Research article

Combination of stationary phase selectivity in SFC method development

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Abstract

Supercritical fluid chromatography was investigated on its capability of separating five, structurally very similar dafachronic acid related steroid hormones, including two pairs of isomeric compounds. Subsequent to a column screening approach, two stationary phases which provided the most promising separations were used for further separation optimization. Finally, none of the two stationary phases were capable of baseline separating all compounds. The elution order of compounds was the same on both stationary phases, but retention factors and resolution differed. Both stationary phases behaved complementary in separable compound pairs. This behavior was used, when the two stationary phases were coupled in series. The combination of stationary phases resulted in improved resolution for all compounds. The order of stationary phases in the coupling had major influence on separation efficiency. Both stationary phases contributed to the overall retention of compounds, resulting in an additive separation. Finally, all investigated compounds could be baseline separated, using this approach. The combination of stationary phases is a powerful option to solve many challenges in separations. Its applicability is mainly due to the unique characteristics of the mobile phase in SFC, which allows to prolong and combine stationary phases without significant losses in separation efficiency.

Introduction

The unique characteristics of its mobile phase make supercritical fluid chromatography (SFC) an interesting and powerful separation technique. But due to the compressibility of the mobile phase, which influences density and selectivity, method development can be a challenging task [1]. Changes in mobile phase flow rate, modifier proportion in the mobile phase (organic co-solvent to promote compound elution), system pressure, temperature and even column length can all affect the selectivity of a separation. Besides that, basic retention mechanisms in SFC are not completely understood, yet [2]. A categorization of stationary phases based on its basic chemistry can be helpful to categorize phases in SFC, but compound behavior in separations is not predictable this way. Therefore column classification schemes, based on linear solvation energy relationships (LSER) or sum of ranking differences (SRD) have been presented, which are very useful to assess column selectivity and to simplify column selection [1, 3–5]. As a consequence, column screening and selection of the most appropriate stationary phase is the first step in SFC method development. The following steps of separation optimization include testing different conditions, e.g. different modifiers and additives, mobile phase gradients, flow rates, column temperatures and back pressure, in order to achieve a sufficient separation. If optimization attempts do not lead to a sufficient separation, the procedure has to be repeated, using a different stationary phase. Especially for chemically and/or structurally similar analytes, this procedure can be time-consuming procedure and a sufficient outcome is not guaranteed.

In this study, the development of a fast separation method for five steroid hormones was aimed. The analytes were part of metabolic pathways outgoing from cholesterol and leading to whether lathosterol or Δ4-dafachronic acid (Figure 1). Dafachronic acids play a key role in larval development and (induced) longevity of the model organism *Caenorhabditis elegans* [6–9]. The set of analytes contained two pairs of isomeric compounds, namely 7-dehydrocholesterol and 4-cholesten-3-one (1, $C_{27}H_{44}O$) and cholesterol and lathosterol (2, $C_{27}H_{46}O$). Both pairs of analytes differ only minor by changes of double bond positions or hydroxyl or keto groups at one position. The analysis of such very similar compounds requires highly efficient technologies and/or intensive separation optimization. Various methods, based on liquid chromatography (LC), utilizing achiral or chiral stationary phases or gas chromatography (GC) coupled with mass spectrometry (MS) have been used for elucidation and

validation of potential precursors in the biosynthetic pathway, but a comprehensive steroid profiling method for *C. elegans* is yet not available [10–14]. The principal applicability of SFC for the separation of hormones and estrogen metabolites has previously been reported [15–17].

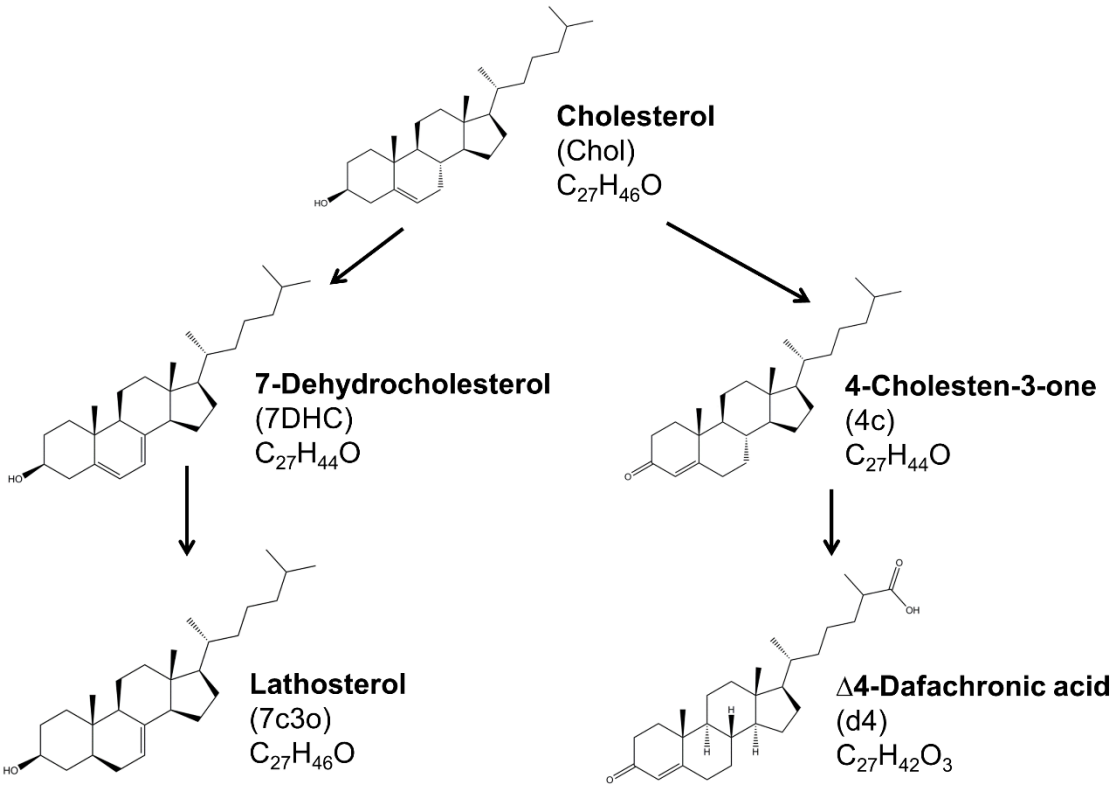


Figure 1: Synthesis pathway of 7DHC, 7c3o, 4c and d4 as metabolites of cholesterol (Chol)

For the set of studied analytes, the method development procedure is presented, including column screening and separation optimization. The separation on two single stationary phases did not lead to sufficient results. Due to the complementary results of both stationary phases a coupling of these, was emphasized to be capable of separating all compounds. Combinations of achiral and chiral or achiral and achiral stationary phases have been used before [16, 18–24]. Anyhow, the combination of two complementary achiral stationary phases is presented as a consequent extension of method development strategies in SFC. The herein provided potential for method development will lead to faster and easier separation optimization for complex samples, containing highly similar analytes.

Materials and methods

Reagents and columns

Acetonitrile, methanol and isopropanol were purchased from VWR (Darmstadt, Germany). Carbon dioxide (CO_2) in 4.5 grade was obtained from Westfalen Gas AG (Muenster, Germany). Cholesterol (Chol), 7-dehydrocholesterol (7DHC) and 4-cholesten-3-one (4c) were purchased from Sigma-Aldrich (Seelze, Germany). Lathosterol (7c3o) was obtained from Steraloids (Newport, RI, USA) and Δ4-dafachronic acid (d4) from Cayman Chemical (Ann Arbor, MI, USA). Eurosphere II C18, C18-A and silica columns (all 150 x 2.0 mm, 5 µm) were provided by Knauer (Berlin, Germany). Waters (Eschborn, Germany) Viridis 2-ethylpyridine column (150 x 2.1 mm, 5 µm) and Phenomenex (Aschaffenburg, Germany) Luna phenyl-hexyl column (150 x 2.0 mm, 5 µm) were both provided by the manufacturers.

Solutes

Standard compounds were dissolved in isopropanol as 1 mg/mL stock solutions. For the column screening and separation optimization experiments, working standard solutions of 40 to 60 µg/mL in acetonitrile were used. To decrease the number of column screening runs, the five compounds were primarily injected as mixtures containing only non-isomeric analytes, or as a mixture of all compounds, when retention order was determined in previous experiments.

Instruments

SFC system

The analytical SFC system (Agilent Technologies, Waldbronn, Germany) consisted of a degasser, binary pump, an autosampler with 10 µL injection loop, a thermostatically controlled column compartment, a diode array detector and a backpressure regulator unit. The column compartment was equipped with a switch-valve which allowed to test up to six columns in the system.

Time-of-flight mass spectrometer

The outlet of the chromatographic system was coupled to a Jet-Stream ESI-time-of-flight mass spectrometer (both Agilent Technologies, Santa Clara, CA, USA). A make-up flow for internal mass calibration was added to the SFC flow before entering the MS. For all experiments positive ESI ionization was used. Data was acquired with Agilent Mass Hunter Acquisition software (B 05.01). For the evaluation of MS and UV data, Agilent Mass Hunter (B 06.00) and ProFinder (B 06.00) software was used.

Separation optimization procedure and methods

The initial screening was performed, using a CO_2 – methanol gradient from 5 to 40 % in 5 minutes. The flow rate was 2.0 mL/min, backpressure was set to 150 bar and column temperature was held constant at 40°C. All steroid standards were injected in all columns and separated under these conditions. The column that provided the most appropriate separation was used for further separation optimization. Different modifiers were tested in the screening method as first optimization step. The most promising modifier was used for further optimization and the gradient slope was subsequently stepwise reduced (5-40%, 5-20% and 5-10% in five minutes) in order to increase compound retention. As last option for optimization, back pressure was reduced to 100 bar. For several experiments the UV detection at 220 nm was favored. MS data was primarily used to investigate the identity of co-eluting compounds. Except for the column screening, separations were repeated twice to assess repeatability of results.

Results and discussion

Generic column screening

For the initial screening, stationary phases were selected on the basis of a LSER classification scheme, in order to obtain high complementarity of selectivities [1]. The column set included a non-polar stationary phase (C18), a polar alkyl phase (hydrophilic end-capped C18-A), an aromatic phase (phenyl-hexyl) and two polar phases (silica and 2-ethylpyridine). All standard compounds were injected on all

columns with general, but identical settings (see experimental section 2.4). Retention times, retention factors, selectivity and resolutions are summarized in Table 1. Strongest retention in general was observed with the C18 column, Chol and 7DHC were not separated, showing identical retention times and 7c3o and 4c eluted very close. The lowest retention was observed for d4. After C18-A and phenyl-hexyl separations some compounds could not be detected. Retention order of detectable compounds was comparable among the two stationary phases, but different to the C18 column. C18 and C18-A separations were not comparable, although there is a high similarity in stationary phase chemistry. However, the introduction of polar groups in non-polar stationary phases may significantly change retention behavior in SFC [25]. The two polar stationary phases, silica and 2-ethylpyridine showed overall lower retention, compared to all other screened stationary phases. Elution order was the same in both columns. 4c eluted first, followed by 7c3o, Chol, 7DHC and highest retention was observed for d4. Latter obtained least retention in C18. Retention factors in separations with the silica column ranged from 0.88 to 2.73 and from 0.75 to 4.55 with 2-EP. Overall, Chol, 7DHC and 7c3o were eluting very closely from both stationary phases.

Table 1: Retention times (RT) and retention factors (k), selectivity (α) and resolution (R) of all investigated analytes obtained from screening experiments with five different stationary phases.

	C18				C18-A				Phenyl-hexyl			
	RT [min]	k	α	R	RT [min]	k	α	R	RT [min]	k	α	R
Chol	1.38	6.67	1.48	0	n.d.	n.d.	n.a.	n.a.	0.66	2.10	n.a.	n.a.
7DHC	1.38	6.67	1.48	4.21	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.a.
7c3o	0.98	4.44	1.39	2.37	0.87	3.49	n.a.	n.a.	0.67	2.16	1.03	0.15
4c	1.03	4.49	1.01	0.57	1.00	4.18	1.20	1.67	0.70	2.29	1.06	0.31
d4	0.79	3.21	n.a.	n.a.	1.09	4.66	1.12	1.18	0.76	2.57	1.12	0.66

	Silica				2-ethylpyridine			
	RT [min]	k	α	R	RT [min]	k	α	R
Chol	0.67	1.86	2.12	3.29	0.79	2.49	1.04	4.79
7DHC	0.71	2.11	1.06	0.28	0.87	2.75	1.10	0.51
7c3o	0.68	2.00	1.07	0.14	0.81	2.39	3.20	0.18
4c	0.41	0.88	n.a.	n.a.	0.41	0.75	n.a.	n.a.
d4	0.82	2.73	1.29	1.27	1.29	4.55	1.66	4.68

The co-eluting compounds in C18 and the not detectable compounds in C18-A and phenyl-hexyl resulted in the decision to a subsequent optimization using polar phases. Since retention was higher in 2-EP, this stationary phase was initially chosen for a further separation optimization.

Separation optimization

Separation optimization of the 2-EP stationary phase focused on 7c3o, Chol and 7DHC, which eluted very closely in the initial screening. Since retention of all analytes was comparably low (k = [2.39; 2.75]), it was aimed to increase retention. Therefore, the organic modifier was changed from methanol to isopropanol, which provided lower elution strength (Table 2). However, 7c3o/4c and Chol/7DHC remained hardly separated (R= 0.48 and 0.94 respectively). It is worth mentioning that already at this stage of optimization, all isomers were separated and only non-isomeric compounds were not fully separated (7c3o/4c and Chol/7DHC). For further optimization, the gradient slope was reduced to 20% and 10% in 5 minutes. This led to increasing retention for Chol, 7DHC and d4. The retention of early eluting 7c3o and 4c was hardly affected. The resolution of the two closely eluting compound pairs remained unchanged. As a consequence, backpressure was reduced from 150 bar to 100 bar. Lower backpressure leads to decreased viscosity of the mobile phase and reduces diffusion coefficients [24], resulting in higher retention times and resolution. As for the change of modifier, effects from reducing backpressure were higher for later eluting compounds. The resolution Chol/7DHC increased to R=1.17, allowing to detect the compounds as two separated peaks in UV chromatograms (Figure 2a). Although 2-EP was suitable for the separation of Chol/7DHC, 7c3o/4c remained unseparated. Therefore, separation optimization was continued with Si, which had shown comparable selectivity to 2-EP in column screening experiments.

Optimization of Si was started again with isopropanol and a 5-40% modifier gradient in 5 minutes. Gradient slope was consequently reduced as in 2-EP experiments (5-20% and 5-10%). After the final optimization step in silica, 7c3o/4c were partly separated (R= 1.06) (Table 2 and Figure 2b). Interestingly Chol/7DHC which was separated by 2-EP, remained unresolved in Si. As a result, each of the stationary phases, was suitable for the separation of one compound pair and both stationary phased showed similar but complementary selectivity. The standard deviations of retention factors, selectivity and resolution of both stationary phases were better than 0.2 (n=3). Separation optimization resulted in a separation of all five investigated compounds by using 2-EP and Si in parallel.

Table 2: Retention times (RT), retention factors (k), selectivity (α) and resolution (R) of the peak pairs and for the separation optimization steps with 2-ethypyridine and silica. Steepness of the mobile phase gradient was altered for 5-40% isopropanol in 5 min. to 5-10% in 5 min. Backpressure was finally reduced from 150 bar to 100 bar.

		Mean RT [min]	St.Dev. [min]	Mean	St.Dev.		k	St.Dev.	α	St.Dev.	R	St.Dev.
2-EP, 5-40% 150 bar	**7c3o**	0.48	0.00	0.08	0.00		1.08	0.01	n.a.	n.a.	n.a.	n.a.
	4c	0.56	0.00	0.10	0.00		1.39	0.01	1.29	0.00	0.48	0.01
	Chol	1.21	0.01	0.08	0.00		4.21	0.02	3.02	0.02	4.35	0.08
	7DHC	1.34	0.00	0.07	0.00		4.73	0.01	1.12	0.00	0.94	0.03
	d4	2.03	0.00	0.06	0.00		7.70	0.00	1.63	0.00	6.09	0.06
2-EP, 5-20% 150 bar	**7c3o**	0.48	0.00	0.08	0.00		1.08	0.01	n.a.	n.a.	n.a.	n.a.
	4c	0.54	0.01	0.10	0.00		1.36	0.01	1.26	0.02	0.47	0.03
	Chol	1.33	0.01	0.10	0.00		4.74	0.04	3.50	0.04	4.76	0.10
	7DHC	1.51	0.01	0.10	0.00		5.59	0.07	1.18	0.02	1.08	0.03
	d4	2.72	0.01	0.08	0.00		10.90	0.12	1.95	0.00	7.74	0.07
2-EP, 5-10% 150 bar	**7c3o**	0.49	0.00	0.08	0.00		1.09	0.05	n.a.	n.a.	n.a.	n.a.
	4c	0.56	0.00	0.10	0.00		1.38	0.04	1.27	0.09	0.45	0.03
	Chol	1.46	0.00	0.13	0.01		5.27	0.13	3.82	0.02	4.55	0.10
	7DHC	1.70	0.00	0.13	0.00		6.30	0.17	1.20	0.06	1.07	0.05
	d4	3.94	0.00	0.15	0.00		15.88	0.37	2.52	0.01	9.51	0.00
2-EP, 5-10% 100 bar	**7c3o**	0.62	0.00	0.11	0.00		1.51	0.02	n.a.	n.a.	n.a.	n.a.
	4c	0.74	0.00	0.14	0.00		2.00	0.06	1.33	0.06	0.54	0.02
	Chol	1.89	0.01	0.15	0.01		6.68	0.11	3.34	0.05	4.63	0.09
	7DHC	2.20	0.00	0.15	0.00		7.82	0.11	1.17	0.03	1.17	0.10
	d4	4.63	0.00	0.15	0.00		17.58	0.21	2.25	0.00	9.46	0.08
Si, 5-40% 150 bar	**7c3o**	0.36	0.00	0.09	0.00		0.51	0.03	n.a.	n.a.	n.a.	n.a.
	4c	0.52	0.01	0.07	0.00		1.22	0.06	2.43	0.25	1.24	0.04
	Chol	0.89	0.01	0.08	0.01		2.80	0.08	2.30	0.05	2.96	0.13
	7DHC	0.95	0.01	0.09	0.00		2.98	0.06	1.07	0.05	0.42	0.02
	d4	1.03	0.01	0.13	0.00		3.31	0.20	1.11	0.05	0.45	0.02
Si, 5-20% 150 bar	**7c3o**	0.36	0.01	0.09	0.01		0.58	0.05	n.a.	n.a.	n.a.	n.a.
	4c	0.52	0.00	0.07	0.00		1.16	0.07	2.00	0.20	1.23	0.04
	Chol	0.93	0.00	0.11	0.00		2.85	0.11	2.47	0.06	2.73	0.04
	7DHC	1.00	0.01	0.11	0.00		3.39	0.08	1.19	0.07	0.34	0.03
	d4	1.11	0.01	0.22	0.00		3.86	0.02	1.14	0.02	0.43	0.03
5-10% 100 bar	**7c3o**	0.47	0.00	0.09	0.01		1.02	0.04	n.a.	n.a.	n.a.	n.a.
	4c	0.63	0.00	0.08	0.00		1.58	0.02	1.55	0.04	1.06	0.04
	Chol	1.12	0.00	0.13	0.00		3.59	0.08	2.27	0.01	2.66	0.07
	7DHC	1.27	0.00	0.14	0.00		4.29	0.01	1.20	0.02	0.63	0.01
	d4	1.50	0.01	0.32	0.01		5.41	0.10	1.26	0.02	0.57	0.02

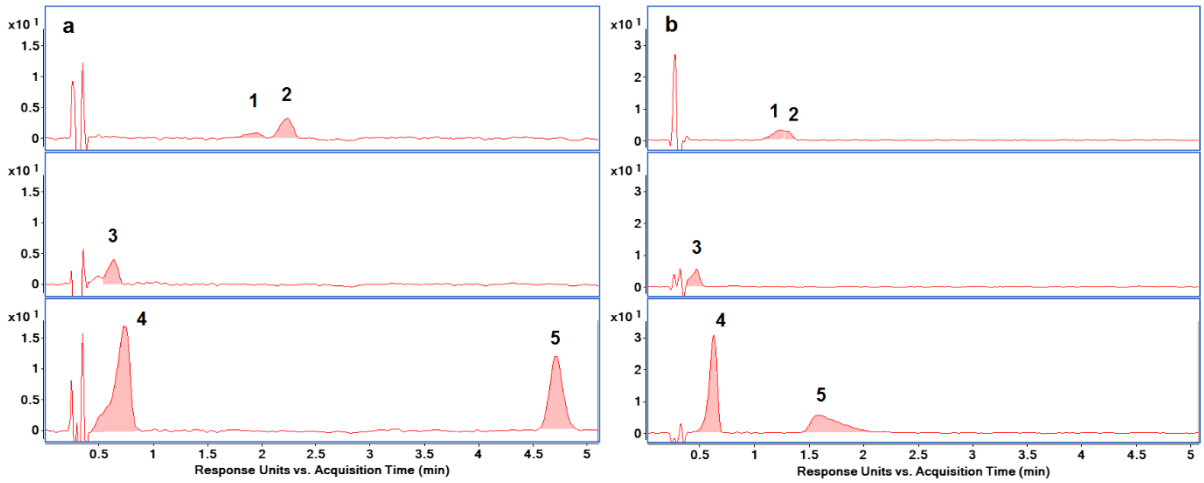


Figure 2: Chromatograms (UV 220 nm) of cholesterol (1), 7DHC (2), 7c3o (3), 4c (4) and d4 (5), separated with 2-ethylpyridine (a) and silica (b). Chromatographic conditions were both 5-10% isopropanol in 5 min. at 100 bar backpressure. 2-EP separation is suitable for Chol/7DHC (1 and 2). The separations obtained from silica (b) were complementary to those with 2-EP. Chol and 7DHC are hardly separated, while 7c3o and 4c, are separated.

Combination of stationary phase selectivities

The outcome of the stationary phase optimization experiments was further investigated on optimization potential. At that point, a sample had to be analyzed on two different stationary phases in parallel, in order to achieve a separation of all compounds. Having to change stationary phases during analyzes was not regarded to be practicable, so alternative approaches were considered. Testing different gradients and additives in the mobile phase and using sub 2-µm or core-shell particles in the stationary phase would have been possible alternatives in separation optimization. Regarding the basic principles, the characteristics of SFC and the complementary separations of 2-EP and Si, coupling columns seemed to be a valuable option to increase practicability. The low viscosity and high diffusivity of the mobile phase in SFC separations result in moderately low pressure decreases along the stationary phase. Pressure drop along the 2-EP column, utilized in this study was 50 bar at 5% modifier and increased to 100 bar at 40% modifier content at constant flow rate of 2 mL/min. Together with the high mobile phase flow rates, these characteristics are ideal for the coupling of stationary phases. The coupling of several stationary phases results in a higher pressure in front of the first column. In contrast to LC, this increased pressure influences the density of the mobile phase, which

may result in changes of retention and/or selectivity [1]. Based on retention factors from the optimization studies, the coupling with the column order silica followed by 2-EP (Si + 2-EP) was expected to be more suitable than the coupling of 2-EP and silica (2-EP + Si). Retention in Si was over all lower than in 2-EP, offering the option to achieve retention in both columns by connecting in the direction Si + 2-EP. The order of columns in serial couplings and the effect on separations has been investigated in several studies, with inconsistent results. In some cases, the order of columns had only minor influence on the separation [19, 21, 26], while in other cases column order clearly affected separations [27, 28]. As a consequence, both serial combinations were tested with a mobile phase gradient from 5 to 10% isopropanol and a backpressure of 100 bar, the most suitable condition during separation optimization with Si and 2-EP.

The hold-up time of the system increased from 0.23 - 0.25 min for single columns to 0.44 - 0.46 min for the serial couplings. For all investigated compounds, the retention times in the coupled set-ups were lower than the sum of individual retention times in Si and 2-EP (Table 3). In both serial couplings, retention factors of all compounds were lower than in 2-EP separations, but higher than in Si. The resolution of Chol/7DHC and 7c3o/4c was improved, independent from column order (Table 3), but highest resolution was observed for Si + 2-EP. Both compound pairs were fully separated by Si + 2-EP (Figure 3a) with a resolution of 1.72 for 7c3o/4c and 1.91 for Chol/7DHC. The coupling of 2-EP + Si was capable of separating Chol/7DHC (Figure 3b). The selectivity of the coupled columns ranged between the selectivities of the single columns. For the Si + 2-EP coupling retention factors and selectivities were found to be comparable to the mean value of individual retention factors and selectivities in single column experiments. This supports the hypothesis that both columns equally contribute to the separation in the coupling. By coupling Si and 2-EP, compounds eluting from Si will still be partially retained in 2-EP, causing additional retention. This effect can be visualized by the retention behavior of 7c3o and 4c. Both compounds were separated in Si + 2-EP but less in the inverse coupling. In single column experiments, separation of these compounds was achieved in Si. So, the main contribution to the separation must be caused by Si. In the 2-EP + Si coupling the compounds were eluted from the 2-EP column and were not retained in the Si column anymore because of too high elution strength of the mobile phase. In the Si + 2-EP coupling, the compounds will be separated in the Si column at first and eluted to the 2-EP column. The mobile phase composition in this column still enabled interactions

between analytes and the stationary phase. Compounds separated in the Si column were not strongly retained in 2-EP (low retention and too high modifier content), while unseparated compounds from Si were retained in 2-EP (higher retention in 2-EP). Such effects were observed for all analytes. The resolution in 2-EP + Si was reduced, compared to Si + 2-EP, because compounds are not retained in the Si column after elution from the 2-EP column. The resolution of compounds separated by Si+2-EP was close to the sum of resolutions from single column experiments. This additive resolution was remarkable, since the coupling of two columns resulted in a higher system pressure. Increased pressured leads to changed mobile phase density and retention [2] in at least the first column of the coupling. The coupling of two stationary phases seems to provide additive separation behavior, instead of one dominating stationary phase. It could clearly be shown that the column order does affect the separation in this case.

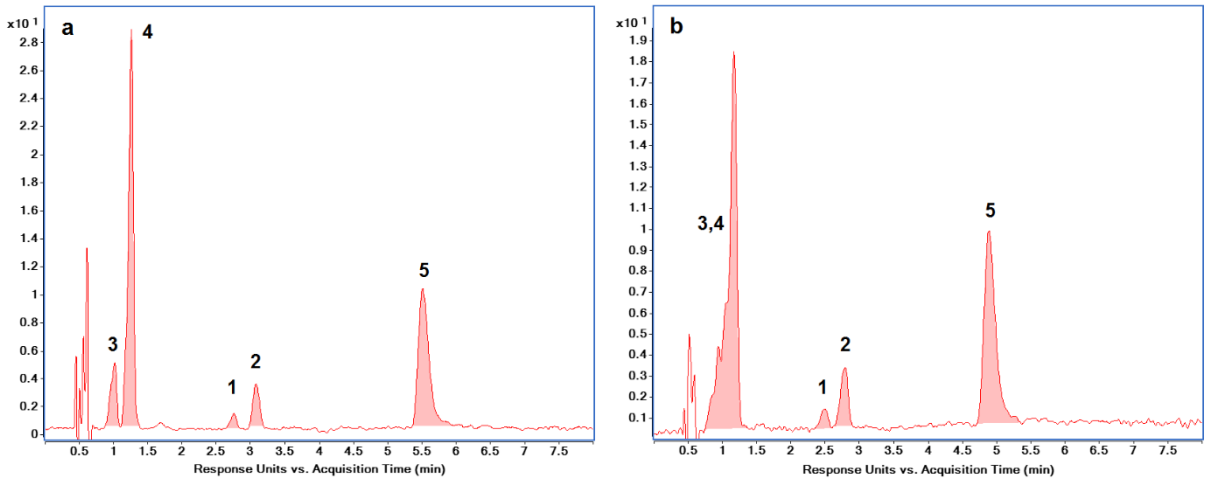


Figure 3: Chromatograms (UV 220 nm) of cholesterol (1), 7DHC (2), 7c3o (3), 4c (4) and d4 (5), separated with the serial coupling of silica and 2-ethylpyridine (a) and 2-ethylpyrindine and silica (b). Chromatographic conditions were both 5-10% isopropanol in 5 min. at 100 bar backpressure. In both cases, Chol and 7DHC are separated, but only the silica + 2-ethylpyridine coupling is capable of separating 7c3o and 4c.

Table 3: Retention times (RT), retention factors (k), selectivity (α) and resolution (R) of the peak pairs for the separations with serially coupled stationary phases silica followed by 2-ethylpyridine and vice versa.

		Mean RT	St.Dev	Mean	St.Dev.		k	St.Dev.	α	St.Dev.	R	St.Dev.
Si+2-EP	**7c3o**	0.85	0.00	0.07	0.03		0.91	0.01	n.a.	n.a.	n.a.	n.a.
5-40% 150 bar	**4c**	1.02	0.00	0.06	0.03		1.29	0.01	1.41	0.02	1.59	0.04
	Chol	1.73	0.00	0.06	0.03		2.86	0.00	2.22	0.01	6.93	0.02
	7DHC	1.84	0.00	0.06	0.03		3.14	0.03	1.10	0.01	1.14	0.06
	d4	2.40	0.00	0.06	0.03		4.40	0.03	1.40	0.00	5.32	0.16
Si+2-EP	**7c3o**	0.84	0.00	0.04	0.03		0.90	0.02	n.a.	n.a.	n.a.	n.a.
5-20% 150 bar	**4c**	1.02	0.00	0.04	0.03		1.30	0.00	1.44	0.03	1.64	0.02
	Chol	1.97	0.00	0.05	0.03		3.43	0.02	2.64	0.01	8.17	0.06
	7DHC	2.16	0.00	0.05	0.04		3.85	0.03	1.12	0.01	1.47	0.07
	d4	3.23	0.01	0.07	0.04		6.26	0.05	1.63	0.00	7.43	0.23
Si+2-EP	**7c3o**	1.04	0.00	0.09	0.04		1.27	0.02	n.a.	n.a.	n.a.	n.a.
5-10% 100 bar	**4c**	1.28	0.00	0.08	0.04		1.78	0.02	1.41	0.04	1.72	0.05
	Chol	2.75	0.00	0.10	0.05		4.99	0.07	2.80	0.01	9.90	0.15
	7DHC	3.08	0.00	0.11	0.05		5.74	0.06	1.15	0.03	1.91	0.02
	d4	5.48	0.00	0.18	0.08		10.97	0.09	1.91	0.00	10.01	0.05
2-EP+Si	**7c3o**	0.80	0.00	0.13	0.00		0.79	0.01	n.a.	n.a.	n.a.	n.a.
5-40% 150 bar	**4c**	0.97	0.00	0.07	0.00		1.19	0.02	1.49	0.02	0.98	0.02
	Chol	1.64	0.01	0.06	0.00		2.71	0.02	2.29	0.02	6.11	0.08
	7DHC	1.76	0.00	0.06	0.00		2.93	0.01	1.08	0.01	1.10	0.09
	d4	2.30	0.00	0.06	0.00		4.14	0.01	1.41	0.00	5.16	0.03
2-EP+Si	**7c3o**	0.80	0.00	0.12	0.00		0.80	0.01	n.a.	n.a.	n.a.	n.a.
5-20% 150 bar	**4c**	0.97	0.00	0.08	0.00		1.18	0.02	1.47	0.02	1.04	0.01
	Chol	1.84	0.00	0.08	0.00		3.15	0.04	2.66	0.02	6.67	0.02
	7DHC	2.02	0.00	0.08	0.00		3.56	0.03	1.13	0.01	1.25	0.02
	d4	3.02	0.00	0.09	0.00		5.82	0.04	1.64	0.00	6.68	0.04
2-EP+Si	**7c3o**	0.94	0.00	0.07	0.00		1.03	0.01	n.a.	n.a.	n.a.	n.a.
5-10% 100 bar	**4c**	1.17	0.00	0.09	0.00		1.53	0.02	1.49	0.03	1.63	0.02
	Chol	2.45	0.01	0.11	0.00		4.30	0.04	2.80	0.00	7.44	0.16
	7DHC	2.73	0.00	0.12	0.00		4.87	0.04	1.13	0.02	1.40	0.03
	d4	4.76	0.00	0.17	0.00		9.25	0.07	1.90	0.00	8.15	0.01

In order to investigate the influence of different mobile phase conditions on separations and to further optimize separations, other previously used separation methods were applied for the couplings. Back pressure was raised to 150 bars and a mobile phase gradient from 5% to 20% isopropanol and 5% to 40% were tested (Table 3). This variation of mobile phase conditions resulted in reduced retention, which was already observed during separation optimization. The resolution decreased with increasing

gradient slope, but this effect was more impacting for the 2-EP+Si coupling than for the Si+2-EP. Even at a gradient from 5-20% in 5 minutes, resolution of 7c3o/4c was decreased to 1.04 (Table 3). The Si+2-EP coupling provided sufficient separations with a gradient of 5-20% modifier in 5 minutes. Both compound pairs of interest were sufficiently resolved (R= 1.64 for 7c3o/4c and 1.47 for Chol/7DHC). Increasing the gradient slope to 5-40% lead to a further loss of retention and resolution of the compound pair Chol/7DHC was insufficient (R= 1.14). The alteration of mobile phase composition and system pressure had significant influence on separations. The increase of modifier concentration lead to a loss of retention and resolution, but the previously observed additive behavior of the Si+2-EP coupling was remained. The reproducibility of separations with coupled stationary phases was in the same range as observed for single column experiments, with retention time standard deviations (n=3) of 0.01 minutes and less (Table 2 and 3).

The coupling of complementary stationary phases can be considered as an alternative approach to solve complex separation challenges. The initial screening of different stationary phases and the separation optimization with the most promising stationary phase should remain the basis of SFC method development. For separation challenges, which cannot be resolved with the primarily selected stationary phase, we advise to utilize data from the initial column screening to assess the complementarity of the other available stationary phases. A combination of stationary phases with complementary selectivity might be a valuable option. The following criteria led to a successful coupling of stationary phases in this study:

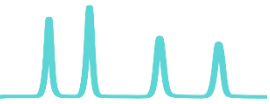
- The set of stationary phases which is tested in the initial screening should contain at least two stationary phases with similar characteristics on the basis of LSER or SRD classification.
- Comparable elution order of compounds on both stationary phases. The coupling of stationary phases with opposing elution order of compounds can lead to impaired separations in the coupling, compared to individual stationary phases
- Coupling of stationary phases due to (net) retention times of the compounds. To preserve the characteristics of individual separations, the (net) retention times of the compounds which should be separated have to be considered. To achieve additive separations, the stationary

phase which provides the fastest elution (lower retention time) should be located in the first position of the coupling.

- Higher system pressures will result in altered separations. The coupling of stationary phases will result in a higher pressure of the mobile phase in the columns. As a result, separations obtained with coupled stationary phases will be comparable but not identical to those achieved in single stationary phases.

Conclusions

Five analytes, among which two pairs of isomeric compounds could be found, were aimed to be separated by SFC. A generic column screening identified two polar phases, silica and 2-ethylpyridine as most suitable for the chromatographic task. After separation optimization, all analytes could be separated by parallel separation with 2-EP and Si, while all isomeric pairs were fully separated. Each stationary phase was capable of separating one (opposite) pair of analytes. Retention order of compounds was identical for the two column and selectivities were complementary. To further optimize separations, both stationary phases were coupled. As a result, both pairs could be separated in a single analysis. It could clearly be shown that the order of columns in the coupling affected separations. The characteristics of the mobile phase in SFC are ideal for the coupling of stationary phases. Even with increased of gradient slope, all compounds remained separated. The coupling of silica and 2-ethylpyridine behaved additive and complementary compared to experiments with single columns. The coupling of Si + 2-EP allowed to baseline separate all five, structurally very similar compounds in less than 6 minutes. Major characteristics from individual separations were still present in the coupling. This powerful approach can help to solve separation challenges and to speed up separation optimization. SFC users should be encouraged to take this option more often into consideration for challenging SFC separations.



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References

[1] Lesellier, E., West, C., The many faces of packed column supercritical fluid chromatography - A critical review. *J. Chromatogr. A* 2015, *1382*, 2–46.

[2] Lesellier, E., Retention mechanisms in super/subcritical fluid chromatography on packed columns. *J. Chromatogr. A* 2009, *1216*, 1881–1890.

[3] West, C., Lesellier, E., Characterisation of stationary phases in subcritical fluid chromatography by the solvation parameter model: II. Compairson tools. *J. Chromatogr. A* 2006, *1110*, 191–199.

[4] West, C., Lesellier, E., Orthogonal screening system of columns for supercritical fluid chromatography. *J. Chromatogr. A* 2008, *1203*, 105–113.

[5] West, C., Khalikova, M.A., Lesellier, E., Héberger, K., Sum of ranking differences to rank stationary phases used in packed column supercritical fluid chromatography. *J. Chromatogr. A* 2015, *1409*, 241–250.

[6] Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., et al., A novel nuclear receptor / coregulator complex controls C . elegans lipid metabolism , larval development , and aging. *Genes Dev.* 2004, 2120–2133.

[7] Hsin, H., Kenyon, C., Signals from the reproductive system regulate the lifespan of C. elegans. *Nature* 1999, *399*, 362–366.

[8] Yamawaki, T.M., Berman, J.R., Suchanek-Kavipurapu, M., McCormick, M., et al., The somatic reproductive tissues of C. elegans promote longevity through steroid hormone signaling. *PLoS Biol.* 2010, *8*, 45–46.

[9] Thondamal, M., Witting, M., Schmitt-Kopplin, P., Aguilaniu, H., Steroid hormone signalling links reproduction to lifespan in dietary-restricted Caenorhabditis elegans. *Nat. Commun.* 2014, *5*, 4879.

[10] Witting, M., Rudloff, H.-C., Thondamal, M., Aguilaniu, H., et al., Fast separation and quantification of steroid hormones Δ4- and Δ7-dafachronic acid in Caenorhabditis elegans. *J. Chromatogr. B* 2015, *978–979*, 118–121.

[11] Li, T.-M., Chen, J., Li, X., Ding, X., et al., Absolute Quantification of a Steroid Hormone that Regulates Development in Caenorhabditis elegans. *Anal. Chem.* 2013, *85*, 9281–9287.

[12] Li, T.-M., Liu, W., Lu, S., Zhang, Y.-P., et al., No Significant Increase in the Δ4- and Δ7-Dafachronic Acid Concentration in the Long-Lived glp-1 Mutant, nor in the Mutants Defective in Dauer Formation. *G3 Genes|Genomes|Genetics* 2015, *5*, 1473–1479.

[13] Aguilaniu, H., Fabrizio, P., Witting, M., The Role Dafachronic Acid Signaling in Development and Longevity in Caenorhabditis elegans: Digging Deeper Using Cutting Edge Analytical Chemistry. *Front. Endocrinol. (Lausanne).* 2016, *7*, 12.

[14] Sardella, R., Carotti, A., Gioiello, A., Lisanti, A., et al., Chromatographic separation of free dafachronic acid epimers with a novel triazole click quinidine-based chiral stationary phase. *J. Chromatogr. A* 2014, *1339*, 96–102.

[15] Hanson, M., Aspects of Retention Behaviour of Steroids in Packed Column Supercritical Fluid Chromatography. *Chromatographia* 1995, *40*, 58–68.

[16] Xu, X., Roman, J.M., Veenstra, T.D., Van Anda, J., et al., Analysis of fifteen estrogen metabolites using packed column supercritical fluid chromatography-mass spectrometry. *Anal. Chem.* 2006, *78*, 1553–8.

[17] Berger, T.A., *Packed Column SFC*, Royal Society of Chemistry, Cambridge 1995.

[18] Alexander, A.J., Staab, A., Use of achiral/chiral SFC/MS for the profiling of isomeric cinnamonitrile/hydrocinnamonitrile products in chiral drug synthesis. *Anal. Chem.* 2006, *78*, 3835–8.

[19] Phinney, K.W., Sander, L.C., Wise, S.A., Coupled achiral/chiral column techniques in subcritical fluid chromatography for the separation of chiral and nonchiral compounds. *Anal. Chem.* 1998, *70*, 2331–5.

[20] Lesellier, E., Analysis of polycyclic aromatic hydrocarbons by supercritical fluid chromatography (SFC). *Analusis* 1999, *27*, 241–248.

[21] Deschamps, F.S., Lesellier, E., Bleton, J., Baillet, A., et al., Glycolipid class profiling by packed-column subcritical fluid chromatography. *J. Chromatogr. A* 2004, *1040*, 115–121.

[22] Abrahamsson, V., Rodriguez-Meizoso, I., Turner, C., Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography. *J. Chromatogr. A* 2012, *1250*, 63–68.

[23] Lesellier, E., Latos, A., de Oliveira, L., Ultra high efficiency/low pressure supercritical fluid chromatography with superficially porous particles for triglyceride separation. *J. Chromatogr. A* 2014, *1327*, 141–148.

[24] Lesellier, E., Efficiency in supercritical fluid chromatography with different superficially porous and fully porous particles ODS bonded phases. *J. Chromatogr. A* 2012, *1228*, 89–98.

[25] Ishibashi, M., Ando, T., Sakai, M., Matsubara, A., et al., High-throughput simultaneous analysis of pesticides by supercritical fluid chromatography/tandem mass spectrometry. *J. Chromatogr. A* 2012, *1266*, 143–148.

[26] Kot, A., Sandra, P., David, F., Selectivity tuning in packed column SFC separation of the sixteen priority polycyclic aromatic hydrocarbons as an example. *J. High Resolut. Chromatogr.* 1994, *17*, 277–279.

[27] Wang, C., Tymiak, A.A., Zhang, Y., Optimization and Simulation of Tandem Column Supercritical Fluid Chromatography Separations Using Column Back Pressure as a Unique Parameter. *Anal. Chem.* 2014, *86*, 4033–4040.

[28] Delahaye, S., Lynen, F., Implementing Stationary-Phase Optimized Selectivity in Supercritical Fluid Chromatography. *Anal. Chem.* 2014, *86*, 12220–12228.

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