclusion

Gradient-elution non-aqueous RP-HPLC method described above allows complete or partial separation of 34 acylglycerols in the hydrolysed blackcurrant seed oil. The α- and γ-linolenic acids, co-eluted in the RP-HPLC, were distinguished, at least in the form of free acids, with the use of LC-NMR method. The identification and quantification of these acids present in one chromatographic peak was based on the analysis of ^1H chemical shifts of the CH_3-end groups related to the chain $C^{15}{=}C^{16}$ and $C^{12}{=}C^{13}$ bonds, respectively. The experimental results indicate a decrease in reaction rate when the lipolysis in the SC-CO_2 medium is repeated. The decrease is most probably caused by a gradual blocking of the immobilized enzyme by produced glycerol, the solubility of which in SC-CO_2 is by order of magnitude lower than that of the oil and its other reaction products. This phenomenon will be further studied with the use of the developed analytical methods.

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