Separation of Free Fatty Acids using Supercritical Fluid Chromatography

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The separation of free fatty acids was performed with supercritical fluid chromatography (SFC) on analytical lab scale apparatus. Experiments were performed using fatty acids, which occur in vegetable oils in different amounts. Free fatty acids were analyzed without derivatization. Several stationary phases of packed columns and types of detectors were used to achieve efficient free fatty acids separation. According to obtained results with SFC, the separation of free fatty acids was compared with results obtained by gas and liquid chromatography.

INTRODUCTION

The lipid components in oils are important for determination the origin and quality of the oil, what is helpful in industrial processing of oils. Oils and fats mainly consist of triglycerides, minor mono- and di- glycerides, free fatty acids and phospholipids, sterols and other fat-soluble components as tocopherols, tocotrienols, carotenoids and sqalene. Triglycerides have three fatty acids bound to glycerol. Due to their structure, fatty acids can be released from glycerol using derivatization procedure. Free fatty acids (FFA) are the bioactive lipid components in oils, especially ω-3 and ω-6 fatty acids, that are characterizing high quality of the oil and have important role on growing pharmaceutical market.
Separation of lipids is usually performed by conventional method with organic solvents, what is due to safety and environmental concerns against government restrictions. Alternative presents carbon dioxide (CO_2) which can be used as supercritical fluid in separation processes, fractionation, membrane system and conversion processes and also particle formation.[1] In separation processes, exactly chromatography, supercritical fluids can act as carrier of substance as in gas chromatography (GC) or dissolve substance as solvents in high performance liquid chromatography (HPLC). Fifty years ago, first mentioned supercritical fluid chromatography (SFC) was using capillary columns and GC-like oven and detector, where high temperature caused increase of diffusivity and on the other hand, degradation of thermal labile substances.[2,3] Improved SFC came 20 years later with packed columns, mixed fluids, composition programming and UV-detection. The stationary phases in packed columns have much higher surphase areas and void volume ratios than capillary columns. Also the usage of polar modifiers, which are incompatible with GC flame ionization detector (FID), mixed with CO_2 causes decrease in retention time of solutes. In SFC system with packed columns the components are similar to LC components, where lower temperatures are reached (operates near critical temperature of fluid). Pressure is controlled by back pressure regulator and accurate flow rates and mobile phase composition can be achieved. The main advantage of SFC over HPLC is lower viscosity and higher diffusivity of supercritical mobile

phases relative to liquids, which leads to faster and more efficient separations. Inert and more volatile CO_2 has good selectivity that can match with reverse phase HPLC.[2]
Numerous applications using SFC as separation method were already published. SFC is already routine separation of pharmaceuticals, chiral separations, separations of substances from natural products and is used as analytical and as preparative scale separation technique. Moreover, some authors have reported the usage of SFC in determining physico-chemical properties of substances. Solubility of several components, also some fatty acids, in supercritical fluid was determined on the base of retention times of substance using SFC.[4] With SFC also the diffusion coefficients of lipids were determined based on Taylor-Aris peak broadening technique.[5]
The aim of this work was separation of underivatized FFA using packed column SFC. Three different stationary phases Nucleosil 100-5 C18, LiChrospher 100 Diol and CN-HP were used for optimization of separation conditions. Previously, the separation was made on HPLC using stationary phase LiChrospher 100 Diol with UV and ELSD detection and on GC using capillary column FFAP and FID. Qualitative and quantitative results obtained by different chromatographic methods applied were compared.

MATERIALS AND METHODS

Materials: Palmitic acid (Cat.No.76122, purity 97%), Palmitoleic acid (Cat.No. 76169, purity 98.5%), Stearic acid (Cat.No. 85680, purity 98.5%), Oleic acid (Cat.No. 75090, purity 99%), Linoleic acid (Cat.No. 62230, purity 99%) and Linolenic acid (Cat.No. 62160, purity 98.5%) were purchase from Fluka. CO_2 (2.5, purity 99.5%) and N_2 (5.0, purity 99.999%) were purchased from Messer, Slovenia. All organic solvents used for analysis were purchased from Merck (Darmstadt, Germany). In experiments, packed columns with stationary phase Nucleosil 100-5 C18, LiChrospher 100 Diol (all from Agilent, USA), CN-HP 60A^{o} (Nova-Pak Waters, USA) and capillary column HP-FFAP (Agilent, USA) were used in separation procedures.

Methods: The separation of free fatty acids was performed using three different chromatographic methods described below:

Gas chromatography. The analyses were performed with GC model 6890 Hewlett-Packard (Pittsburgh, PA, USA) with FID temperature set at 300°C and column (HP-FFAP 30 m x 0.25 mm x 0.25 μm). The oven time-temperature profile was as follows 120°C (1min), 25°C per min to 180°C (1min), 5°C per min to 220°C (10 min), 5°C per min to 230°C (30 min). The carrier gas was helium with total flow through the column 64.0 mL/min. The samples were analyzed in *n*-hexane solutions.[6]

Liquid chromatography. An Agilent 1200 Series system was used coupled with UV-Vis diode array detector and Evaporative Light Scattering Detector. As mobile phase acetonitrile (A) and water (B) was used with following gradient: 30% of A at 0 min, linear gradient to 100% of A to 20 min. Flow rate of mobile phase was 1 ml/min at 45°C. Separation of fatty acids was carried out through packed column Agilent LiChrospher 100 Diol (250 x 4.6 mm ID, 5 μm particles size) and detected at 210 nm on DAD. The conditions on ELSD were following: temperature of evaporation 55°C, gain 12, filter 3 and pressure 3.5 bar.

SCF chromatography. SFC system (Figure 1) consists of a reservoir for mobile phase (CO_2 tank), a unit for establishing, maintaining and controlling pressure (NWA PM-101 and PR-102), an optional unit for adding a modifier (Binary pump, Agilent 1100), Rheodyne

switching valve, separation column (different length and stationary phases) with electrical heating, UV-VIS diode array detector (Agilent 1100 with high pressure micro flow cell) for determining concentration of substance in the outlet stream, the unit for expansion of mobile phase (NWA PE-103) and flow meter.

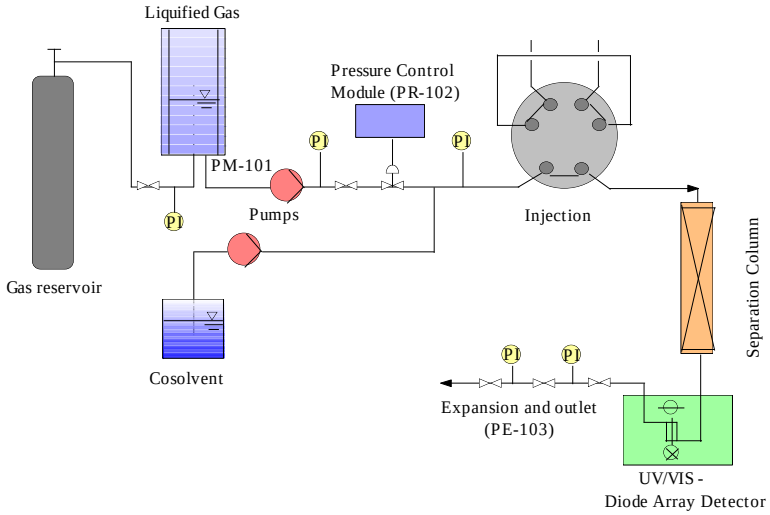


Figure 1: Flow scheme of laboratory scale SFC.

All three systems were controlled by Agilent Chemstation program, with which the quantification (resolution, retention factor, selectivity and efficiency) of separated peaks was obtained.

RESULTS

The standard method for determination of free fatty acids content in oils is a titration method. There only total content of FFA can be determined. For obtaining quantitative and qualitative information on free fatty acids composition gas and liquid chromatography are usually applied (Figure 3). Usually, before analysis the derivatization of bounded fatty acids into methyl- or ethyl- ethers was made, which are easier to detect.[7] In this work, fatty acids were analyzed without derivatization, in oils they were untied from triglycerides by saponification procedure.

The separation of FFA was also performed with SFC on analytical lab scale apparatus. The most useful mobile phase in SFC is carbon dioxide due to its physical and chemical properties. Carbon dioxide as SCF has low critical pressure and temperature, chemical inertness and low viscosity that results in high column separation efficiencies. As alternative method for identification and separation of FFAs, SFC has some advantages and some similarities with gas or with liquid chromatography. SFC can be used to perform separation of FFA at lower temperature than GC and with higher sensitivities than LC. Experiments were performed using fatty acids and oils containing different amounts of FFA. FFAs were analyzed without derivatization. Several stationary phases of packed columns were used to achieve good free fatty acids separation.

Separations were performed in pressure range from 100 to 300 bar and at temperatures 26, 29, 30, 32 and 35°C, the flow rate through the column was in range of 0.75 to 1.25 L/min STP. Better results were achieved by adding organic solvent, in our case methanol, to mobile phase – CO_2. UV detection was performed at wavelength 205 nm. Three different stationary phases

were used as shown in Figure 2. Stationary phases LiChrospher 100 Diol and CN-HP were chosen as normal, with polar modified silica gel phases, which have better chromatographic properties for separation of substances than usually used silica or reverse phase columns. In Figure 2a can be seen that separation on stationary phase Nucleosil C-18 does not give any results. Unsatisfying results were obtained also with CN-HP column, what can be seen in Figure 2c. More experiments were done on column with stationary phase LiChrospher 100 Diol (Figure 2b), which was also used in HPLC method. The separation of palmitic, oleic and linolenic acid was achieved by using methanol as mobile phase modifier (Figure 2b). The ratio between CO_2 flow and modifier flow was 200:1.

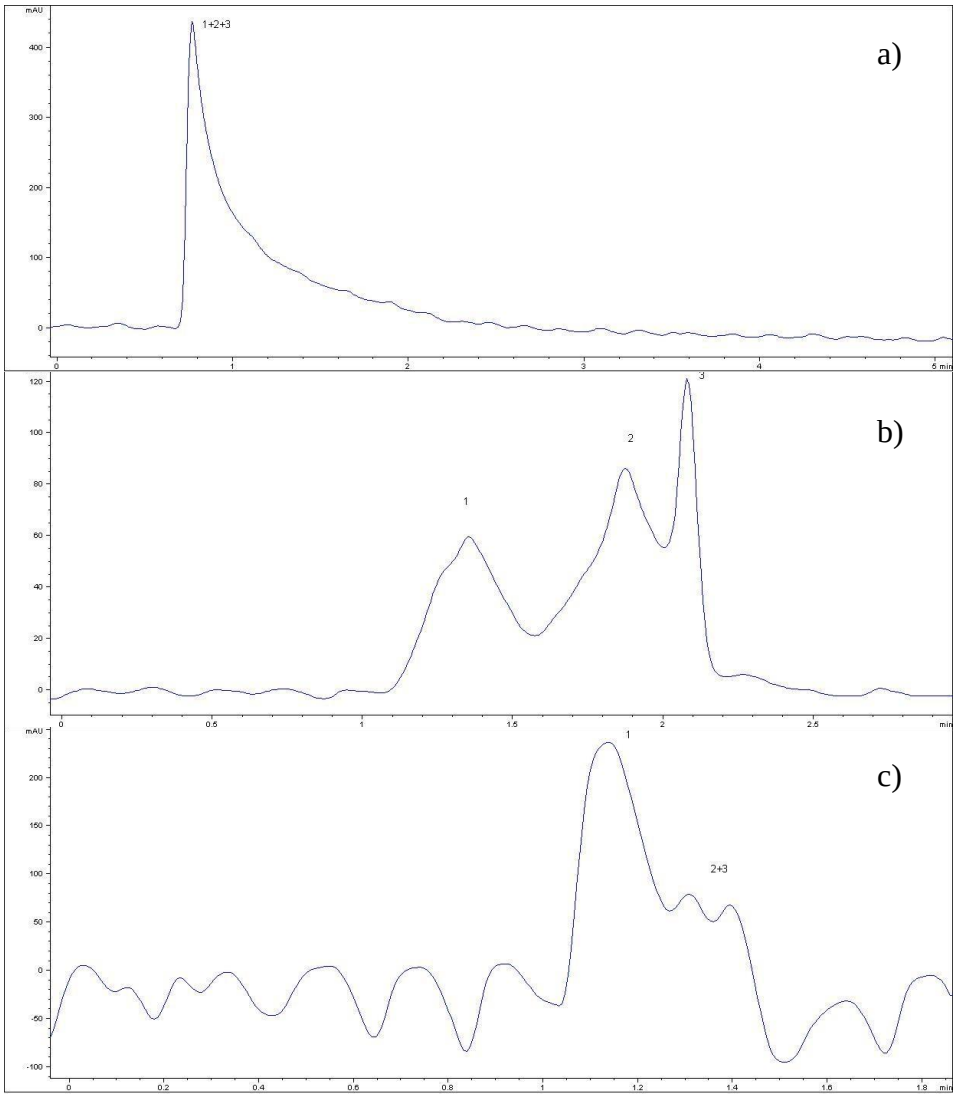
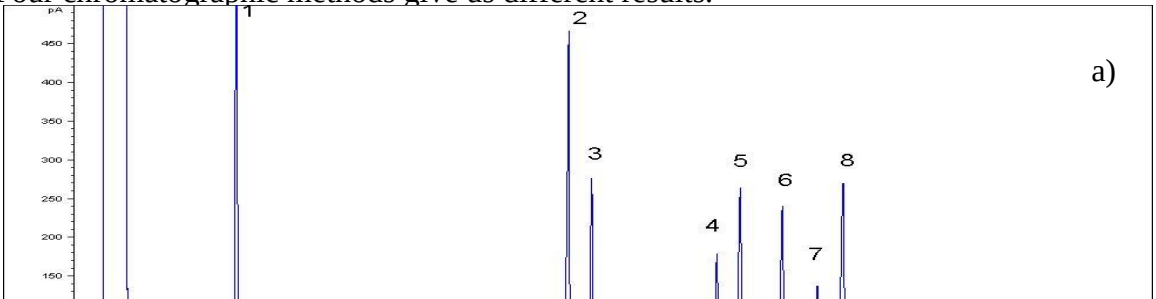


Figure 2: Comparison of fatty acids separation on SFC using different stationary phases of packed columns: (a) Nucleosil 100 C-18, (b) LiChrospher 100 Diol, (c) CN-HP. Separation of FFA in mixture of 1- palmitic acid, 2- oleic acid and 3- α-linolenic acid.

Separation of FFA using different analytical techniques is shown in Figure 3. Optimal separation methods were developed on GC with FID (Fig. 3a), on HPLC coupled with UV (Fig. 3b) and ELSD detector (Fig. 3c) and already mentioned SFC with UV detector (Fig. 3d). Four chromatographic methods give us different results.



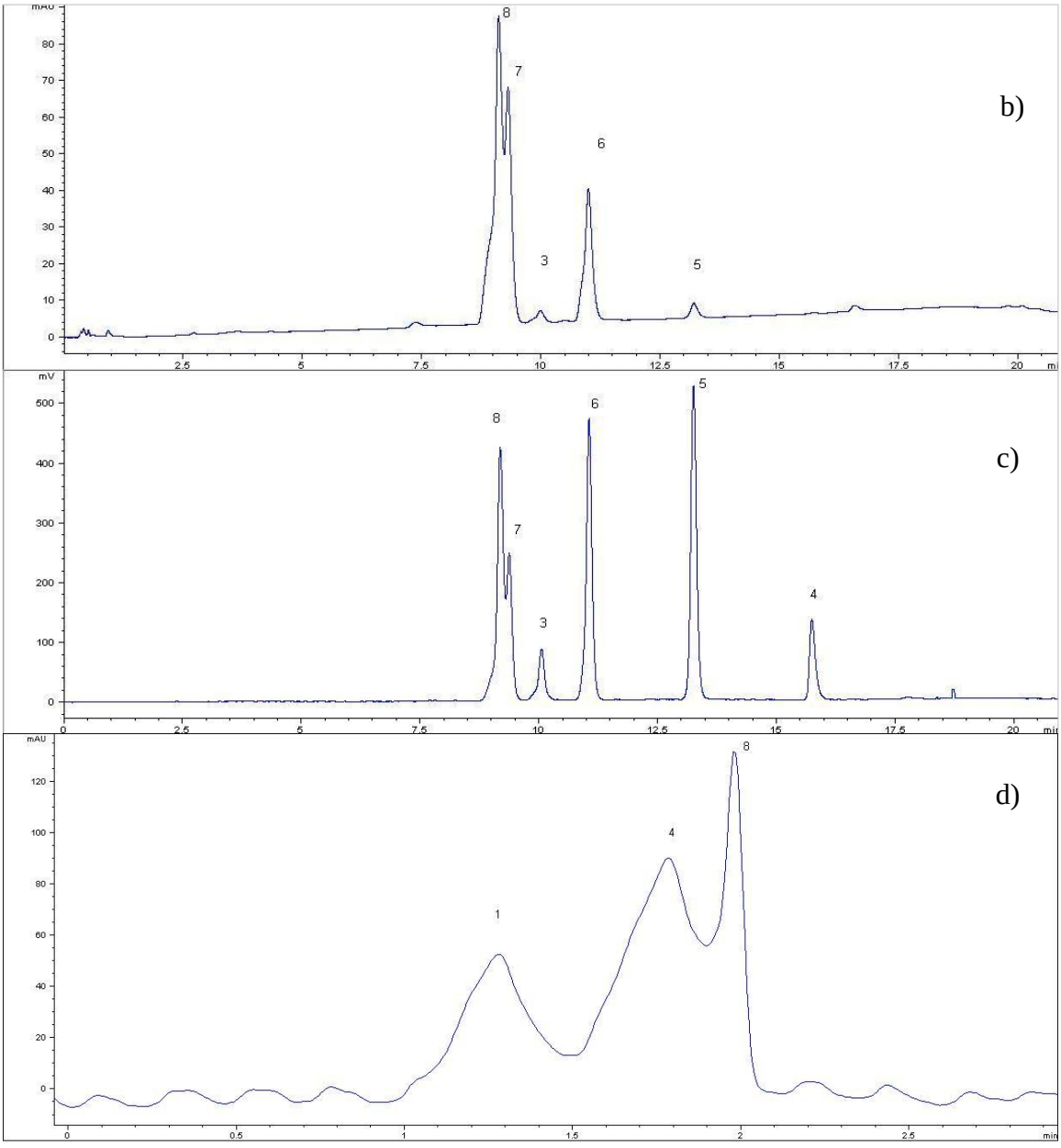


Figure 3: Separation of free fatty acid 1- heptanoic acid, 2- palmitic acid, 3- palmitoleic acid, 4- stearic acid, 5- oleic acid, 6- linoleic acid, 7- γ-linolenic acid, 8- α-linolenic acid, 9- eruric acid with (a) GC-FID, (b) HPLC-UV, (c) HPLC-ELSD, (d) SFC-UV.

Table 1: Quantification of peak separations: the comparison between chromatographic methods.

	GC-FID	HPLC-UV	HPLC-ELSD	SFC-UV	Sep.criteria
resolution	4.44 – 83.41	0.65 – 6.24	0.79 – 19.25	0.71 – 1.44	> 1.5
retention factor	-	3.83 – 4.83	3.87 – 8.94	-	1 - 10
selectivity	1.03 – 3.05	1.03 – 1.23	1.03 – 1.22	1.11 – 1.45	> 1.1
efficiency	36205 - 332485	12450 - 26687	22449 - 777356	471 - 4388	> 10000

The calculated quantities of separation are shown in Table 1. The range is taken from current method of all separated peaks. Separation criteria are presented In Table 1 in last column. As can be seen, more or less all separation methods achieved the criteria. Separation performed on GC-FID has excellent resolution and efficiency, where number of plates in column is calculated. Separation on HPLC-UV has in comparison with HPLC-ELSD worse separation values, due to low absorbance of fatty acids with linear structure chain. According to unseparated peaks of α- and γ-linolenic acid (Fig. 3b and c), the length of the column and their particle size should be further optimized. The SFC separation has resolution below separation criteria, but the selectivity was achieved. Because of low retention times, the efficiency of the column was bad. The retention times obtained by using HPLC method instead of GC decreased by factor 2, but using SFC the retention times were decreased 10-times compared to HPLC and 20-times compared to GC performance. The resolution and efficiency can be improved by changing detection like in case of HPLC method.

CONCLUSION

According to performed experiments it can be concluded that:
- the separation is possible to perform with different chromatographic methods,
- the selection of stationary phase is most important,
- SFC has advantages regarding time of analysis and selectivity,
- disadvantages of efficiency can be improved by detection method as seen in HPLC analysis using ELSD and
- no FA derivatization procedures were needed, what is time and costs facility.

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