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Ultra-fast separation of estrogen steroids using subcritical fluid chromatography on sub-2-micron particles

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ABSTRACT

Estrogen steroids, represented by estradiol and its related substances, include both structurally very close and simultaneously different analogs. Their separation still remains an analytical challenge. Subcritical fluid chromatography (SbFC) on sub-2-micron particles was found to be an appropriate tool to obtain fast and efficient separation of nine target analytes. Among the four tested stationary phases charged hybrid modified with PFP (pentafluorophenyl) moiety was found to be the most convenient providing the fastest separation within 1.6 min using quick gradient elution with carbon dioxide and methanol as an organic modifier. However, complete separation was obtained also on other tested phases including bare hybrid stationary phase, hybrid stationary phase modified with 2-EP (2-ethylpyridine) and also C18, which is less typical in SbFC. The baseline separation on the latter columns was achieved by means of a temperature increase, a change in organic modifier type and gradient time increase respectively.

Quantitative performance was evaluated at optimized conditions and method validation was accomplished. Excellent repeatability of both retention times (RSD < 0.15%) and peak areas (RSD < 1%) was observed. The method was linear in the range of 1.0–1000.0 μg/ml for all steroids with the lowest calibration point being an LOQ, except for Δ-derivatives, that provided better sensitivity and thus LOQ of 0.5 μg/ml. The sensitivity was sufficient for the analysis of real samples although it was still five times lower compared to UHPLC-UV experiments.

1. Introduction

Supercritical fluid chromatography (SFC) is a separation technique widely used in pharmaceutical industry for chiral [1–3] and achiral separations [4–7] in both analytical and preparative scales. Supercritical conditions are reached when the substance occurs above its critical temperature and pressure. Such a mobile phase demonstrates lower viscosity and higher diffusivity relative to liquids. Therefore, minimum height equivalent to a theoretical plate values about similar to LC are obtained at linear velocities three to five-times greater than in LC [8]. Low critical values of carbon dioxide (temperature 31.1 °C and pressure 73.8 bar) allowed it to become the most preferred mobile phase in SFC. Due to its lipophicility, carbon dioxide enables elution of only a limited number of species. For the analysis of less lipophilic compounds the addition of an organic modifier is necessary in order to increase solvating power of carbon dioxide. It is necessary to emphasize, that the addition of an organic modifier dramatically increases the critical values of carbon dioxide. Therefore, under commonly used chromatographic conditions at about 120–150 bar and about 40 °C the mobile phase does not exist anymore in supercritical state, when higher percentage of organic modifier is added [9]. Therefore the technique would further be referred to as a subcritical fluid chromatography (SbFC), once the organic modifier was used, which has previously also been referred to as packed-column SFC.

SFC and SbFC have been already denoted as interesting methods for the separation of steroid compounds and were applied for the analysis of corticosteroids [10–12], estrogens [13,14], androstenone [15] and selected synthetic mixtures of steroids from various classes [16–18]. Hanson [19] and Berger et al. [20] evaluated retention behavior of steroids of different polarities and structures in SbFC using various stationary phases including bare silica and silica modified with phenyl, nitro, cyanopropyl, diol, C18, amino [19] and diol, cyanopropyl, sulfonic acid, C18 and phenyl respectively [20]. The importance of stationary and mobile phase polarity have also been discussed by Hanson besides temperature and pressure variations and their influence on selectivity using isocratic elution [21].

However, most of the works date back to 80s–90s and the separation runs were still in the range of 8–10 min or much longer (> 20 min), which is not anymore the state of the art in these kinds of analyses. Apparently, capillary SFC methods provided even longer analysis time (30–40 min) due to low solvating power

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of CO_2 mobile phases [18]. Only some selected androgen steroids were successfully separated in 3 min [22] or in less than 1 min analysis [6] using ultra-fast SbFC on sub-2-micron particles. A group of estrogen steroids that include both structurally very close and different analogs simultaneously, i.e. estradiol and its related substances, which should be monitored during QC (quality control) of the drug product according to the requirements of the drug master file (Fig. 1), represents an analytical challenge. They have been analyzed previously using LC-UV with only a partial success, as the separation of the main critical pair of estrone and Δ-estrone has not been reached even when using more than 20 min gradient analysis on different stationary phases [23]. Other approaches common for the analysis of estrogen steroids include LC or GC coupled to tandem mass spectrometry (MS/MS) [24–27]. When MS/MS detection is applied, the baseline separation of individual components is not critical except for the isomeric species, such as e.g. α- and β-estradiol, which still remains fairly challenging. Therefore, even these relatively modern LC or GC–MS/MS approaches often required substantially long analysis times (usually 15–20 min) [28]. The introduction of UHPLC enabled to shorten the analysis times bellow 10 min [29,30].

Recently, new SFC platforms and new stationary phases have been commercially introduced in order to extend application potential and reliability of SFC and SbFC methods. Similarly to UHPLC, sub-2-μm particles are of great importance, as they enable highly efficient and ultra-fast separations [6,22]. The aim of this work was to develop ultra-fast SbFC method for the separation of the group of structurally similar/different steroids. Individual parameters that influenced the separation and selectivity in the SbFC method are pointed out. The amount of contribution of these parameters to the change in method selectivity is discussed in detail. Quantitative aspects have still remained challenging in SFC and SbFC applications due to low repeatability of both retention times and injection process, which was critical especially with the old type of SFC instruments. The method repeatability, validation and the applicability to real sample is also shown.

2. Experimental

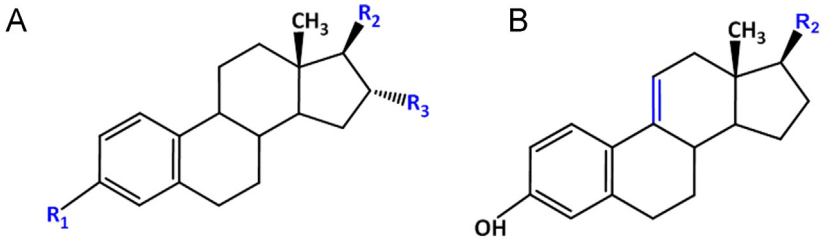
2.1. Chemicals and reagents

Reference standards of steroids were used for the purpose of this study. Estriol (99.9%), β-estradiol (>98%), estrone (>99%) and ethinylestradiol (≥98%) were obtained from Sigma-Aldrich, Czech Republic. α-estradiol hemihydrate (98.8%), estradiol-3-methylether (98.3%), and estradiol-17-acetate (99.3%) were obtained from Vetranal, Germany. 1,3,5,(10),9(11)-estratetraen-3, 17-β-diol designated as Δ-estradiol and 1,3,5,(10),9(11)-estratetraen-3-ol, 17-on designated as Δ-estrone were obtained from Steraloids, USA. Methanol, ethanol, propan-2-ol, acetonitrile gradient grade and tetrahydrofuran HPLC grade were provided by Sigma-Aldrich.

2.2. Ultra high performance subcritical fluid chromatography

The supercritical fluid chromatography system Acquity UPC^2 (Waters, Milford, USA) consisted of Acquity UPC^2 binary solvent manager, Acquity UPC^2-FL sample manager, Acquity UPC^2 convergence manager, Acquity column manager and Acquity UPC^2 PDA detector.

All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject 1 μL. Methanol was used as a needle wash solvent. The separation was performed using four SFC dedicated stationary phases including: Acquity UPC^2 BEH, Acquity UPC^2 BEH 2-EP, Acquity UPC^2 CSH PFP and Acquity UPC^2 HSS C18 SB, all of them at 100 × 3.0 mm and 1.7 μm particles. Gradient elution was performed using CO_2 (>99.995%, LindeGas, Czech Republic) and various modifiers including methanol, ethanol, propan-2-ol and acetonitrile at flow-rate 2.5 ml/min. The temperature was optimized in the range of 40–90 °C. The BPR (back-pressure regulator) pressure was typically set-up to 2000 psi and the variations were observed in the range of 1500–2500 psi. UV detection was performed at 225 nm.



	compound		R1	R2	R3	Log *P*	pK$_a$
1	methoxy-estradiol	EST-me	-OCH$_3$	-OH		5.03	15.07
2	estradiol 17-acetate	EST-ac	-OH	-O-CO-CH$_3$		4.98	10.26
3	estrone	ESN	-OH	=O		4.40	10.25
5	α-estradiol	α-EST	-OH	··OH		3.91	10.27
6	ethinylestradiol	et-EST	-OH	-OH, -CΞCH		4.00	10.24
7	β-estradiol	β-EST	-OH	-OH		3.91	10.27
9	estriol	ESTRI	-OH	-OH	-OH	2.91	10.25
4	Δ-estrone	d-ESN		=O		3.62	10.41
8	Δ-estradiol	d-EST		-OH		3.59	10.39

Fig. 1. The structures of estrogen steroids included in this study and their physico-chemical properties. Most of the steroids are derived from structure (A), except for Δ-estradiol and Δ-estrone, that are derived from structure (B).

2.3. Preparation of standard solutions

The stock standard solutions of all steroids were prepared in tetrahydrofuran due to its good compatibility with SbFC mobile phases (aprotic solvent, non-H-bond donor capability) providing good peak shapes and solubility of the analytes. The concentrations of the stock solutions were 1.0 mg/ml or 10.0 mg/ml for the linearity measurements. They were combined in mixed working standard solution and further diluted also with tetrahydrofuran.

2.4. Preparation of gel samples

Oestrogel gel formulation (Laboratories Besins International, Paris, France) containing 60 mg/100 g of estradiol was used as a real sample for the evaluation. The gel samples were extracted using a mixture of THF/ethylacetate (1:1). 2.00 g of gel were accurately weighed into a centrifugation tube. 10 ml of extraction media were added and the tube was closed with the screw cap. The extraction was performed on ultrasonic bath for 20 min. The samples were centrifuged at 4000*g* for 5 min and the supernatant was used for the analysis after the filtration through PTFE membrane (0.20 μm).

2.5. Method validation

The method was validated in terms of accuracy and precision that were evaluated at three concentration levels (100, 10 and 1 μg/ml) in three replicates according to the requirements of ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines [31]. Method linearity was evaluated using serial dilutions of stock solutions in the range of 0.5–1000.0 μg/ml with tetrahydrofuran, injected always in triplicates. Method sensitivity was expressed as LOQ, which was calculated based on *S*/*N* ratio and correlated to the values of accuracy and precision. Method selectivity was assured by the calculation of peak purity using Empower software (Waters). System suitability test (SST) including repeatability of retention times and peak areas, peak asymmetry and resolution was an important part of the evaluation procedure.

3. Results and discussion

SFC and SbFC methods development for the separation of complex mixtures containing components that vary in physico-chemical properties is typically based on gradient elution, similarly as in LC. The elution strength of a supercritical fluid mobile phase depends on a density of the fluid. The density can be changed by the variation in the pressure, temperature, and also with an addition of an organic modifier, which has further influence to critical values of the mobile phase and solvent strength.

3.1. The influence of stationary phase

Tested stationary phases included currently available SFC stationary phases containing sub-2-micron particles. These stationary phases differed both in selectivity and polarity representing very polar (bare hybrid phase developed as bridged ethyl hybrid (BEH) and the same hybrid phase modified with 2-ethylpyridine (2-EP) group), moderately polar (charged hybrid phase modified with PFP group) and non-polar (C18 modified silica) stationary phases [32]. For polar phases an increase in analyte polarity should lead to an increase in retention, while an increase in molar volume should decrease it corresponding to the behavior in normal-phase liquid chromatography [32,33]. Non-polar stationary phases represented mostly by alkylsiloxyne-bonded phases favor dispersive interactions between the solutes and the stationary phase, which increases with increasing alkyl chain [34]. Moderately polar stationary phases often including aromatic moieties are characterized by charge transfer, dispersion and π–π interactions, depending on the specific type of stationary phase [32,35]. The selectivity of PFP modified stationary phase has been only scarcely examined in SFC and SbFC applications using sub-2-micron particles [6].

Similarly to other chromatographic techniques, the choice of stationary phase in SbFC has the strongest impact on the selectivity of separation as it is demonstrated in Fig. 2 and as was expected due to the different nature of selected stationary phases [32]. The experiments were performed at the same chromatographic conditions described in Section 2.2 using gradient elution from 5% to 30% of methanol as an organic modifier at 2.5 ml/min, 55 °C and 2000 psi on all stationary phases. At selected gradient conditions a complete resolution of all nine components of the mixture was obtained on charged hybrid column modified with PFP group in 1.6 min (Fig. 2A). Compared to other tested stationary phases, PFP modified phase demonstrated the lowest retentive capabilities, which could be attributed to low polarizability and lack of dispersive interactions at this phase [35]. On the other hand, the strongest retention of steroids was observed on C18 stationary phase, probably due to strong dispersive interactions and some contribution of π-interactions [34]. Although significant changes in steroid retention were observed among the four columns due to different stationary phase properties, important selectivity changes, such as reversed elution order, were observed to a lesser extent. The elution order was identical on charged hybrid PFP and on a bare hybrid column (Fig. 2A and D), which is somewhat surprising considering the differences in the stationary phases and interaction mechanisms. Slight differences in selectivity were observed on 2-EP modified hybrid column (coelution of peaks α-estradiol and β-estradiol, reversed order of α-estradiol and ethinylestradiol) and on silica based C18 modified column (partial coelution of the peaks of estrone and Δ-estrone and later elution of the peak of methoxyestradiol), Fig. 2C and B. These findings contradict partially the previously published studies focused on separation of steroids on various stationary phases modified with substantially different chemistries (pure silica and silica modified with phenyl, nitro, cyanopropyl, diol, C18, amino, and sulfonic acid), where the elution order was not influenced by stationary phase chemistry and the elution order changes were not observed at all, in spite of the variability of used phases [19,20].

In early 90s SFC and SbFC analyses of steroids was recommended to be performed using only polar stationary phases, such as diol or cyanopropyl with polar organic modifiers. Non-polar polar stationary phases, including C18 or phenyl phase demonstrated significant peak tailing similarly as using of less polar organic modifiers (such as acetonitrile or tetrahydrofuran) [20]. Due to the huge progress that have been made in the development of stationary phases, substantially improved peak shapes were now observed for all examined steroids on all stationary phases included in this study, both polar and non-polar. Even though not exactly the same stationary phases were tested, for charged hybrid modified with PFP group, for hybrid stationary phase modified with 2-EP and for phenyl phase similar type of interactions including especially π–π interactions might be supposed. The values of peak asymmetry were close to 1.0 for all analytes on all tested stationary phases (Table 1, Fig. 2) compared to the findings of Hanson, who found asymmetry values for estrone 1.60, estriol 1.70, β-estradiol 1.65 and α-estradiol 1.85 on a phenyl modified silica based stationary phase and gradient elution with MeOH as an organic modifier [19]. Moreover, in our study, the values of peak asymmetry were not found to be dependent on the

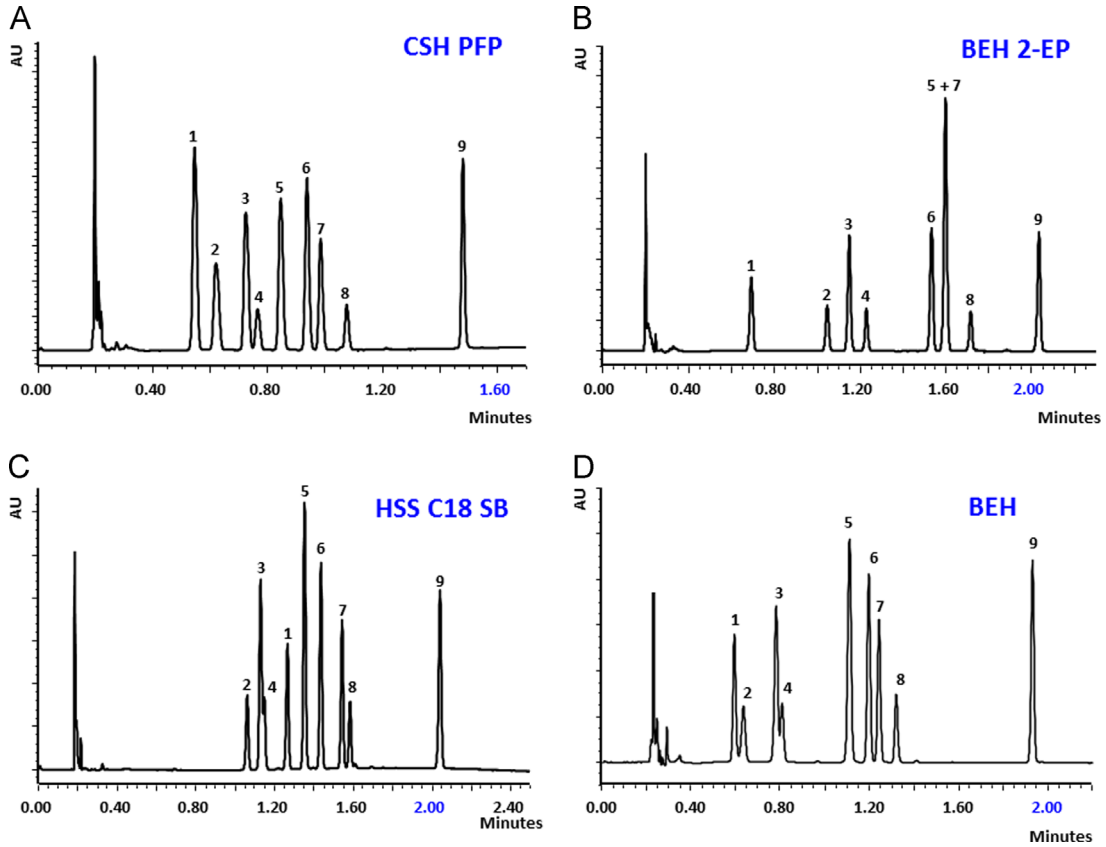


Fig. 2. The separation of estrogen steroids on four tested stationary phases: (A) Acquity UPC^2 CSH PFP, (B) Acquity UPC^2 BEH 2-EP, (C) Acquity UPC^2 HSS C18 SB, (D) Acquity UPC^2 BEH using gradient elution with 5–30% of methanol in 3 min at 2.5 ml/min. The temperature was set at 55 °C and BPR pressure to 2000 psi. (1) methoxy-estradiol, (2) estradiol-17-acetate, (3) estrone, (4) Δ-estrone, (5) α-estradiol, (6) ethinylestradiol, (7) β-estradiol, (8) Δ-estradiol, and (9) estriol.

Table 1
The results of resolution, peak asymmetry and peak capacity for tested stationary phases. Gradient elution was performed using MeOH from 5% to 30% in 3 min on all stationary phases. The temperature was set-up at 55 °C and BPR pressure to 2000 psi.

Analyte	CSH PFP			BEH 2-EP			BEH			HSS C18 SB		
	R_s	A_s	P_c	R_s	A_s	P_c	R_s	A_s	P_c	R_s	A_s	P_c
Estradiol methylether	NA	1.16	119	NA	0.92	111	NA	1.05	127	4.97	0.92	137
Estradiol acetate	2.94	1.18	99	12.75	0.98	111	2.22	0.89	94	NA	0.95	127
Estrone	3.62	1.22	111	3.80	0.95	111	4.84	NA	105	NA	NA	NA
α-Estradiol	3.10	1.18	119	2.54[a]	0.90[a]	119[a]	9.53	0.95	105	3.93	0.93	137
Ethinylestradiol	3.57	1.21	127	11.93	0.91	119	3.21	0.99	119	3.92	0.93	148
β-Estradiol	2.09	1.18	137	2.54[a]	0.90[a]	119[a]	1.81	0.98	127	5.10	0.91	137
Estriol	18.85	1.18	161	12.50	0.85	119	25.21	0.95	137	20.24	0.91	127
Δ-Estrone	1.58	1.19	137	3.09	0.98	119	0.97	NA	109	NA	NA	127
Δ-Estradiol	4.01	1.08	137	4.55	0.94	119	3.18	1.10	119	1.89	0.94	137

The values of resolution (R_s) were not available (NA) for the first peak eluted in chromatogram and for the peaks that were only partially separated. The values of asymmetry (A_s) were not available (NA) for the peaks that were only partially separated. P_C – peak capacity.

[a] The value is given for the sum of the peak of α- and β-estradiol, that fully coeluted.

presence of free hydroxyl and keto groups in the tested group of estrogens contrary to the previous findings (Table 1) [19].

The peak capacities obtained at tested SbFC conditions ranged within 94–161 (Table 1) and were quite comparable among the four tested stationary phases except for few cases (e.g. peak of estradiol acetate on PFP and bare hybrid stationary phase). The peak resolution on charged hybrid modified with PFP and hybrid stationary phase modified with 2-EP was always >1.5, which means complete baseline separation. In case of 2-EP stationary phase the pair of α- and β-estradiol fully coeluted. On silica based C18 and on bare hybrid stationary phase estrone and Δ-estrone were not perfectly separated, thus the resolution could not be calculated or corresponded only to 0.97 respectively. Thus, the critical pairs of tested estrogen steroid mixture included α-/β-estradiol and estrone/Δ-estrone. The isomers of estradiol were successfully separated with more than baseline resolution on all tested stationary phases except for 2-EP modified hybrid stationary phase (Fig. 2B, Table 1), where they completely coeluted. The elution order of the two isomers was reversed compared to the previously published results [19], which speculated the mechanism of polar interactions of α-estradiol (having slightly higher apparent polarity) with the free silanol groups of silica based stationary phases enabling thus substantially stronger retention of α-estradiol. These interactions are expected to be limited on all

kinds of hybrid stationary phases. Estrone and Δ-estrone were completely separated on charged hybrid stationary phase modified with PFP group and on 2-EP modified hybrid phase, while only partial separations were observed on the remaining two stationary phases (Fig. 2). An enhanced selectivity of PFP and 2-EP stationary phases towards the separation of steroids could be attributed to π–π interactions between the aromatic functional moieties of these stationary phases and steroid skeleton. Further improvement of the separation of the two critical pairs of peaks on other tested stationary phases was obtained using various approaches as will be discussed in following paragraphs. Charged hybrid stationary phase modified with PFP group was found to be particularly interesting for the analysis of estrogen steroids.

3.2. The influence of organic modifier

The applicability of CO_2 as a mobile phase in SFC is limited due to its low polarity. In analysis of steroids it was recognized to be ineffective already in early 80s and 90s. Therefore, methanol was the most practically used organic modifier due to better peak symmetries in both gradient and isocratic elution providing adequate solvent strength for the elution of steroid mixtures. Other organic modifiers, such as acetonitrile or tetrahydrofuran provided very poor peak shapes, which might be explained by missing H-bond donor capacity [11,19,20]. Other alcohol modifiers were usually not tested. Organic modifiers tested in this study included methanol, ethanol, propan-2-ol and acetonitrile. Their effect on retention and selectivity was evaluated on the four stationary phases.

It is important to note, that the prediction of the influence of the organic modifier is quite difficult. A positive effect of alcohol type modifier change on the separation selectivity of nine estrogens was observed on hybrid stationary phase modified with 2-EP (Fig. 3A). With methanol, the critical pair of peaks 5 and 7, i.e. α- and β-estradiol, did not separate at all. With the addition of ethanol, partial separation was observed, while with propan-2-ol almost baseline separation was obtained. Solvating power of carbon dioxide decreased with the decreasing polarity of the solvent as follows: propan-2-ol < ethanol < methanol. The highest retention factors were then obtained with acetonitrile. However, using acetonitrile as an organic modifier led to substantial distortion of peaks (data not shown), which is in agreement with the previously published results [20]. Therefore, its application was only favorable in mixtures with alcohols to change further the polarity and thus finely tune the selectivity.

However, a higher retention obtained by changing of methanol for ethanol or propan-2-ol did not necessarily mean only an improvement in separation selectivity as it is shown for C18 stationary phase (Fig. 3B). The critical pair of peaks 3 and 4 being estrone and Δ-estrone, the pair most challenging for separation was to partially split with methanol and subsequently fully coelute

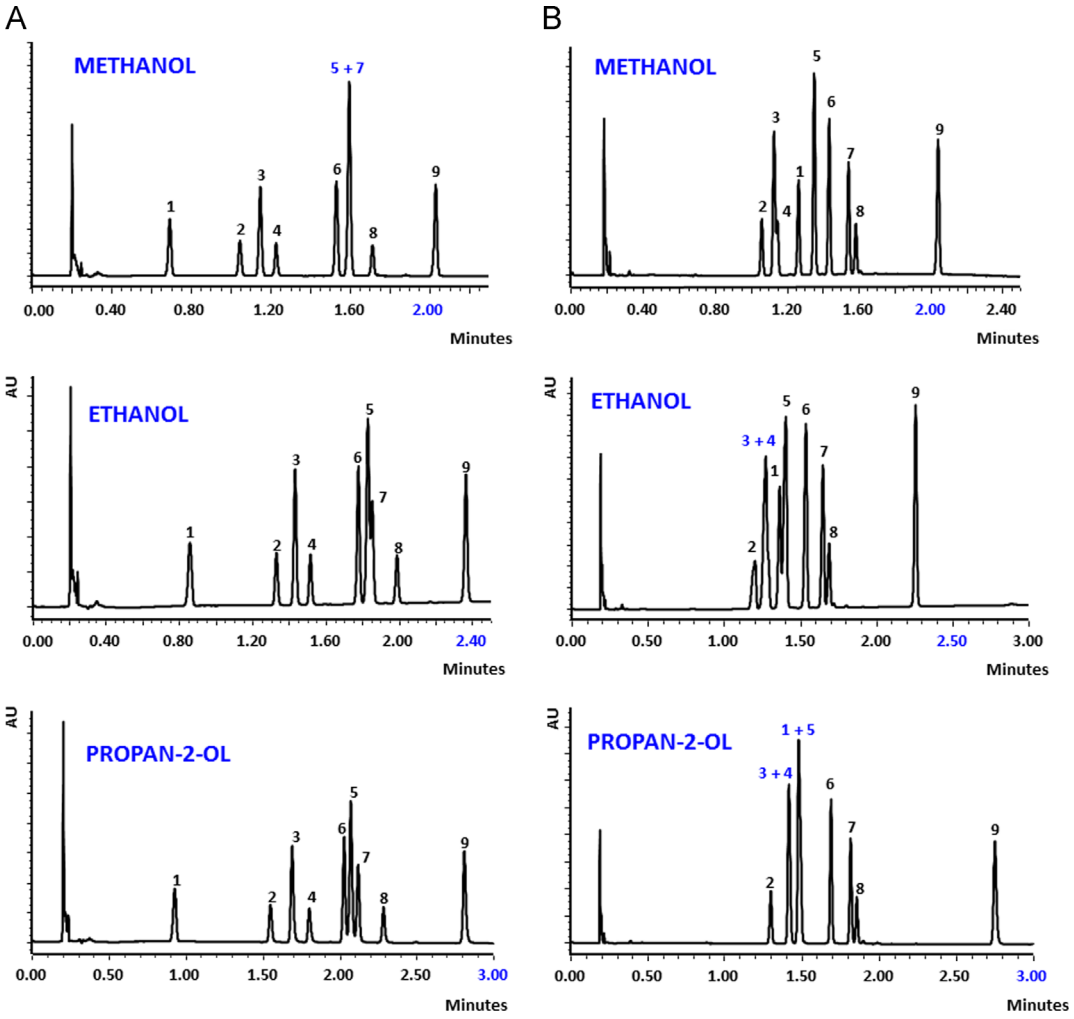


Fig. 3. The influence of organic modifiers methanol, ethanol and propan-2-ol on the separation of estrogen mixture. (A) Acquity UPC² BEH 2-EP, (B) Acquity UPC² HSS C18 SB using gradient elution with 5–30% of methanol in 3 min at 2.5 ml/min. The temperature was set at 55 °C and BPR pressure to 2000 psi. Peak numbers correspond to Fig. 2.

with the addition of ethanol or propan-2-ol. Moreover, another coelution of methoxyestradiol and α-estradiol was observed when propan-2-ol was used as an organic modifier. Similar effect was observed on charged hybrid stationary phase modified with PFP ligand. A coelution of Δ-estrone and α-estradiol and estrone and α-estradiol was observed with ethanol and propan-2-ol respectively, while the baseline separation was observed with methanol (data not shown). Practically no effect on separation selectivity when changing the alcohol type was observed on bare hybrid stationary phase (data not shown). Therefore, the influence of an organic modifier type on the separation selectivity must be always optimized experimentally. Methanol appears to be a good modifier of the first choice. Except for common alcohols, their mixtures with acetonitrile can provide further interesting selectivity.

3.3. The influence of gradient set-up

The elution in SbFC can be performed both isocratically and by means of gradient elution. In LC the change of gradient slope can significantly contribute to the change of separation selectivity. In SbFC, the gradient of density and solvent strength is formed with an addition of an organic modifier. Moreover, when increasing the modifier content the retention is decreased also due to a competition between the modifier and analytes for the interaction sites on the stationary phase. Practically, the concentration of organic modifier is usually kept below 40% or less in order to assure supercritical conditions or at least reasonable viscosity and diffusion coefficients (subcritical conditions) [9]. Nevertheless, already small concentrations of polar modifiers can substantially increase the solvent strength to enable elution of steroid compounds [11,19,21].

Similarly to LC, for the method development in SbFC it is convenient to perform gradient screening, especially when structurally different species are analyzed. Typically, gradient elution starting from about 2–5% up to 30–40% of organic modifier (if this is allowed by the pressure limits of the instrumentation and the stationary phase at given flow-rate and BPR pressure) is employed. However, the change in gradient conditions (initial and final compositions of mobile phase) had almost no importance in SbFC if the selected gradient window was wide enough to cover the elution of all the analytes in the mixture (Fig. 4A). Once the analytes were eluted in a certain modifier concentration window (5–15%, 3 min gradient time), i.e. at the certain mobile phase density/solvent strength, it was impossible to change further the selectivity of the separation. Thus changing the final composition of mobile phase to 25 or 35%, while keeping the same gradient time had no more significance as the density/solvent strength of mobile phase played the crucial role and the analytes would always be eluted within 5–15% concentration range with the same selectivity pattern. It was only possible to improve resolution by increasing the gradient time or to speed-up an analysis by adjusting the final composition of the mobile phase in gradient needed to elute exactly all the analytes in a certain gradient time. In this way the adjustment of the separation of nine steroids on charged hybrid phase modified with PFP was made. The initial gradient elution performed from 5–30% of MeOH in 3 min revealed that all the components were eluted within 1.6 min with about 17% of MeOH. After the gradient adjustment to these conditions practically the same selectivity was obtained in much shorter period of time (1.6 min). Similar behavior was observed also on other three stationary phases with slightly different MeOH concentrations needed for the elution of analytes.

On the other hand, if the compounds were not retained enough/were not eluted, i.e. if the gradient window was not selected appropriately, the change in gradient profile might have played some role, as it is demonstrated on bare hybrid stationary phase (Fig. 4B). The decrease of MeOH content at initial conditions of gradient allowed for the improvement of the resolution of the pairs methoxy-estradiol/estradiol-17 acetate and estrone/Δ-estrone. Therefore, for the initial screening and method development for the mixtures containing compounds of different physico-chemical properties it is convenient to set-up quite wide elution window, i.e. 2–40%.

3.4. The influence of pressure

When using an organic modifier as a component of the mobile phase in SbFC, both critical temperature and pressure are rapidly elevated with increasing amount of the modifier [9]. The separation is performed in subcritical conditions with mobile phase being a mixture of liquefied CO_2 gas and an organic solvent rather than supercritical fluid. As a consequence, under these conditions the solvating power and the retention can hardly be controlled by changing the pressure because the temperature and the pressure are below the critical values of binary mixture fluid and the densities do not change much by the pressure [9].

As the density of supercritical fluid chromatography increases with the pressure, the elution strength is increased and the retention times become shorter. As expected, the influence of the pressure had a greater effect on retention times than on the separation selectivity. Any substantial change in selectivity of separation was observed on the four tested stationary phases at 55 °C in tested pressure range of 1500–2500 psi (data not shown). In only one case the completely coeluted peaks 5 and 7 (α/β-estradiol) on 2-EP modified hybrid stationary phase demonstrated partial splitting. However, baseline resolution could not be reached by further decrease in pressure due to the instrumentation limits. Moreover, some decrease of peak capacity in the range of 3–15% has been observed on all tested stationary phases. Therefore, the pressure variations did not play an important role in the SbFC method development and optimization. However, pressure stability is extremely important for the repeatability of the retention times.

3.5. The influence of temperature

As the elution strength of a supercritical mobile phase depends on a density of the fluid, higher elution strength is obtained at lower temperature, when the density of supercritical fluid is higher, which is contrary to the retention behavior in LC separations. Elevated temperature thus increases the retention in SFC. However, as was already mentioned in Section 3.4, it is important to keep in mind the mobile phase state. Due to the presence of an organic modifier both critical temperature and pressure of CO_2 increase substantially [9]. Therefore, the influence of temperature is of minor importance, when the separation is performed at higher percentage of organic modifier, i.e. in subcritical conditions. The influence of temperature on the selectivity of separation in SbFC is difficult to predict, similarly as in LC separations. Moreover, the temperature set-up is often limited by the stability restrictions of the stationary phase and a system back-pressure limits (lower temperatures).

Using sub-2-micron particles and defined gradient conditions, the temperature could have been changed within the range of 40–90 °C. Lower limit was defined by the resistance of sub-2-micron particles and pressure limit exceeding maximum system pressure at given flow-rate 2.5 ml/min and BPR pressure at 2000 psi, while higher limit was selected to observe the behavior of the stationary phases under SbFC conditions at elevated temperature and to examine, if elevated temperature might improve the selectivity of separation in SbFC methods. The selection of 90 °C as an upper limit was not in a perfect accordance with the data on

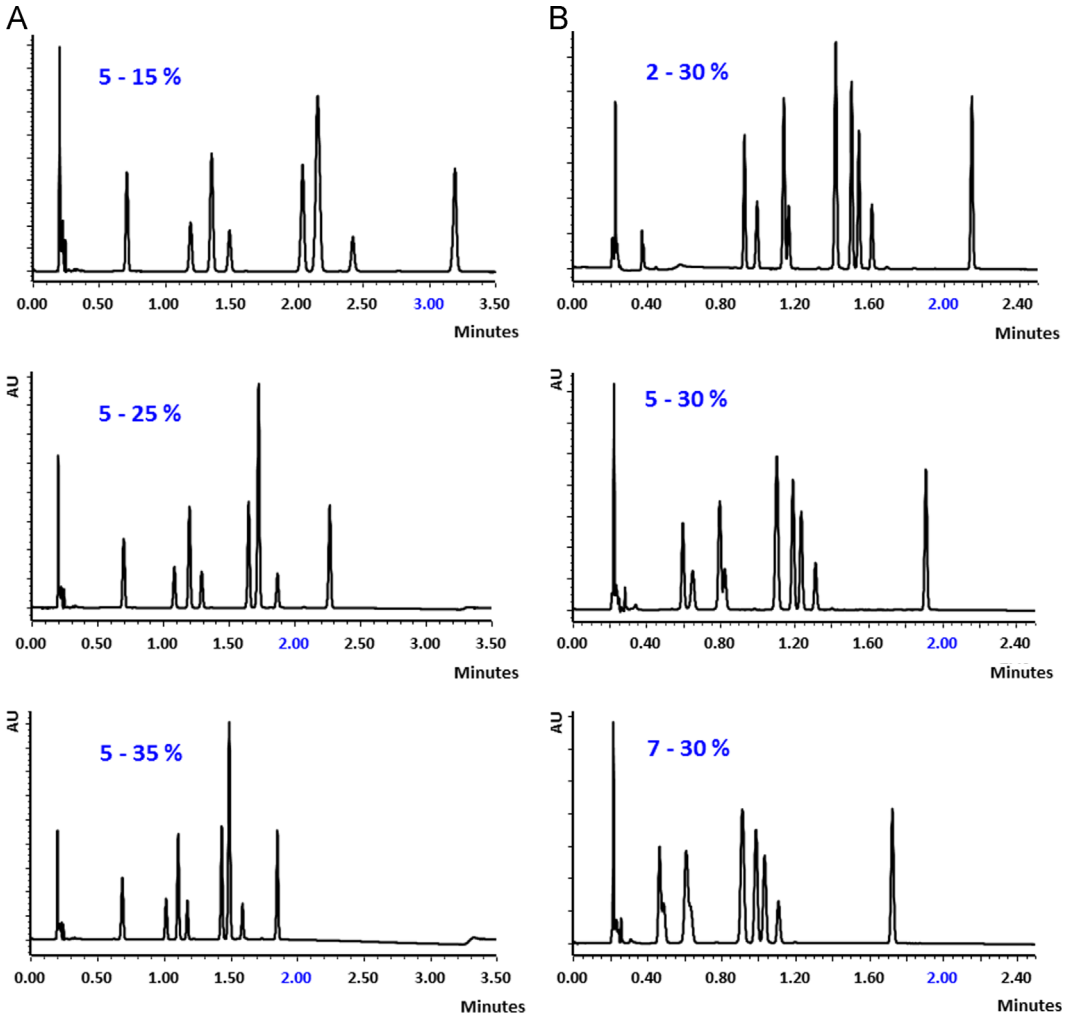


Fig. 4. The influence of a gradient slope on the separation of mixture of estrogen steroids. (A) The change in the final composition of the mobile phase (15%, 25% and 35%) on Acquity UPC^2 BEH 2-EP, (B) the change in the initial composition of the mobile phase (2%, 5% and 7%) on Acquity UPC^2 BEH. The analyses were performed using MeOH as an organic modifier, the temperature was set-up at 55 °C and BPR pressure to 2000 psi. Peak order corresponds to Fig. 2.

stationary phase stability of the manufacturer. However, it is necessary to point out, that the stability of these columns was initially tested at LC conditions and has not been further verified under SFC or SbFC conditions. The stability tests of stationary phases under SFC conditions were not the purpose of this study, as further results revealed elevated temperature not to be highly useful.

In our experiments, elevated temperature was used on all four tested stationary phases. These experiments were only performed in short term scale, therefore no significant changes in column performance were observed, except for silica based C18 column. Generally, peak capacities observed on tested stationary phases increased with increasing temperature ranging within 90–160, except for silica based C18 stationary phase, where the decrease in peak capacity of 7–18% was observed at the temperature higher than 70° C. Other stationary phases provided an increase in peak capacity ranging from 12% to 20% for charged hybrid modified with PFP, 6% to 16% for bare hybrid phase and no change or increase of 6–13% in peak capacity for hybrid phase modified with 2-EP within the temperature range from 50 to 90 °C.

Three different cases of the influence of the temperature on the selectivity of separation are shown in Fig. 5: an improvement of the separation selectivity on bare hybrid stationary phase when increasing the temperature from 50 °C (A) to 90 °C (B), a decrease in selectivity of separation on C18 column when increasing the temperature from 50 °C (A) to 90 °C (B) and finally no effect on hybrid column modified with 2-EP group and on charged hybrid stationary phase modified with PFP group (data not shown). The influence of the temperature on selectivity was thus not straightforward to predict. The fluid in SbFC is more compressible, when the content of MeOH is lower, thus with the elution of the first peaks in chromatogram. Therefore, there should be more effect on retention and selectivity for these early eluted peaks due to the influence of temperature to density compared to later eluting peaks, where the fluid is much less compressible. This aspect was tested using van't Hoff's plots for all tested steroids on the four stationary phases. In our study the analytes were usually eluted between 8% and 23% of MeOH. However, any substantial difference or significant relationship between the behavior of the early and later eluting peaks as a function of temperature has not been observed. The influence of the temperature on the retention of individual analytes was actually random.

Hanson referred decreasing selectivity of the separation of four steroids on polar stationary phases (silica and diol) at elevated temperatures [21]. Greater influence was observed on polar stationary phases than on non-polar stationary phases. However, in these experiments only isocratic elution with different percentage of methanol (up to 16%) was applied. Silanol groups and polar organic modifier that provided coverage of the polar stationary phase were considered to be playing the major role in this phenomenon. A reversed situation was observed in our study, i.e. an

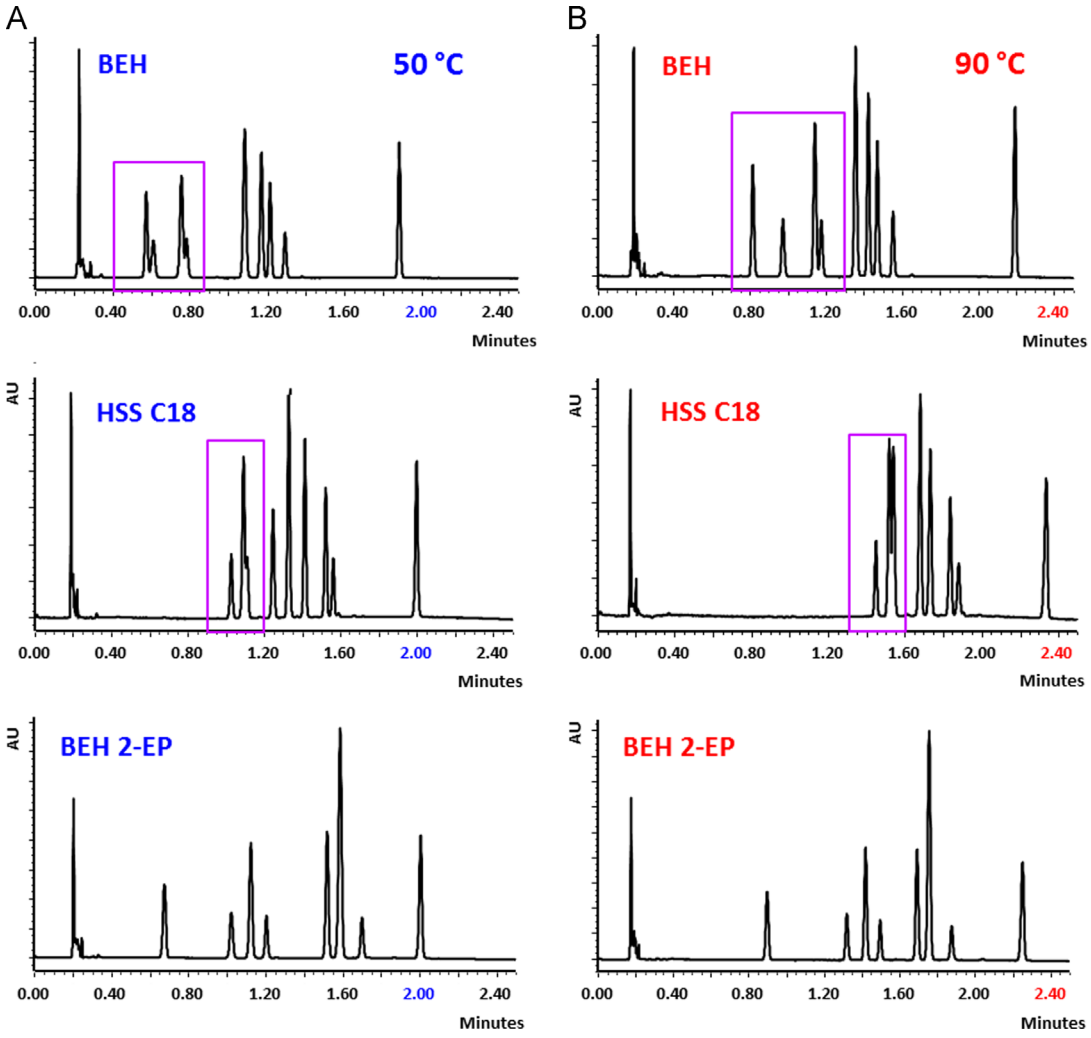


Fig. 5. The influence of the temperature on SbFC separation of estrogen steroid mixture. (A) 50 °C on Acquity UPC^2 BEH, Acquity UPC^2 HSS C18 SB and Acquity UPC^2 BEH 2-EP stationary phase, (B) 90 °C on Acquity UPC^2 BEH, Acquity UPC^2 HSS C18 SB and Acquity UPC^2 BEH 2-EP stationary phase with MeOH as an organic modifier. The BPR pressure was set-up to 2000 psi. Peak order corresponds to Fig. 2. The separation of critical pairs of peaks is highlighted with rectangle.

increase of selectivity was obtained on polar hybrid stationary phase with elevated temperature. This was probably due to lower amount of free silanols and gradient elution with higher percentage of methanol (up to about 23%), which both prevented from methanol/surface equilibrium to change the selectivity more significantly.

3.6. Quantitative analysis and method validation

Even though SFC/SbFC has already demonstrated a great potential in separation tasks, there is still a lack of quantitative reports. Initially, the SFC method's sensitivity and repeatability used to be important issues that did not enable SFC to compare with LC [36]. With the instrumental development and the introduction of sub-2-micron particles particles similar results to LC or UHPLC respectively are now possible to obtain.

Quantitative analysis of nine steroids was performed at optimized conditions on charged hybrid column modified with PFP group at 55 °C and 2000 psi using methanol as an organic modifier. Gradient analysis was adjusted as described in Section 3.3 in order to get analysis time of 1.6 min. Table 2 displays the results of System suitability test showing the values obtained for the repeatability of peak retention times and areas, resolution and peak asymmetry. Excellent repeatability was obtained for both retention times (RSD < 0.15%) and peak area (RSD < 1%). Very good peak shapes were obtained, as is demonstrated by the values of peak asymmetry close to 1.0 for all compounds. The resolution was > 1.5 for all separated peaks.

The method sensitivity was evaluated based on the signal-to-noise ratio (S/N). The concentration corresponding to $S/N \geq 10$ and providing sufficient accuracy and precision was determined as a limit of quantitation, which corresponded to 1.0 μg/ml for most of analytes and was two times lower for Δ-derivatives. Such sensitivity was sufficient for the evaluation of the presence of impurities in real sample of pharmaceutical gel preparations even though it was still five times lower compared to the UHPLC method (see Table 2). The LOQ was the lowest point of calibration curves, which demonstrated very good linearity ($r^2 > 0.9990$) in the range of 1.0–1000.0 μg/ml and 0.5–1000.0 μg/ml respectively for Δ-derivatives respectively. Accuracy and precision were evaluated at three concentrations levels, i.e. 1, 10 and 100 μg/ml using Oestrogel gel samples. The results of accuracy ranged within 96.1–103.5% and the method's precision within 0.4–4.5% of RSD. Method selectivity was assured by calculation of peak purity using Empower software. No interferences from the gel matrix were observed. As all the validation parameters met the requested requirements, the fast SbFC-UV method could be applied for the quantitative analysis of real samples.

Table 2
Method validation and SST: the results of SST (repeatability, resolution and peak asymmetry) and method linearity for the SbFC method. A comparison of sensitivity is shown also for the UHPLC method in the last column of the table.

Analyte	R_s	A_s	linearity	LOQ [μg/ml]	S/N	t_r [RSD %]	A [RSD %]	LOQ UHPLC [μg/ml]
Estradiol Methylether	2.41	1.08	0.9995	1.0	19	0.13	0.59	0.2
Estradiol acetate	2.85	1.08	0.9994	1.0	11	0.12	0.54	0.2
Estrone	9.17	1.10	0.9993	1.0	18	0.11	0.34	0.2
α-Estradiol	2.66	1.09	0.9993	1.0	15	0.09	0.65	0.2
Ethinylestradiol	3.31	1.16	0.9997	1.0	14	0.10	0.37	0.2
β-Estradiol	1.94	1.09	0.9994	1.0	14	0.08	0.67	0.2
Estriol	12.68	1.08	0.9994	1.0	14	0.06	0.62	0.2
Δ-Estrone	1.88	1.01	0.9993	0.5	20	0.10	0.23	0.1
Δ-Estradiol	3.64	0.96	0.9993	0.5	23	0.07	0.38	0.1

R_s – resolution, A_s – asymmetry, and A – peak area.

4. Conclusion

Fast and efficient SbFC separation of the mixture of estrogen steroids was obtained on four SFC dedicated stationary phases with sub-2-micron particles. Among the four stationary phases, charged surface hybrid modified with PFP group outperformed all other tested stationary phases and demonstrated the best potential for the separation of the target group of steroids. Quantitative performance of the optimized method demonstrated very good results in terms of repeatability of both peak areas (RSD < 1.0%) and retention times (RSD < 0.15%). Further method validation provided good results for all tested parameters including linearity, accuracy and precision. The LOQs were determined at 1.0 μg/ml and 0.5 μg/ml, which was five times lower compared to the UHPLC-UV method, but sufficient for reliable determination of impurities in real Oestrogel gel samples.

The influence of the individual parameters of the SbFC method was evaluated on the four stationary phases. The choice of the stationary phase and an organic modifier were the two critical factors for successful method development. However, the influence of the organic modifier must be always evaluated experimentally. Alcohol-type modifiers are preferred due to better efficiency of separation and symmetrical peaks. Methanol is a good organic modifier of the first choice. Other parameters, including gradient set-up, temperature and pressure could only be used for further fine tuning and adjustments. Almost any changes in selectivity were observed when pressure was changed in the range of 1500–2000 psi and only slight changes in selectivity were observed when the temperature was changed in the range of 40–90 °C. Concerning gradient set-up, if the gradient window is selected wide enough (such as 2–35%), not any more changes in selectivity are expected.

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References

[1] K. De Klerck, D. Mangelings, Y.V. Heyden, J. Pharm. Biomed. Anal. 69 (2012) 77.
[2] M. Maftouh, C. Granier-Loyaux, E. Chavana, J. Marini, A. Pradines, Y.V. Heyden, C. Picard, J. Chromatogr. A 1088 (2005) 67.
[3] K. De Klerck, D. Mangelings, D. Cliquq, D. De Boever, Y.V. Heyden, J. Chromatogr. A 1234 (2012) 72.
[4] C. Brunelli, Y. Zhao, M.-H. Brown, P. Sandra, J. Chromatogr. A 1185 (2008) 263.
[5] A.J. Alexander, T.F. Hooker, F.P. Tomasella, J. Pharm. Biomed. Anal. 70 (2012) 77.
[6] A.G.-G. Perrenoud, J.-L. Veuthey, D. Guillarme, J. Chromatogr. A 1266 (2012) 158.
[7] A.J. Alexander, L. Zhang, T.F. Hooker, F.P. Tomasella, J. Pharm. Biomed. Anal. 78-79 (2013) 243.
[8] D.R. Gere, Science 222 (1983) 253.
[9] M. Saito, T. Nitta, Fundamental properties of supercritical fluids relevant to chromatography and extractions, in: M. Saito, Y. Yamauchi, T. Okuyama (Eds.), Fractionation by Packed-column SFC and SFE, Principles and Applications, VCH, New York, 1994, pp. 27–60.
[10] K. Yaku, F. Morishita, J. Biochem. Biophys. Methods 43 (2000) 59.
[11] K. Yaku, K. Aoe, N. Nishimura, T. Sato, F. Morishita, J. Chromatogr. A 773 (1997) 277.
[12] D.W. Later, B.E. Richter, D.E. Knowles, M.A. Andersen, J. Chromatogr. Sci. 24 (1986) 249.
[13] N.K. Jagota, J.T. Stewart, J. Pharm. Biomed. Anal. 10 (1992) 667.
[14] X. Xu, J.M. Roman, T.D. Veenstra, J. Van Anda, R.G. Ziegler, H.J. Issaq, Anal. Chem. 78 (2006) 1553.
[15] M. Tuomola, M. Hakala, P. Manninen, J. Chromatogr. B 719 (1998) 25.
[16] J.S. Loran, K.D. Cromie, J. Pharm. Biomed. Anal. 8 (1990) 607.
[17] E. Huang, J. Henion, J. Chromatogr. A 511 (1990) 257.
[18] C. Baiocchi, G. Giacosa, M.A. Roggero, E. Marengo, J. Chromatogr. Sci. 34 (1996) 399.
[19] M. Hanson, Chromatographia 40 (1995) 58.
[20] T.A. Berger, J.F. Deye, J. Chromatogr. Sci. 29 (1991) 280.
[21] M. Hanson, Chromatografia 39 (1994) 431.
[22] Ch. Gourmel, A.G.-G. Perrenoud, L. Waller, E. Reginato, J. Verne, B. Dulery, J.-L. Veuthey, S. Rudaz, J. Schappler, D. Guillarme, J. Chromatogr. A 1282 (2013) 172.
[23] L. Havlíková, L. Nováková, L. Matysová, J. Šícha, P. Solich, J. Chromatogr. A 1119 (2006) 216.
[24] Y.K.K. Koh, T.Y. Chiu, A. Boobis, E. Cartmell, J.N. Lester, M.D. Scrimshaw, J. Chromatogr. A 1173 (2007) 81.
[25] M. Pedrouzo, F. Borrull, E. Pocurull, R.M. Marcé, Talanta 78 (2009) 1327.
[26] X. Peng, Z. Wang, C.h. Yang, F. Chen, B. Mai, J. Chromatogr. A 1116 (2006) 51.
[27] P. Hoffmann, M.F. Hartman, T. Remer, K.-P. Zimmer, S.A. Wudy, Steroids (2010) 1067.
[28] H. Tomšíková, J. Aufartová, P. Solich, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, L. Nováková, Trends Anal. Chem. 34 (2012) 35.
[29] L. Sun, W. Yong, Y. Chu, J.-M. Ling, J. Chromatogr. A 1216 (2009) 5416.
[30] V. Kumar, N. Nakada, M. Yasojima, N. Yamashita, A.C. Johnson, H. Tanaka, Chemosphere 77 (2009) 1440.
[31] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Q2 (R1): Text on Validation of Analytical Procedures, US FDA Federal Register, 2005.
[32] C. West, E. Leselier, J. Chromatogr. A 1191 (2008) 21.
[33] C. West, E. Lesellier, J. Chromatogr. A 1110 (2006) 200.
[34] C. West, E. Lesellier, J. Chromatogr. A 1110 (2006) 181.
[35] C. West, E. Lesellier, J. Chromatogr. A 1115 (2006) 233.
[36] M. Saito, J. Biosci. Bioeng. 115 (2013) 590.