Scientific paper

Supercritical Fluid Chromatography and Scale up Study

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Abstract

The influence of process parameters on supercritical fluid chromatography (SFC) was investigated together with scaling-up to the preparative scale. Within this scope, separation of model compounds (caffeine and theophylline), dependencies on pressures, temperatures, types and concentrations of the modifiers, and types of stationary phases, were examined separately. Experiments were performed on analytical scale and on pilot preparative scale SFC apparatus. Bare silica and silica 2-ethylpyridine were used as stationary phases, with CO_2/methanol or CO_2/ethanol at elevated pressures and temperatures as mobile phase. Observations obtained on the analytical scale were used for scaling-up to preparative scale. The aim of this study is evaluating the influences of process parameters when separating model compounds using SFC, and s practical demonstration of the scaling-up method of SFC along with the operation of a pilot preparative to production scale SFC apparatus, which is one of the bigger similar apparatuses worldwide.

Keywords: Supercritical fluid chromatography; Scaling-up; Preparative supercritical fluid chromatography

1. Introduction

Supercritical fluid chromatography (SFC) is one of the separation processes, which has been intensively investigated and developed over recent years. The abbreviation SFC refers to supercritical state, but separation could also be performed in other regions of p-T diagrams.[1,2] Beside the supercritical region, SFC could be carried out in subcritical region *i. e.* under conditions where CO_2 is liquid (operating conditions lower than and T_c or T_c and p_c).[2,3]

It is mainly applied within analytical or semi-preparative scales for the separations and characterization of bioactive compounds,[4] like pharmaceuticals.[5–7] SFC is also applied for detecting and separating impurities in pharmaceuticals.[8] Pharmaceuticals often contain one or several chiral centers. Such compounds could be separated using SFC on chiral stationary phases. Over recent years several authors have reviewed the separating of enantiomers using SFC on chiral stationary phases.[9–13] Several studies have been published on chiral compound separation[14–18] and semi-preparative SFC.[19,20] It is possible with semi-preparative SFC to obtain several 100 mg of product per day but with proper scaling-up to preparative scale or production scale it is possible to obtain up to several kg of product per day. SFC is relatively well-developed within the field of analytical applications. Recently, growing interest has been observed in applying SFC in the preparative scale. Among others, several authors[21–25] have described scaling-up to preparative SFC. Kamarei *et al.* compared the preparative SFCs of chiral compounds to preparative liquid chromatography (prep-LC).[26] SFC in comparison to LC provides faster separation, where comparable yields are obtained at higher optimal injection volumes. Faster separation and higher injection volumes contribute greatly towards higher productivity.[26]

Although, SFC offers several advantages over other chromatographic techniques, it cannot replace them completely, due to the limitations of supercritical fluids.

Pressure and temperature directly control the density of the mobile phase and consequently influence other physical-chemical properties such as viscosity, diffusivity, solubility *etc.*[1,27] Viscosity and solubility increase with increasing density of the mobile phase. In contrast, diffusivity decreases with increasing density. Solubility strongly influences the retention factor in SFC. The retention behavior could be estimated from solubility data, mobile phase velocity, and adsorption mechanism.[27]

In the case of polar compounds' separations, CO_2 is modified with organic solvents, usually with methanol or ethanol and in some cases acetonitrile. When the mixture

is under supercritical conditions both components of the mobile phase are completely miscible. However, SFC is often carried out in a subcritical state of the mixture. Consequently, process parameters have to be adjusted properly in order to maintain one-phase, and to avoid any decreasing of the efficiency.

Packing material of the column obstructs the flow-rate of the mobile phase, which leads to pressure and density drop along the column. Consequently, the mobile phase is expanded and slightly cooled during the expansion. If the temperature within the column is not constant, separation efficiency is decreased.[28,29] Density drop has a notable effect on column efficiency within the region with high compressibility.[30,31]

The aim of this work was to evaluate the influences of process parameters on the separation of model compounds caffeine and theophylline, and to select the stationary phase, modifier and the optimal range of the process parameters for performing SFC on pilot preparative scale apparatus. Further, the operation of pilot preparative to the production scale SFC apparatus, which is one of the biggest similar apparatuses worldwide, is discussed, together with the background of the scale-up method.

2. Experimental Section

2. 1. Materials

2. 1. 1. Chemicals

CO_2 (purity> 99.5%) used for SFC was supplied by Messer (Ruše, Slovenia). Methanol (J. T. Backer ®, Netherlands) HPLC grade (No. 9093-01) and ethanol (Merck, Darmstadt, Germany) of chromatography grade (No. 111727) were used as a modifiers. Caffeine (Ph. Eur, anhydrous, No. 27602) was supplied by Fluka Analytics (Buchs, Switzerland) and theophylline (> 99%, anhydrous, No. T1633) was supplied by Sigma-Aldrich Chemie (Buchs, Switzerland).

2. 1. 2. Stationary Phases

Lichrospher® SI60 (Merck, Germany) and Viridis™ Silica 2-ethylpirydine SFC (Waters, MA, USA) columns, lengths of 250 mm and 4.6 mm *i. d.,* packed with 5 μm were used for preliminary research on analytical scale SFC.

Bulk material LiChroPrep® Si60 supplied by Merck (Darmstadt, Germany), was used as packing material of prep-SFC.

In addition, LiChroPrep® Si60 was packed for our demands in the columns (100 mm and 250 mm in length and 4.6 mm *i.d.*) and was tested in analytical scale SFC.

2. 2. Apparatus

2. 2. 1. Analytical Scale SFC

Investigation of process parameters' influences was performed on analytical scale SFC apparatus designed in partnership with New Way of Analytics (Lörrach, Germany). The apparatus flow-chart is shown in Fig. 1. This apparatus consist of a high pressure CO_2 pump New Ways of Analytics (Germany), modifier pump Series 1100 Agilent Technologies (CA, USA), manual sample injector Rheodyne 7725 (IDEX Health & Science, CA, USA), column in the oven, UV/ diode array detector (DAD) Series 1100 Agilent Technologies (CA, USA), and reducing valve.

CO_2 from the cylinder is liquefied and enters a high pressure CO_2 pump, where inlet pressure is generated. The constant mass flow-rate of the CO_2 is adjusted and maintained with a reducing valve. The inlet pressure was oscillating at ± 5 bar CO_2 and the modifier are mixed, before the mobile phase enters the injection valve. The volumetric flow-rate of the CO_2 was measured with gas flow meter at ambient conditions. Volumetric flow rate of the modifier was adjusted by a modifier pump. Afterwards, the mass flow-rate of the CO_2 and modifier were calculated.

The mobile phase proceeds through the injection valve and chromatographic column to the DAD, where the compounds from the injected sample are detected, and furthermore characterized.

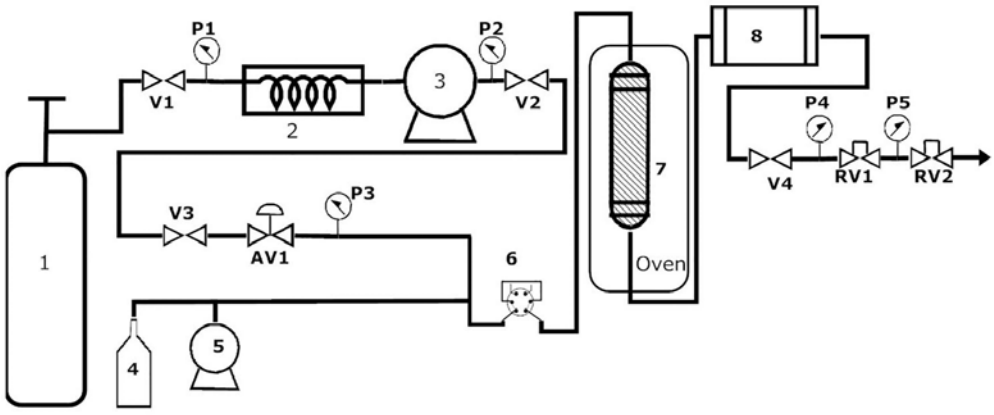

Fig. 1. Flow chart of analytical scale SFC apparatus 1 – CO_2 tank, 2 – cooler , 3 – CO_2 pump, 4–modifier tank, 5 – modifier pump, 6 – injection valve, 7 – column, 8 – detector, V1–V4 – valves, AV1 – automated valve, P1–P5 – pressure gauge, RV1 and RV2 – reducing valves

2. 1. 3. Preparative Scale SFC

Based on results obtained on analytical SFC the separation of model compounds was scaled-up to pilot preparative to the production scale SFC apparatus, which is one of the biggest such apparatus worldwide and was designed in partnership with New Way of Analytics (Germany). This apparatus consists of a CO_2 tank, cooler, CO_2 pump URACA (Germany), heater, by-pass loop, modifier tank and pump (LEWA, Germany), sample tank and pump (Maximator, Germany), column (1m in height and 0.1 m *i.d.*), on-line UV detector, two separators for collecting fractions of the sample and one separator for collecting the modifier, automated valve (AV5, Fig. 2), evaporator, and a condenser for CO_2 recirculation. Fig. 2 presents a flow- chart of the pilot preparative to production scale SFC.

The maximum capacity (flow-rate) of the CO_2 pump is approximately 400 kg CO_2 per h. Adjusting the pressure and flow within the system is not a straightforward procedure. CO_2 from the tank enters the cooler, where it is liquefied completely. Liquid CO_2 enters the three piston CO_2 pump which compresses the CO_2 and transports it through the system. The high pressure pump is the source of pressure and flow of the mobile phase, but additional automated valves are required for proper adjustment.

The CO_2 flow is split at the exit of the pump into two streams; main stream and stream through bypass loop. The CO_2 pump has to operate at a certain rotation speed in order to produce the desired mass flow-rate, but the pressure which is generated during this procedure, is insufficient. Therefore, the column inlet pressure is additionally adjusted using closing automated valves (BP1 and BP2 in Fig 2) on the bypass loop. Additionally, an automated valve (AV4) is located behind separators (S1 and S2).

Before CO_2 is mixed with the modifier, it is heated in the heater. The temperature of the CO_2 has to be higher in the heater than that expected in the column, due to heat losses when mixing with modifier and passing the column.

The column is equipped with a piston, which allows the uses of various quantities of the stationary phase. The piston under compressed CO_2 minimizes the particles' distances and holds the stationary phase in place.

Eluted compounds are detected on-line with a UV detector. Based on on-line detection, delayed timing is determined for pneumatically opening the valves (AV1, AV2, and AV3) of the separator, in order to collect eluted compounds. The separators (S1 or S2) are composed of two major parts. The first part is the suction pressure vessel with piston. The second part is a heated high pressure collecting vessel, where CO_2 is decompressed and recycled back into the CO_2 system. Operating the separators requires 3 steps. First, in the preparation step, the pressure, lower than the operating pressure, is introduced above the piston of the first part. This step is required, to avoid rapid decompression of the mobile phase at fraction collection. In the second step, fraction collection is performed to the suction pressure vessel. In the third step the fraction from the suction part is redirected into the collective vessel. By decompressing the fraction, particles of the collected compound precipitate.

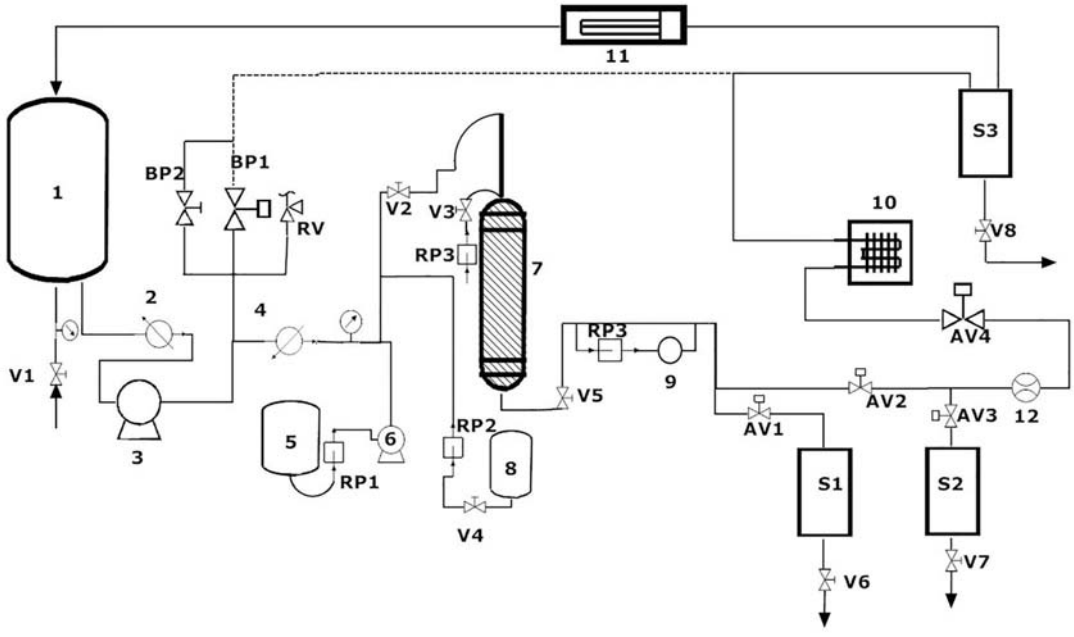

Fig. 2. Flow chart of pilot preparative to production scale SFC apparatus for SFC:1 – CO_2 tank, 2 – CO_2 cooler, 3 – CO_2 pump URACA, 4 – CO_2 heater, 5 – modifier tank, 6 – modifier pump LEWA, 7 – column with piston, 8 – Sample tank, 9 – UV detector, 10 – evaporator, 11– condenser of CO_2, 12 – flow meter; V1-V8 – opening valves, RP1–RP3 – pneumatic pump Maximator, AV1–AV4 automated valves, RV – safety relieve valve, BP1 and BP2 – bypass loop valves. S1–S3 – separators

The mobile phase has residues of solvent flowing through the evaporator, before it reaches the third separator (S3). In this separator rapid decompression of the mobile phase occurs. Gasified CO_2 proceeds from the separator to the condenser, where it is liquefied and returned to the tank. Meanwhile, modifier is collected in the separator.

2. 3. Experiments

2. 3. 1. Sample Preparation

Known amounts of the model compounds' standards (caffeine or theophylline) were dissolved separately in volumetric flasks in either ethanol or methanol, depending on the type of modifier used. Small portions of each solution were transferred into another volumetric flask and dissolved in order to prepare a sample mixture.

2. 3. 2. Determination of Process Parameters

First, solubility data of the compounds within supercritical CO_2 were obtained from the literature[32–34] and analyzed. The maximum solubility of caffeine in SC-CO_2 was roughly 5×10^{-4} mole per mole of CO_2 at operating conditions which were close to the maximal operating parameters of the equipment (p = 250 bar and T = 65 °C). The solubility of theophylline in SC-CO_2 was approximately one order of magnitude lower than the solubility of the caffeine. It was noted, that the solubility of the compounds increased with increasing density. High solubility of the solutes in CO_2 is achieved at relatively high density, hence high pressures are required, which exceeds the limitations of the equipment. Therefore, a modifier was added, in order to increase the solvent strength of the mobile phase at lower conditions.

Experiments of analytical scale SFC were performed on the bare silica and silica 2-ethylpyridine (2-EP) stationary phases with dimensions (250 × 4.6 mm, 5 μm particle size) and bare silica stationary phase packed for our demands (100 × 4.6 mm and 250 × 4.6 mm; particle size 15–25 μm). The influences of the pressure and temperature of the mobile phase, and the type and mass fraction of the modifier in the mobile phase on the separation were investigated. First separation was carried out on bare silica stationary phase at 35 °C, inlet pressure 160 bar and outlet pressure 130 bar without any modifier. In the following experiments methanol was added at 35 °C and inlet pressure 160 bar (outlet pressure 130 bar). Single test compounds were injected for qualitative analysis of the compounds in the mixture. The mass fraction of methanol in the mobile phase was increased from 0% up to 15%. Later, the experiments were performed at different inlet pressures ranging from 125 bar up to 225 bar at a constant temperature of 35 °C and with constant mass flow-rate (3 g/ml) and content (15%) of the mobile phase in order to observe pressure influence on the separation with SFC. Pressure range and modifier content in the mobile phase were changed in a similar manner, when separations were carried out under the other isothermal conditions (45 °C and 55 °C). Afterwards, similar investigation on SFC separation was performed with ethanol using a slightly changed range of inlet pressures (160 bar to 225 bar).

After examination of the separations on bare silica stationary phase with 5 μm particles, multiple experiments were conducted on different types of stationary phase. Pressure and temperature range, and mass fraction of modifier were identical as in the case of separations on bare silica. It should be noted, that separations performed on 2-EP stationary phase permitted higher concentrations of modifier in the mobile phase (up to 20.5% *w/w*), due to the specific adsorption behavior of the tested compounds.

Separations, performed on 100 mm and 250 mm custom-packed columns and methanol as modifier, were mainly carried out at conditions with inlet pressures bellow 180 bar, because the test compounds co-eluted, when applying higher operating pressure. Similar results were obtained when ethanol was used on 100 mm column. Only in the case of the 250 mm column and ethanol as modifier, no co-elution was obtained at inlet pressures higher than 180 bar.

In order to determine selectivity, dead time (t_0) was measured, afterwards. Certain small amount of benzene was dissolved in methanol. This solution was injected into the column. Retention time of eluted benzene was dead time. Dead time examinations were performed at pressure of 200 bar and temperature of 35 °C on silica stationary phase, with methanol and ethanol as modifier, and on 2-EP stationary phase with methanol as modifier. It was assumed that influences of the type of modifier and the process parameters on t_0 could be neglected, due to small changes in t_0 value.

2. 3. 3. Development of Preparative SFC

First, the process parameters range was determined, regarding the preliminary experiments performed on the analytical scale SFC apparatus, and regarding the operational limitations of the preparative scale SFC apparatus.

Rajendran suggested two approaches for scaling-up of SFC, traditional approach and rational design approach.[35] Rational design involves three stages: system characterization, computer modeling and design, and scale-up. Rational design provides better results for simple (ideal) systems but it is not ideal for scaling-up of real systems, which are more complex. When a system is characterized, the rational design approach suggests the computer modeling for optimization. Objective functions are limited to physical constrains and system characteristics. It

is worth noting that this suggested approach does not provide simple results for real systems, where many of the system' characteristics are unknown. In the traditional approach, the maximum amount of injected sample is determined. The traditional approach is simple, however it does not provide an optimal solution, because it does not explore the full operating domain.[35] In the presented work traditional approach was used. The maximal amounts of sample per injection were measured on analytical scale SFC apparatus on selected column, with ethanol as modifier at 35°C and inlet pressure 160 bar, by increasing the concentrations of the test compound in each sample, and later by increasing of the volume of the injection loop until overloading. Extremely high values of UV absorbance (high compounds concentration), and overlaying peaks (lowering the selectivity), were the criteria for overloading. Further, the velocities of the mobile phase have to be constant, for ensuring that the following relationship in equation (1) is satisfied

$$\frac{Q_{prep}}{Q_{analyt}} = \left(\frac{d_{prep}}{d_{analyt}}\right)^2 \quad (1)$$

where and are flow rates of the mobile phase and diameter of the column, respectively. Additionally, equation (2) has to be considered when calculating the injection volume

$$\frac{V_{inj.prep}}{V_{inj.analyt}} = \left(\frac{d_{prep}}{d_{analyt}}\right)^2 \quad (2)$$

where $V_{inj.prep}$ and $V_{inj.analyt}$ are the injection volumes on the preparative scale and analytical scales, respectively.

In the presented work, firstly, the operating conditions based on literature solubility data were investigated on the analytical equipment. The region of operating conditions was determined regarding on results.

After evaluation of the process parameters was completed and the operating conditions had been selected, separation was performed on the pilot preparative scale SFC apparatus. System characteristics of the tested compounds required additional experiments on pilot SFC apparatus.

3. Results and Discussion

3. 1. Determining the Influence of Process Parameters on Analytical Scale SFC

3. 1. 1. Influence of the Modifier

Experiments were carried out using different compositions of the mobile phase to achieve separation of model compounds, caffeine and theophylline. When pure CO_2 was used, at a temperature 35 °C and inlet pressure 160 bar (outlet pressure 130 bar at mass flow-rate 3 g/mm), the elution of theophylline was unachieved, due to strong adsorption of the compound on the surface of the stationary phase. When the 2% *(w/w)* methanol was added to the mobile phase, the compound has eluted with relatively long retention time and showed an irregular chromatographic peak (Fig. 3).

When the mass fraction of methanol in the mobile phase was increased to 5% (*w/w*), theophylline has eluted with reduced retention time (7.56 min) and a more symmetrical peak (Fig. 4). An additional increase of mass fraction of the modifier up to 15% *(w/w)* is reducing retention time significantly (3.9 min). When both the tested compounds were jointly injected, a decrease in the resolution was observed at higher mass fractions of the modifier in the mobile phase (Table 1). The influence of the amount of methanol in mobile phase on the separation of the tested compounds on 2-EP stationary phase is presented in Fig. 5.

Table 1. Influence of modifier on SFC separation 2-EP stationary phase p_{in} = 200 bar, p_{out} = 165 bar, 35 °C CO_2, flow rate 3 g/min; caffeine (1), theophylline (2); $t_{r,i}$ – retention time, k'_i – retention factor, R_s –resolution, α - selectivity

Modifier	$t_{r,1}$	$t_{r,2}$	k'_1	k'_2	Rs	α
Ethanol (%)						
5	5.258	10.45	9.516	19.9	12.82	2.09
10	2.456	3.289	3.912	5.578	4.67	1.43
15	1.728	2.114	2.456	3.228	2.49	1.31
20.5	1.324	1.518	1.648	2.036	1.83	1.24
Methanol						
5	2.534	4.871	4.068	8.742	11.48	2.15
10	1.448	2.134	1.896	3.268	4.6	1.72
15	1.206	1.535	1.412	2.07	2.31	1.47
20.5	0.871	1.085	0.742	1.17	1.52	1.58

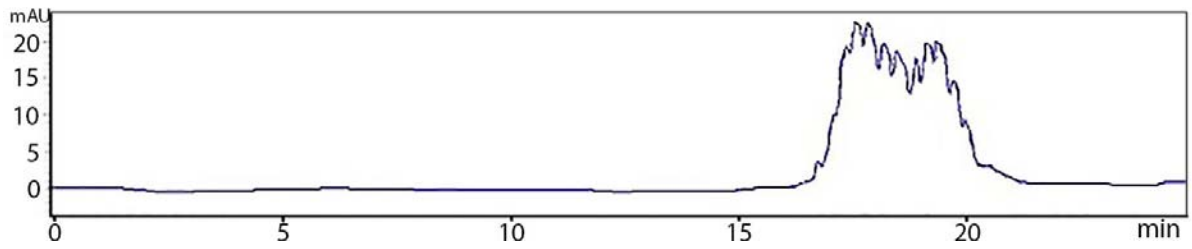

Fig. 3. Chromatogram of theophylline at inlet pressure 160 bar, 35 °C and 2% (*w/w)* of methanol, bare silica column

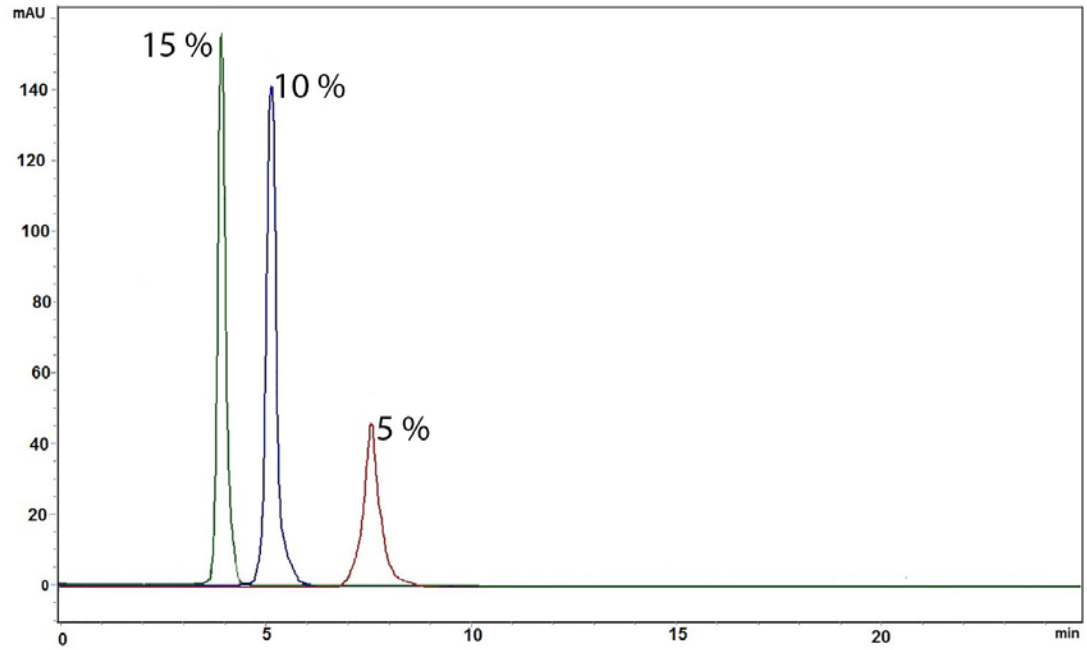

Fig. 4. Combined chromatograms of theophylline at inlet pressure 160 bar and 35 °C at different mass fractions of ethanol; 5%, 10%, and 15% on bare silica column

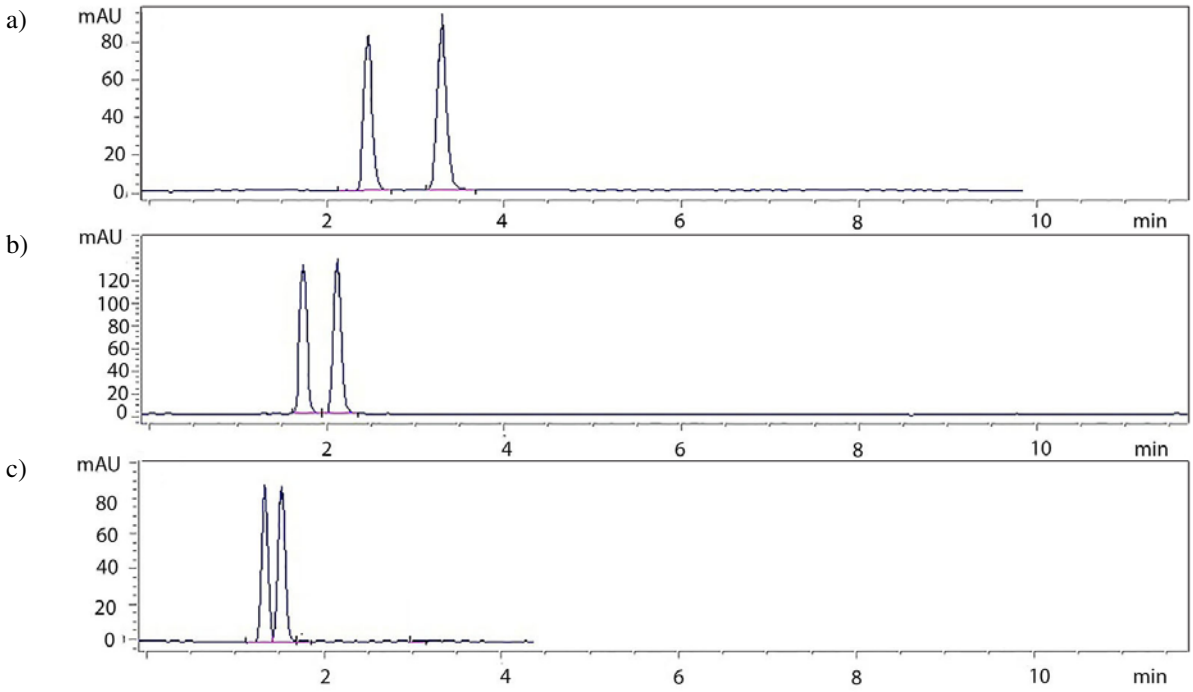

Fig. 5. Chromatograms of caffeine and theophylline at inlet pressure 200 bar and 35 °C at different mass fractions of ethanol; a) 10%, b) 15% and c) 20% on silica 2-EP stationary phase; 1st peak caffeine 2nd peak theophylline

Similar effects occurred for modifiers investigated, *i. e.* methanol and ethanol. It could be noted, that the increase in modifier mass fraction in the mobile phase, had significant impact on the separation resolution. Retention time, selectivity and resolution were decreased by increasing the modifier content, which can be observed from Fig. 5 and Table 1.

The content of the modifier in the mobile phase had significant influence on the dielectric constant (polarity) of the mobile phase and consequently influenced the solubility of the compounds in the mobile phase.

The main difference between both modifiers used is presented in Fig. 6, Fig. 7 and Table 1. Retention time

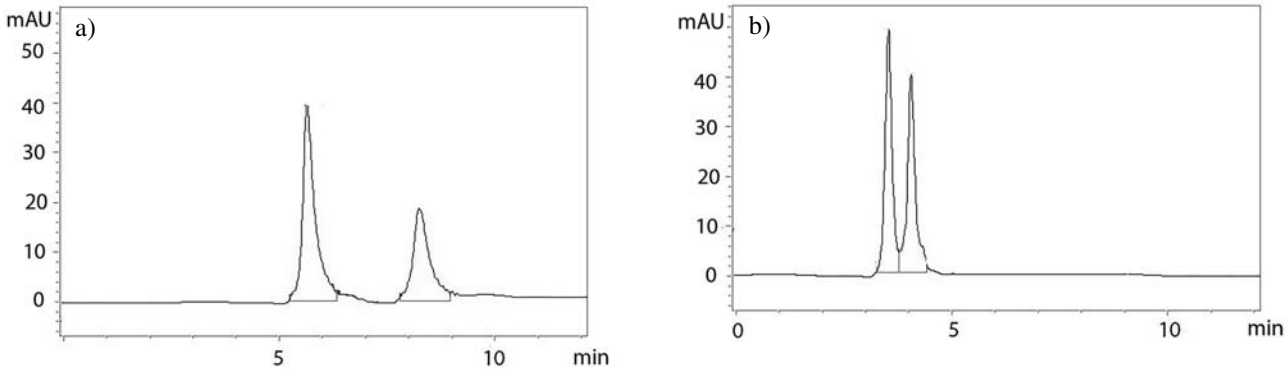

Fig. 6. Comparison of chromatograms of theophylline and caffeine, p_{in}= 200 bar, T= 35 °C, 15% of ethanol (a) and methanol (b), bare silica; 1st peak theophylline, 2nd peak caffeine

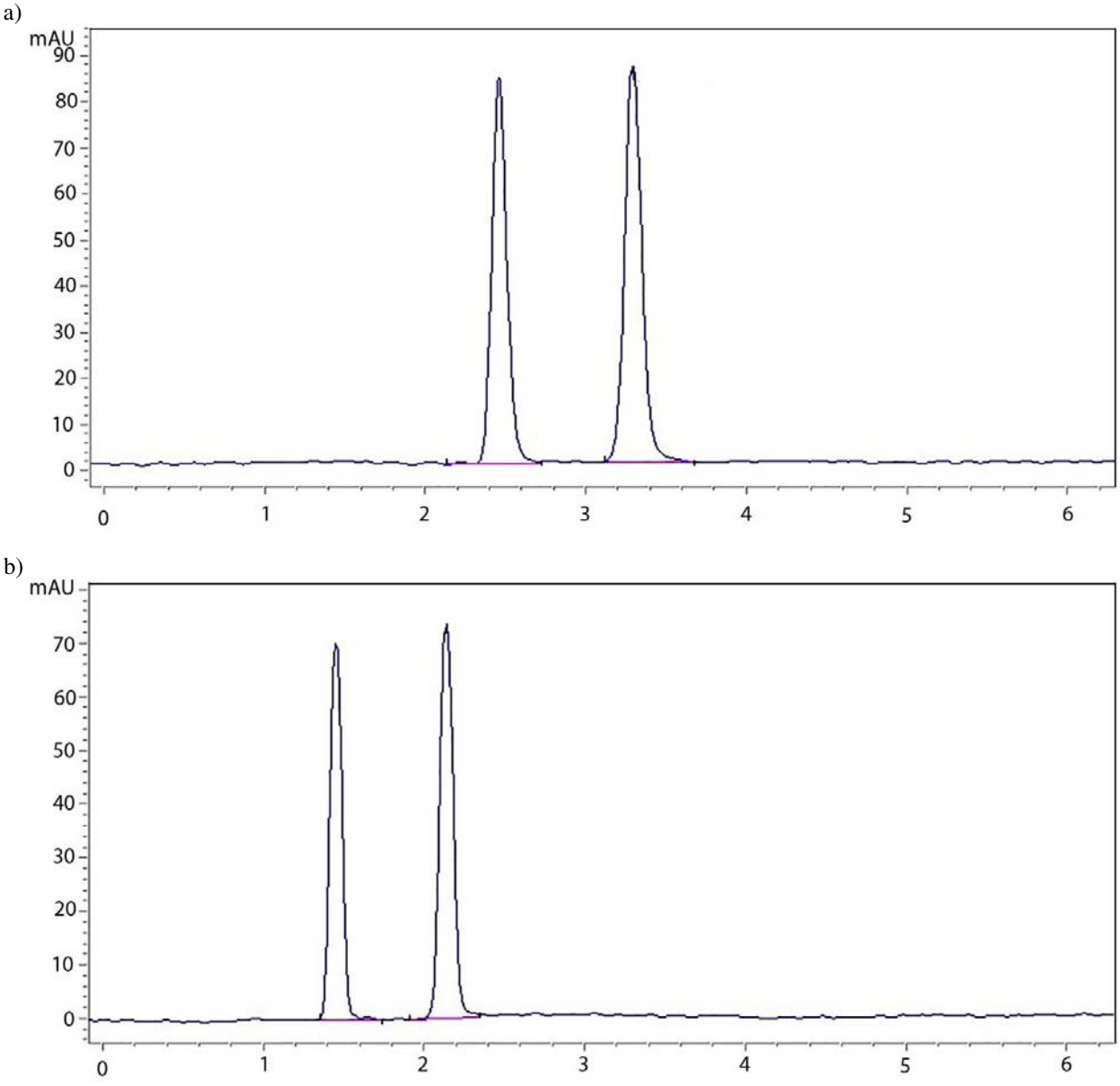

Fig. 7. Comparison of chromatograms caffeine and theophylline p_{in}= 200 bar, T= 35 °C, 10% of ethanol (a) and methanol (b), on 2-EP stationary phase, 1st peak caffeine, 2nd peak theophylline

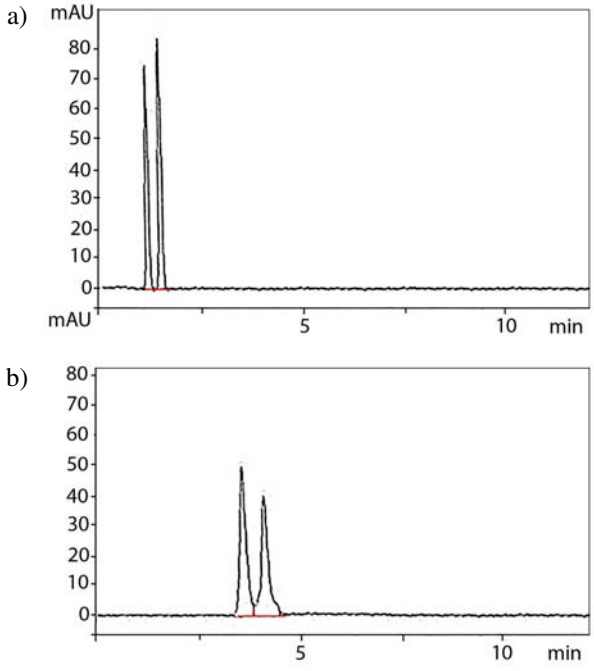

Fig. 8. Comparison of chromatograms caffeine and theophylline p_{in}= 200 bar, *T*= 35 °C, 15% of methanol (a) 2-EP: 1st peak caffeine, 2nd peak theophylline, (b) bare silica: 1st peak theophylline, 2nd peak caffeine;

and resolution were higher in the case of ethanol as modifier. This phenomenon was more intense using the bare silica column (Fig. 6). Oppositely, separations with methanol provided more symmetrical peaks. This effect was more notable on chromatograms which corresponded to the bare silica stationary phase. Similar influences on the different types of the modifier were observed for separations performed on the silica 2-EP stationary phase.

If the chromatograms in Fig. 8 are compared, significant differences can be observed between both stationary phases. Elution order is reversed the 2-EP column. Additionally, retention times are lower. For comparable resolution (Fig. 6a and Fig. 7a) lower mass fraction of the modifier was used in the case of 2-EP. When methanol was used as modifier, lower mobile phase pressure was required. Furthermore, if inlet pressure above 220 bar was applied at temperature of 35 °C, the compounds co-eluted in case of methanol as modifier. Similarly compounds co-eluted when using more than 20% (w/w) of methanol.

It can be concluded that ethanol is a better alternative as modifier in preparative scale SFC separations, due to its higher resolution. Methanol was, in more of the cases, the better alternative for analytical applications of SFC.

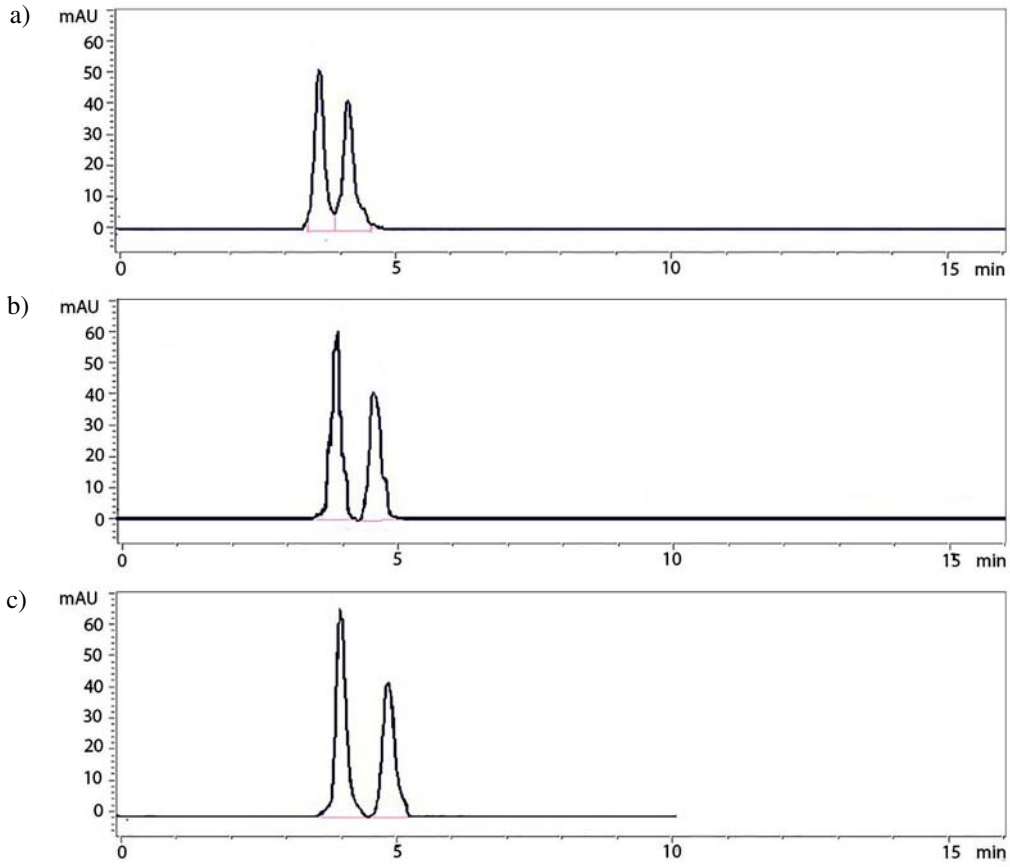

Fig. 9. Chromatograms of theophylline and caffeine *T*= 35 °C, 15% of methanol, a) inlet pressure 200 bar, b) inlet pressure 160 bar, c) inlet pressure 125 bar on bare silica column; 1st peak theophylline, 2nd peak caffeine

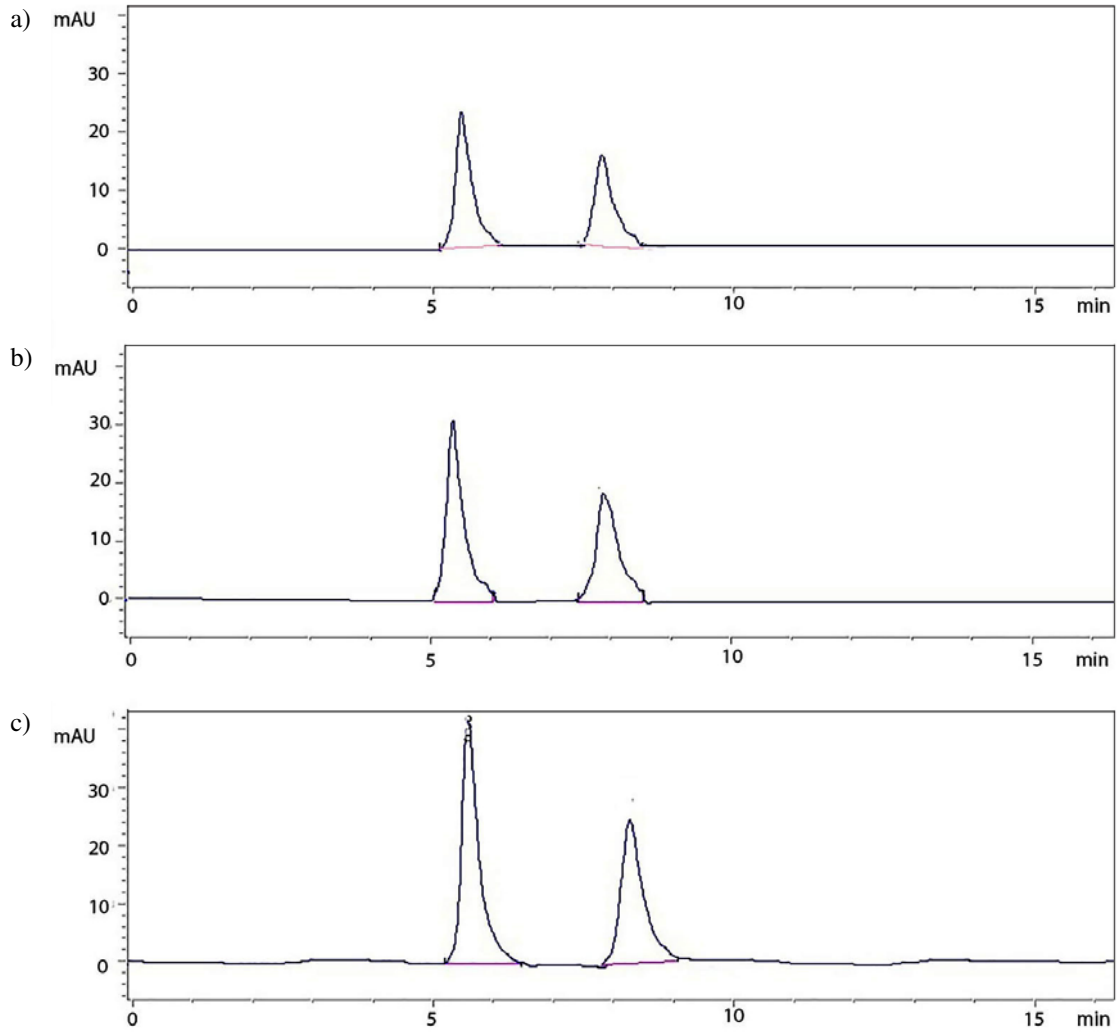

Fig. 10. Chromatograms of theophylline and caffeine; T= 35 °C, 15% of ethanol, a) inlet pressure 250 bar, b) inlet pressure 225 bar, c) inlet pressure 200 bar, bare silica; 1st peak theophylline, 2nd peak caffeine

3. 1. 2. Influences of the Pressure and Temperature

The next set of experiments were conducted at 35 °C, with 15% of methanol or ethanol and different inlet pressures from 125 to 200 bar (outlet pressure was from 95 to 170 bar, at constant mass flow of the mobile phase 3 g/min), for methanol and inlet pressure from 200 bar to 250 bar (outlet pressure from 170 bar to 215 bar) for ethanol. When the pressure was increased at constant temperature and constant mass flow of the mobile phase, the density increased and thus the solubility of the compounds in mobile phase increased. Consequently, retention time reduced with increasing pressure (Fig. 9 and Fig. 10). From Table 2 it could be observed that resolution and selectivity decreased with increasing pressure at constant temperature and methanol as modifier on the silica column. Selectivity slightly increased when pressure was increased in the cases of ethanol on silica column and the 2-EP column and methanol on 2-EP column. However, selectivity decreased in case of separations with methanol as modifier on the silica stationary phase. Selectivity and resolution decreased when increasing the temperature. Unexpected behavior was observed only when ethanol as modifier was used on a 2-EP column at constant pressure and different temperatures. Similar effect of pressure was observed for all isotherms and with both ethanol and methanol as modifiers. Retention times of compounds increased when the temperature was increased (Fig. 11).

Similar separations were performed on the 2-EP stationary phase. Similar effect on retention behavior was observed as in case of the bare silica column, when pressure and temperature were changed. As noted previously, the compounds eluted with lower retention times and lower resolutions, when 2-EP was used as stationary phase (Fig. 5, Fig. 6 and Fig. 7). The chromatographic peaks were more symmetrical for the tested compounds on 2-EP. Further, the major difference between both stationary phases was the reversed order of elution for both tested compounds.

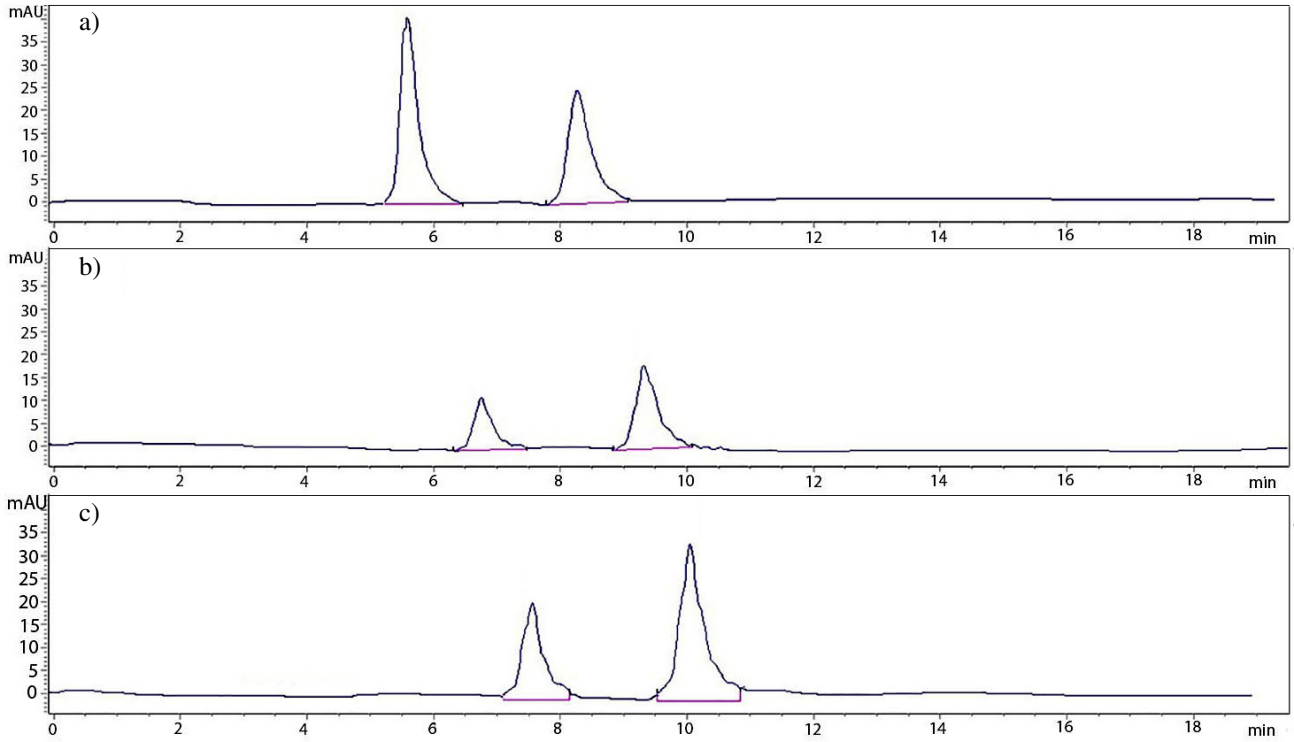

Fig. 11. Chromatograms of theophylline and caffeine (2); p = 200 bar, 15% of ethanol, a) T = 35 °C, b) T = 45 °C, c) T = 55 °C, bare silica; 1st peak theophylline, 2nd peak caffeine

The mass fraction of the modifier in the mobile phase had the greatest influence on compound retention, selectivity and resolution. Pressure and temperature (density) effects were less significant within this region of operation. According to the guidelines in literature,[3] the experimental results and equipment limitations, operating parameters were selected as follows: inlet pressure is ranging from 150 bar to 250 bar and temperatures in the column from 35 °C to 65 °C. For the tested compounds, the optimal mass fraction of ethanol in mobile phase was between 5–10% (*w/w*).

It is worth to note that the 2-EP stationary phase is better alternative for analytical applications in this case, because it contributes to shorter analysis time, therefore to

Table 2. Influence of pressure and temperature on retention time, retention factor, resolution and selectivity on bare silica: theophylline (1), caffeine (2), and 2-EP: caffeine (1), theophylline (2); t_{ri} – retention time, k'_i – retention factor, R_s –resolution, $\acute{a}$ – selectivity

Stationary phase	T(°C)	Modifier (%)	p_{in} (p_{out})	$t_{r,1}$	$t_{r,2}$	k'_1	k'_2	R_s	α
bare silica	35	Methanol	125 (95)	3.96	4.83	4.66	5.91	2.73	1.27
		15%	160 (130)	3.88	4.56	4.54	5.51	2.14	1.21
			200 (170)	3.59	4.11	4.12	4.87	1.98	1.18
	35	Ethanol	200 (170)	5.63	8.23	6.51	9.97	5.2	1.53
		15%	225 (193)	5.36	7.86	6.14	9.48	4.58	1.54
			250 (215)	5.09	7.62	5.78	9.16	4.85	1.58
	45		200 (170)	6.75	9.31	8	11.41	4.5	1.43
	55		200 (170)	7.55	10.04	9.07	12.39	4.36	1.33
2- EP	35	ethanol	200 (170)	2.46	3.29	3.92	5.58	4.67	1.43
		10%	225 (192)	2.33	3.2	3.66	5.41	4.95	1.48
			250 (213)	2.23	3.14	3.46	5.29	5.18	1.53
	45		200 (170)	2.49	3.47	3.99	5.93	5.21	1.49
	55		200 (170)	2.71	3.87	4.43	6.73	4.43	1.52
	35	methanol	200 (170)	1.45	2.13	1.9	3.27	4.6	1.72
	45	10%	200 (170)	1.53	2.22	2.06	3.43	4.65	1.67
	55		200 (170)	1.63	2.36	2.26	3.72	4.96	1.65

an increase in production. Furthermore, compounds could co-elute at higher sample concentrations, due to low values of resolution.

3. 2. Results of Preparative SFC

The bare silica stationary phase was selected for scale-up, due to higher selectivity, hence resolution. Additionally, operating conditions, type of modifier and concentration of modifier in the mobile phase were selected.

Separations on pilot preparative scale SFC apparatus were performed on the silica stationary phase with particle size distribution from 15–25 μm, in order to avoid greater pressure drops. Preliminary testing was performed on an analytical scale with custom-packed columns with having packing material 15–25 μm. Non-efficient separation with overlaying chromatographic peaks was obtained, when methanol was used as the modifier on the custom-packed column. Fully separated peaks were obtained on the custom-packed column (length 250 mm) when ethanol was used as modifier. In Fig. 12 a chromatogram of theophylline and caffeine, is shown at inlet pressure 160 bar 35 °C and 10% (*w/w*) of ethanol as modifier. The effect of the process parameters was similar as in those separations performed on an analytical scale and discussed in the previous section.

Separations performed on column with smaller particles provided more efficient separations (more symmetrical peaks and smaller width on the half of the peak), while bigger particles provided lower pressure drops.

In the next stage maximum masses of the tested compounds per injection were investigated on an analytical scale as described in the experimental section. Maximum concentrations of the tested compounds within the sample were identical to the concentrations at saturation.

Before operating on the pilot scale, the column of the pilot preparative SFC was packed with stationary phase, where the height of the packing material was similar to the length of the column (~250 mm) used during analytical scale SFC. Inner diameter of the column was 100 mm.

A sample with known concentrations of tested compounds was injected in the mobile phase and transported to the column of the pilot preparative scale SFC, at inlet pressure 160 bar and heater temperature of 45 °C, 10% (w/w) of ethanol at mass flow-rate of the mobile phase 1.1 kg/min. Under the given conditions no separation was obtained due to co-elution. This was probably a consequence of the fact that the column was not equipped with a heater and therefore the temperature in the column was not constant and was lower than in the heater. Further, stationary phase could be compressed during operation with the column piston and consequently the height of the stationary phase was lower.

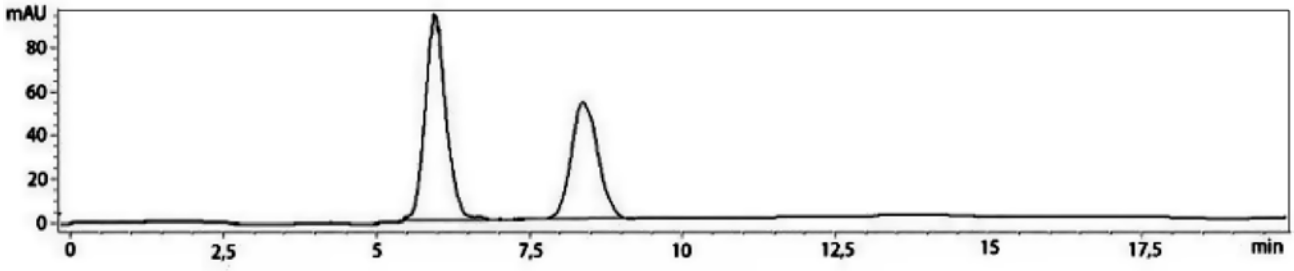

Fig. 12. Chromatogram of theophylline and caffeine obtained on analytical scale SFC at 160 bar, 35 °C, 10% ethanol as modifier on bare silica 15–25 μm; 1st peak theophylline, 2nd peak caffeine

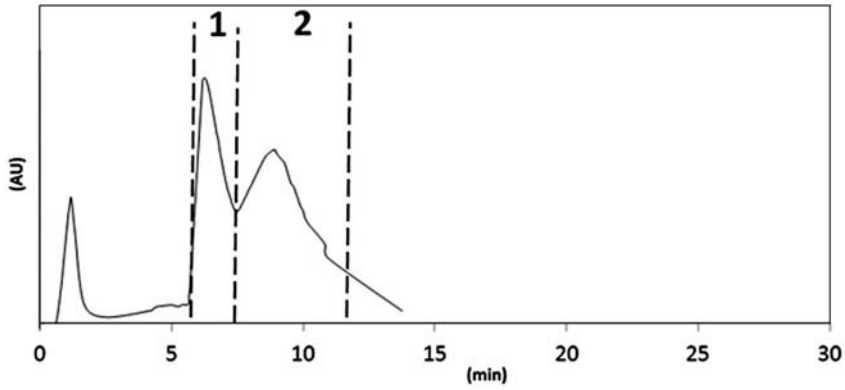

Fig. 13. Chromatogram obtained on pilot preparative to production scale SFC, 1st fraction and 2nd fraction; temperature of the column was 45 °C, column inlet pressure was 180 bar. Modifier was added after injection at mass fraction of approximately 6%, mobile phase flow was approx. 1.2 kg/h (measured on the flow meter),

No separation was obtained either, when the temperatures and pressures were changed. When the height of the stationary phase was increased (~350 mm) and temperature in the column was increased to 45 °C, separation with partially resolved peaks was obtained.

Although, the height of the stationary phase in the column was higher, compounds co-eluted, and the peaks were slightly overlaid. The chromatogram in Fig. 13 was obtained at inlet pressure 180 bar, temperature 45°C and 6% of ethanol at a mass flow-rate of the mobile phase of 1.2 kg/min. The concentration of both compounds in the injected sample was 50% (*w/w*). In order to reduce modifier consumption modifier pump was powered on after injection of the sample. Even so, collection of the fractions was possible.

The fraction in separator S1 contained around 95% of theophylline and 5% caffeine, while the fraction in separator S2 contained 85% of caffeine and 15% of theophylline. The peaks on the chromatogram (Fig. 13) correspond to ethanol, theophylline (1), and caffeine (2).

Even though separation was achieved, further investigations have to be performed in order to improve separation using pilot preparative scale SFC apparatus.

4. Conclusions

Supercritical fluid chromatography is a very promising method for separating of many compounds. It is successfully applied in analytics and it has great potential even in preparative to production scales. In presented work operation of pilot preparative scale SFC apparatus was investigated. The influence of process parameters on the separation of caffeine and theophylline was studied using analytical scale apparatus and subsequently preparative scale apparatus was used for fractionation of the both compounds. It can be concluded that the type of the modifier is important for SFC applications. Ethanol, which is in comparison to methanol less polar, provided more resolved peaks. This is favorable for preparative SFC applications. Oppositely, methanol is used in most analytical applications.

The content of the modifier in the mobile phase has a much more significant impact on retention behavior than density, hence pressure and temperature alone, because dielectric constant (solubility) is increased more by the addition of a modifier. Too high a content of the modifier in the mobile phase causes co-elution of the compounds. Furthermore, too high content of the modifier is not economical, due to increase of utility costs.

The traditional approach was used for scaling-up. Even though, the method is relatively simple, often no optimal results are achieved. Therefore, additional experiments were required on pilot preparative SFC apparatus to obtain separation. As a result the initial mixture, which contained 50% of both compounds, was separated into two fractions that contained 95% of theophylline and 85% of caffeine. The practical demonstration of scale-up procedure on the pilot preparative scale SFC can be used for scale-up of real systems *e. g*. fractionation of the plant extracts.

It can be concluded, that preparative SFC is a very promising method for fractionation and purification of bioactive compounds within food or pharmaceutical industries in the future.

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Povzetek

Raziskali smo vpliv procesnih parametrov na kromatografijo s superkritičnimi fluidi (SFC) skupaj z povečevanjem obsega na preparativno merilo. V tem okviru smo posamezno raziskali odvisnost tlaka, temperature, tipa in koncentracije so-topila, ter tipa stacinarne faze na separacijo testnih komponent (kofeina in teofilina) s SFC. Eksperimente smo izvedli na napravah za SFC v analitičnem in pilotnem preparativnem merilu. Separacije smo izvajali na dveh stacionarnih fazah: čisti silica in silica 2-etilpiridina. Kot mobilno fazo smo uporabili CO_2/metanol or CO_2/etanol pri povišanih tlakih in temperaturah. Rezultate eksperimentov iz SFC naprave v analitičnem merilu smo uporabili za povečevanje obsega na preparativno merilo.
Cilj študije je bila evaluacija vpliva procesnih parametrov na separacijo testnih komponent in praktični prikaz povečevanja obsega in obratovanja pilotne preparativne naprave, ki sodi med večje podobne naprave v globalnem merilu.