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Enhanced capabilities of separation in Sequential Injection Chromatography – Fused-core particle column and its comparison with narrow-bore monolithic column

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ABSTRACT

In the Sequential Injection Chromatography (SIC) only monolithic columns for chromatographic separations have been used so far. This article presents the first use of fused-core particle packed column in an attempt to extend of the chromatographic capabilities of the SIC system. A new fused-core particle column (2.7 μm) Ascentis® Express C18 (Supelco™ Analytical) 30 mm × 4.6 mm brings high separation efficiency within flow rates and pressures comparable to monolithic column Chromolith® Performance RP-18e 100-3 (Merck®) 100 mm × 3 mm. Both columns matches the conditions of the commercially produced SIC system – SIChrom™ (8-port high-pressure selection valve and medium-pressure Sapphire™ syringe pump with 4 mL reservoir – maximal work pressure 1000 PSI) (FIAlab®, USA). The system was tested by the separation of four estrogens with similar structure and an internal standard – ethylparaben. The mobile phase composed of acetonitrile/water (40/60 (v/v)) was pumped isocratic at flow rate 0.48 mL min^{-1}. Spectrophotometric detection was performed at wavelength of 225 nm and injected volume of sample solutions was 10 μL. The chromatographic characteristics of both columns were compared. Obtained results and conclusions have shown that both fused-core particle column and longer narrow shaped monolithic column bring benefits into the SIC method.

1. Introduction

Sequential Injection Chromatography (SIC) is a young and developing technique belonging to the liquid chromatographic methods. Introduction and first application of SIC technique was in 2003 by Šatínský et al. [1]. The method is based on highly versatile sequential injection analysis (SIA) manifold which enable programmable and controllable flow of liquids with controlled dispersion [2,3].

Chromatographic trend in flow methods has been developed by several workgroups over the years. Manifolds named as Flow Injection Analysis-Chromatography (FIA-C) and Multi-Syringe Chromatography (MSC) were presented (based on Flow Injection Analysis, Multi-Syringe Flow Injection Analysis and Multi-Commutated Analysis manifolds). Various applications show the advantages and variability of the manifolds but define their limits too. The first applications and experience were concluded in review [4], while later SIC works were published by Chocholouš et al. [5,6]. Cerdà with several co-workers (González-San Miguel, M. Fernández, F. Maya, C. Fernández and M.A. Obando) established MSC system [7–14]. Capitán-Vallvey with co-workers (J.F. García-Jiménez, M.C. Valencia and J.B. Claver) employed Chromolith® monolithic pre-column (5 mm × 4.6 mm i.d.) into FIA-C [15–19]. Masini with co-workers (L.B.O. dos Santos, M. Rigobello-Masini and C.M.C. Infante) presented separations on SIC [20–22]. The early SIC manifolds build from "common" flow parts had suffered from leaking and limited flow rate until the commercial SIChrom™ manifold (FIAlab®, USA) with all parts designed for the resistance to chromatographic work pressures and organic solvents was introduced, thus the manifold enables to use some of columns typical for common separations in HPLC. However, all developed methods show interesting variations of the flow manifold, but their chromatographic parts are behind the modern chromatographic needs – column choice, separation speed, selectivity and efficiency. So far, the chromatographic part of SIC has been represented by short or moderately long monolithic columns (5, 10, 25 or 50 mm of length and 4.6 mm of internal diameter).

Presented work describes a new application of fused-core particle column and its comparison with recently introduced 100 mm × 3 mm monolithic column in SIC. The superficially porous particles (fused-core) packed columns were first commercially introduced in 2007. Nowadays, they are represented by Halo™

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from Advanced Materials Technology, Ascentis® Express from Supelco, Poroshell from Agilent Technologies and Kinetex® from Phenomenex. Ascentis® Express columns are filled with 2.7 μm superficially porous silica solid core particles (solid core made of fused silica of 1.7 μm diameter, which is impenetrable by analytes, and shell – 0.5 μm thick layer of porous silica gel) in contrast to common totally porous particles with 5 μm or smaller 3, 2 or 1.7 μm diameter. The separation efficiency of these columns is comparable to sub-2 μm particle columns with the same kind of stationary phase but under working-pressure of 3 μm particles [23]. These features are mostly caused by much shorter diffusion path of analytes in porous-shell particles (thus accelerated mass transfer, the C term of the Van Deemter curve) in contrast to longer diffusion path in totally porous particles. The small sized and superficially porous particles (improves the rate of mass transfer and reduces the eddy diffusion effect, resulting smaller plate high and higher optimum linear velocity) and small column dimensions result in small peak broadening.

Monolithic columns with narrow inner diameter represent other trend in LC technique. They bring lower dead volume together with faster separations without increased working pressure within typical (recommended) flow rates. Narrow-bored columns (2 and 3 mm i.d.) dramatically reduce inner volume (2 mm i.d. presents 19% and 3 mm i.d. presents 43% of volume of common 4.6 mm i.d. column) that brings more effective usage of mobile phase [24]. Monolith sorbent has a porosity exceeding 80% and a bimodal pore structure (macropores and mesopores), which gives improved chromatographic performance in terms of separation and column back-pressure (equivalent to columns packed with 11 μm particles). The efficiency of monolithic columns is comparable to that of columns packed with particles with diameter between 3 μm and 4 μm. Recent chromatographic trends and detailed comparisons of new columns in HPLC were reviewed by renowned authorities Guillarme et al. [25], Fekete et al. [26], Pietrogrande et al. [27] and Saunders et al. [28]. All of them concluded the bright future for superficially porous particle columns and advantageous properties of newer narrow bore monoliths. Both trends proved a lot of interesting properties in HPLC and from some of them SIC might profit too.

The estrogens are group of steroid compounds functioning as the primary female sex hormones with primary estrogen estradiol. Metabolism of estrogens is represented by number of structures that are more or less similar. The chosen estrogens for column testing are represented by α-estradiol, β-estradiol, ethinyl-estradiol and estrone (Fig. 1), the structures are different only in substitutions in position 17 of steroid structure, thus suited for testing of chromatographic performance of columns. Ethylparaben – common preservative in food and drug chemistry was chosen as an internal standard.

2. Experimental

2.1. Apparatus

Commercially available SIChrom™ instrument (FIAlab® Instruments Inc., Bellevue, WA, USA) with S17 PDP syringe pump with 4.0 mL reservoir (Sapphire™ Engineering, MA, USA) and an 8-port high-pressure stainless-steel selection valve C5H (Valco Instrument Co., Houston, TX, USA) was used in the presented work. Flow lines were made of 0.25 mm and 0.50 mm I.D. PEEK tubing. Samples were aspirated through the 8-port selection valve and then delivered to the chromatographic column and to the flow-cell of the detector. The manifold was equipped with fiber-optic CCD UV-VIS detector USB 4000 (Ocean Optics Inc., Dunedin, FL, USA), SMA connector Ultem® micro-volume (9 μL) Z-flow cell with optical path of 20 mm (FIAlab® Instruments Inc., Bellevue, WA, USA), deuterium UV light source DH-2000 (Ocean Optics Inc., Dunedin, FL, USA) and SMA connector ended fiber optic cables with core diameter 600 μm (CeramOptec®, East Longmeadow, MA, USA). In one of the pump outlet a manometer that enables real time monitoring of the system pressure and 1000 PSI relief valve as a system pressure safety valve were mounted. The 20 PSI relief valve behind the flow cell prevented the spontaneous flow of mobile phase from the flow cell when the pump is stopped. The whole SIC system was controlled with PC equipped with FIAlab® 5.9 software (FIAlab® Instruments Inc., Bellevue, WA, USA). Manifold setup is depicted in Fig. 2.

Direct sample separation was performed on porous shell particle reverse-phase column Ascentis® Express C18 (Supelco™ Analytical) 30 mm × 4.6 mm, 2.7 μm (equipped with Opti-Guard® C18 1 mm guard pre-column) and monolithic reverse-phase column Chromolith® Performance RP-18e 100-3 (Merck®) 100 mm × 3 mm (equipped with Chromolith® RP-18e 5–3 mm guard pre-column). All experiments were performed at laboratory temperature (25 °C).

2.2. Chemicals

Working standards of β-estradiol (B-EST), 17-α-ethinylestradiol (ET-EST), estrone (ESTN), ethylparabene (EP) (obtained from Sigma–Aldrich–Fluka) and 17-α-estradiol-hemihydrate (A-EST) (Riedel-de Haën) were used for the purpose of this project. Acetonitrile and methanol (Chromasolv®, for LC) were obtained from Sigma–Aldrich®. Millipore Milli-Q RG ultra pure water (Millipore™ s.r.o., Czech Republic) was used for preparation of the solutions.

Mobile phase for separation of all substances on both particle and monolithic columns was acetonitrile/water (40/60 (v/v), no pH adjustment). Mobile phase was degassed before use by sonication for 5 min. Working standards were dissolved in acetonitrile at concentration of 0.5 mg mL^{-1} and all were stored at 5 °C for 1 month.

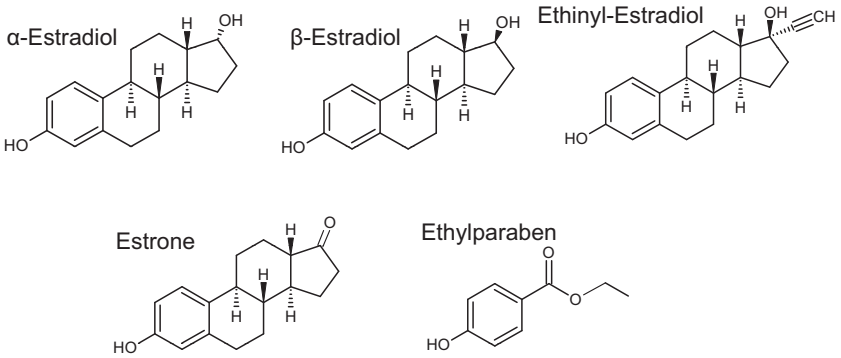

Fig. 1. Chemical structures of used estrogens and internal standard ethylparaben.

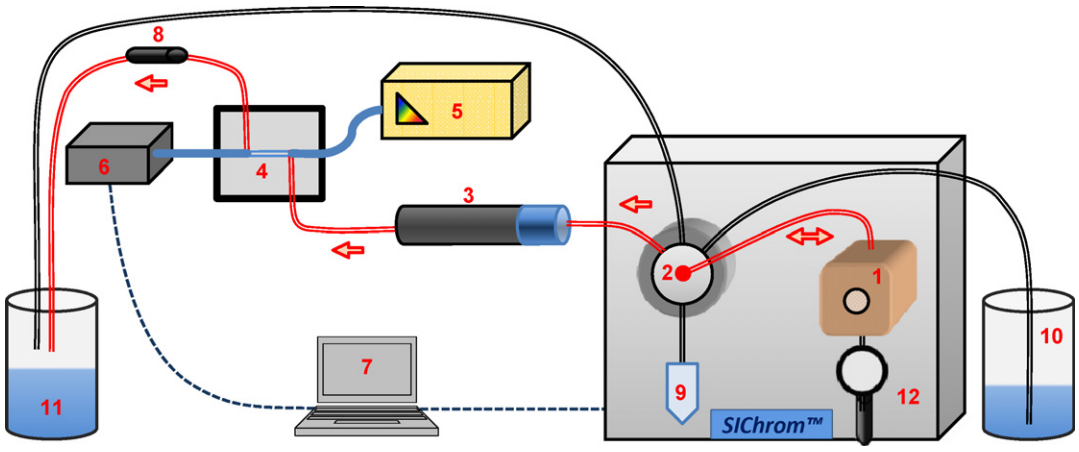

Fig. 2. Scheme of SIC setup for determination of 4 estrogens with IS. 1, Sapphire syringe pump; 2, eight-port selection valve; 3, chromatographic column; 4, Z-flow cell; 5, UV lamp; 6, CCD UV-VIS detector; 7, computer with FIAlab software; 8, relief valve 20 PSI, post column; 9, sample; 10, mobile phase; 11, waste; 12, manometer with relief valve – system pressure safety valve 1000 PSI.

The tested sample was a mixture of working standard solutions of four estrogens (B-EST, A-EST, ET-EST and ESTN) and an internal standard EP diluted with mobile phase. The final concentrations of the analytes in sample were 20.0 μg mL^{-1} of each estrogen and 4.0 μg mL^{-1} of EP. This solution was used for all measurements on both columns – chromatographic characterization and evaluation of analytical parameters of the SIC process.

3. Results and discussion

Separation on fused-core particle column was compared with separation on monolithic column. Determination of four estrogens and EP was used to verify theoretical aspects of these new approaches. Development was focused on optimization of the chromatographic conditions for simple, fast, high performance as well as low cost analysis.

3.1. Method development

The first priority for performing a fast and effective separation is the selection of appropriate stationary phase. The SIC method was developed on Ascentis® Express C18 30 mm × 4.6 mm column with regards to HPLC method developed by Vrkočová on Zorbax SB C18 (50 mm × 4.6 mm, 1.8 μm) column [29]. The separation was performed by isocratic elution. Methanol based mobile phases were tested with less satisfactory results and higher back pressure while acetonitrile based mobile phases showed better separation and peak symmetry of the analytes. Then the optimization was focused on finding an appropriate acetonitrile/water composition to achieve a good separation of all compounds together with a short time of whole analysis – the optimal ratio 40/60 (v/v) was chosen. The pH adjustment of mobile phase did not bring any better results so the pH was left unchanged after mixing of solvents.

For a comparative study monolithic columns of various diameters and lengths were tested. The development was started with shorter monolithic columns (Merck® Chromolith® FastGradient RP-18e 50 mm × 2 mm and Merck® Chromolith® Flash RP-18e 25 mm × 4.6 mm) but with unsatisfactory results. The separation of analytes was incomplete with stronger mobile phases or the time of analysis was very high accompanied with high consumption of mobile phase. The third tested monolithic column Chromolith® Performance RP-18e 100 mm × 3 mm column performed complete separation of all analytes, reasonable analysis time and mobile phase consumption, therefore the column of length 100 mm was chosen for a comparison with the fused-core particle column.

Mobile phase flow-rate was set to 0.48 mL min^{-1} with the aim to achieve the highest efficiency of both columns and fast analysis, the system-pressures within the separation were 750 PSI with particle column and 660 PSI with monolithic column. The mobile phase volume of 2.2 mL for particle column and 3.0 mL for monolithic column (35% more) was necessary for elution of all separated analytes within one analysis cycle. The injected volume of analyzed solutions was 10.0 μL with regards to recommended and tested capacity of the columns. From the UV spectra of all analyzed compounds, the optimal detection wavelength 225 nm that provided sufficient sensitivity was chosen. The other wavelengths used were 215 nm and 280 nm for better peak identification within method development. The detector was set to higher scanning rate (achieved 2.5 Hz) and integration time was 200 ms. The internal standard was chosen from the analytes with retention characteristics similar to estrogens. EP showed the best properties (eluting first, not interfering and very good detector response). The proposed system with porous-shell particle column enabled successful separation of target analytes in the time 5.7 min (0.53 min for aspiration of mobile phase to the pump reservoir, 0.02 min for aspiration of sample, 4.59 min for elution with mobile phase). The separation done with monolithic column took 7.5 min (0.72 min aspiration of mobile phase, 0.02 min for aspiration of sample, 6.3 min for elution with mobile phase) – 30% longer time of analysis. Column details and characteristics of measurement are summarized in Table 1. The sequence of particular steps of the SIC control program for separation of all substances (a single cycle) is described in Table 2.

3.2. Chromatographic characteristics and figures of merit

Separation of B-EST, A-EST, ET-EST, ESTN and EP was carried out under mentioned conditions. Chromatograms were processed and peak heights were used for data evaluation. Basic chromatographic parameters were calculated from experimental data, such as retention time, peak symmetry, number of theoretical plates, height equivalent to a theoretical plate (HETP) and peak resolution (following the Ph. Eur.). The characterizations of separation processes on both columns are given in Table 3.

The measurement showed good results for all analytical parameters (linearity, sensitivity, repeatability, selectivity and precision). Linearity was established with a series of working solutions prepared by diluting the stock solution with 40% acetonitrile to the final concentrations. It was obvious that the quite low molar absorption coefficient of estrogens enables only narrow linear calibration

Table 1
The details of tested columns and characteristics of SIC measurement.

	Ascentis® Express C18	Chromolith® Performance RP-18e	Difference AE to ChP (%)
Stationary phase packing	Fused-core particles ø 2.7 μm (core 1.7 μm and shell 0.5 μm)	Monolithic rod 100 mm × 3 mm	
Length (mm)	30	100	30
I.D. (mm)	4.6	3.0	153
System dead volume (mL)	0.28	0.71	39
Volume of mobile phase for one analysis (mL)	2.2	3.0	73
Flow rate (mL min^{-1})	0.48	0.48	
Linear velocity of mobile phase (cm s^{-1})	0.085	0.112	76
Time of one analysis (min)	5.7	7.5	76
Time of elution (min)	4.6	6.3	73

Table 2
The sequence of particular steps of the SIC control program (a single cycle) for estrogens separation on the fused-core particle column (AE) and on the monolith column (ChP) – in colons.

Action	Unit	Parameter
Mobile phase aspiration	Selection valve	Valve port 2 – mobile phase reservior
	Pump	Aspiration flow rate 4.2 mL min^{-1}
	Pump	Volume 2.2 (3.0) mL
Sample aspiration	Selection valve	Valve port 4 – sample reservoir
	Pump	Flow rate 0.6 mL min^{-1}
	Pump	Volume 0.01 mL
Mobile phase dispension–separation of the sample	Selection valve	Valve port 3 – column
	Pump	Flow rate 0.48 mL min^{-1}
	Pump	Volume 2.21 (3.01) mL

Table 3
Characterization of SIC process performed on particle column (AE) and its comparison with monolith column (ChP).

	EP		B-EST		A-EST		Et-EST		ESTN	
	AE	ChP	AE	ChP	AE	ChP	AE	ChP	AE	ChP
Retention time (min)	1.55	2.80	2.36	3.94	2.88	4.57	3.24	5.16	3.69	5.72
Peak symmetry	1.67	1.50	1.67	1.38	1.50	1.50	1.30	1.30	1.25	1.30
Number of theoretical plates	1288	2106	1875	2632	1930	4595	2482	4919	2377	3890
Height equivalent to a theoretical plate (μm)	23.3	47.5	16.0	37.9	15.5	21.7	12.1	20.3	12.6	25.7
Peak resolution	4.06	4.06	2.15	2.14	1.42	1.48	1.58	1.63		

range and was much lower than molar absorption coefficient of ethylparaben. Six working solutions were used for calibration of both methods. The lowest concentration was excluded for Ascentis column because of higher noise of the baseline than in analysis with monolithic column and the highest concentration was excluded for monolithic column because of the imperfection of peak shape caused the error of quantification. Each working solution was injected in triplicate and the mean value of peak height was used for the calibration curve. The limit of detection (LOD) was calculated as concentration when the signal to noise ratio 3 was obtained from analysis of the standards, and the limit of quantification (LOQ) was defined as concentration when the signal to noise ratio 10 was obtained from analysis of the standards. The analytical parameters of measurement with both columns are in Table 4. The representative sequential injection chromatograms showing successful separation of all analytes on the fused-core particle column is shown in Fig. 3 and on the monolithic column is shown in Fig. 4.

4. Discussion

Separation of five analytes was successfully reached with both columns, but by different ways – column length, diameter and format of stationary phase. The efficiency of separation was clearly described by number of theoretical plates where the monolithic column reached higher numbers, but HETP (where is the column length taken into account) turned the advantage to the side of the fused-core particle column. Resolutions of peaks showed the same results for both columns. This means that the use of short fused-

Table 4
Validation results and analytical parameters of SIC process on particle column (AE) and its comparison with monolithic column (ChP).

	EP		B-EST		A-EST		ET-EST		ESTN	
	AE	ChP	AE	ChP	AE	ChP	AE	ChP	AE	ChP
Calibration range (μg mL^{-1})	0.6–10.0	0.3–5.0	3.1–50.0	1.6–25.0	3.1–50.0	1.6–25.0	3.1–50.0	1.6–25.0	3.1–50.0	1.6–25.0
Equation of calibration-slope	0.0044	0.0036	0.0075	0.0054	0.0055	0.0051	0.0076	0.0071	0.0052	0.0049
Equation of calibration-intercept	−0.0371	−0.0100	0.0275	0.0239	0.0211	0.0042	0.0281	0.0058	0.0180	0.0523
Correlation coefficient	0.997	0.999	0.998	0.999	0.998	0.999	0.998	0.999	0.999	0.999
Limit of detection (μg mL^{-1})	0.2	0.1	1.0	0.5	1.0	0.5	1.0	0.5	1.0	0.5
Limit of quantification (μg mL^{-1})	0.6	0.3	3.1	1.6	3.1	1.6	3.1	1.6	3.1	1.6
System precision (%)[a]	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Repeatability of time t_R (%)[a]	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0

[a] Relative standard deviation (R.S.D.) values were calculated for repeated standard injections (n = 6) – concentration 20.0 μg mL^{-1} of each estrogen and 4.0 μg mL^{-1} of EP.

Table 5
Advantages and drawbacks of tested columns from the perspective of SIC method.

Approach in SIC	Advantages	Drawbacks
Monolithic column (Chromolith® RP-18e 100 mm × 3 mm)	Low resistance of sorbent allow use all lengths of Chromolith HPLC columns	Range of working pH 2–7.5
	Flow rates up to several ml min^{-1}	Limited choice of column chemistries
	Higher separation efficiency of sorbent attainable by SIC	Higher price
	Modern narrow bore columns	
Fused-core column (Ascentis® Express C18 30 mm × 4.6 mm, particle 2.7 μm)	Moderate resistance of sorbent – same level as monolithic rod (100 mm × 3 mm)	Resistance of sorbent require solvents with lower viscosity for mobile phases
	Moderate inner volume	Flow rate limited to 0.6 mL min^{-1}
	Higher separation efficiency than monolithic column (low HETP)	Lower flow rates in SIC do not allow the highest separation performance of sorbent
	High mass capacity	
	Range of working pH 2–9	
	Wider choice of column chemistries	
	Wider choice of column dimensions	
	Lower price	

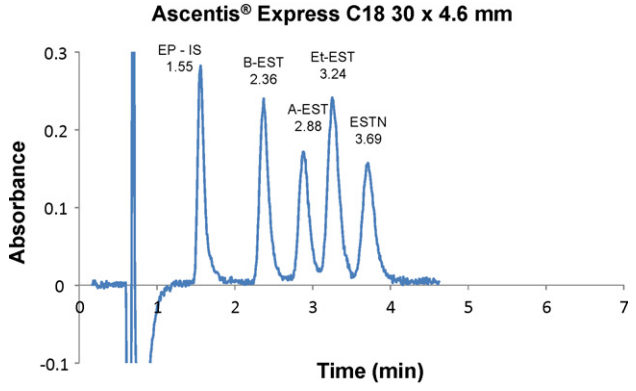

Fig. 3. SIC chromatogram of the separation of 4 estrogens with IS on fused-core particle column. The mobile phase acetonitrile/water (40/60 (v/v)), flow rate 0.48 mL min^{-1} (volume 2.2 mL). UV detection at 225 nm.

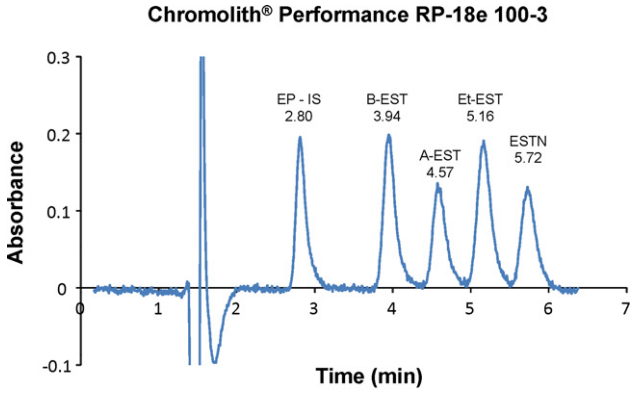

Fig. 4. SIC chromatogram of the separation of 4 estrogens with IS on monolithic column. The mobile phase acetonitrile/water (40/60 (v/v)), flow rate 0.48 mL min^{-1} (volume 3.0 mL). UV detection at 225 nm.

core particle columns with lower dead volume and with the same flow rate results in similar separation efficiency to that of longer and narrow-bore monolithic columns. The features of new approaches in SIC are discussed in Table 5.

5. Conclusion

The novel approaches in SIC system (chromatography in flow systems) were successfully developed and used for fast and effective chromatographic analysis. Two columns with different format of stationary phase – fused-core particle and monolithic (with the same stationary phase RP-C18) were compared in terms of separation efficiency in SIC. The complete separations of mixture of four estrogens with similar structure and internal standard EP were achieved under optimal conditions. High separation efficiency was achieved however the used flow rate 0.48 mL min^{-1} is not high. It is obvious that higher mobile phase flow rates (1.0–2.5 mL min^{-1}) could be achieved only with short (25 or 50 mm) and 4.6 i.d. monolithic columns when the limit of 1000 PSI is not passed.

In summary, the SIC manifold for the first time equipped with fused-core particle or longer monolithic column provides higher performance of separation compared to SIC methods with previous used monolithic columns. This work in flow methods can be considered as a broadening of chromatographic abilities of the SIC and following the trends in the rapid chromatographic separations. Improved performance of chromatography and typical features of SIC – discontinuous flow and easy sample handling, easy operation and portability of instrument enabled reduction of cost per analysis.

Drawbacks of SIC system are still in lower precision of syringe pump, lower performance of USB CCD spectrophotometric detector and flow-lines (wider I.D. of tubes), basic processing software and overall "experimental-LEGO" build-up of SIC manifold compared to latest HPLC systems.

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