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Generic chiral method development in supercritical fluid chromatography and ultra-performance supercritical fluid chromatography

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ABSTRACT

The development of chiral separation methods in pharmaceutical industry is often a very tedious, labour intensive and expensive process. A trial-and-error approach remains frequently used, given the unpredictable nature of enantioselectivity. To speed-up this process and to maximize the efficiency of method development, a generic chiral separation strategy for SFC is proposed in this study. To define such strategy, the effect of different chromatographic parameters on the enantioselectivity is investigated and evaluated. Subsequently, optimization steps are defined to improve a chiral separation in terms of resolution, analysis time, *etc.* or to induce separation when initially not obtained. The defined strategy proved its applicability and efficiency with the successful separation of a novel 20-compound test set. In a second stage, the method transfer from a conventional to an ultra-performance SFC system is investigated for the screening step of the separation strategy. The method transfer proved to be very easy and straightforward. Similar enantioresolution values, but slightly shorter analysis times were obtained on the ultra-performance equipment. Nevertheless, even more benefit may be expected in ultra-performance SFC when customized sub-2 μm chiral stationary phases will become available.

1. Introduction

Over the past years, much attention has been paid to sub- and supercritical fluid chromatography (SFC) in the context of chiral separations [1–4]. By exploiting the benefits of sub- and supercritical fluids, fast and efficient enantioseparations can be obtained in SFC. Simply returning to ambient conditions evaporates the primary eluent, carbon dioxide (CO_2), from the mobile phase after analysis. Hence, SFC can deliver a significant reduction in waste generation and – disposal compared to conventional high-performance liquid chromatography (HPLC) [5]. The higher flow rates, that can be applied in SFC, allow higher productivities relative to HPLC, which is an important asset in a pharmaceutical industrial environment to accelerate the drug development process [6,7]. Given these properties, SFC has become a predominant technique for (preparative) enantioresolutions [2,3,6–8].

As for all separation techniques, chiral method development is also in SFC quite labour intensive. Enantioselectivity remains unpredictable and the best way to achieve appropriate separation conditions is by experimental trial-and-error. To make method development more efficient and faster, generic separation strategies can be utilized [9–12]. These strategies screen a chiral compound on a limited number of complementary chromatographic systems (stationary + mobile phase combinations) in order to find the most suitable system, showing the best enantioselectivity. Depending on the outcome of this screening, optimization steps guide the user further to obtain the desired separation. In case the desired separation could not be achieved, one is referred to screen in another separation technique.

A generic screening approach in SFC, that allows a fast selection of an appropriate chromatographic chiral separation system for diverse chiral mixtures, was proposed earlier [13]. Polysaccharide-based chiral stationary phases (CSPs) were used in this screening because of their broad enantiorecognition capabilities and easy availabilities [3,14]. However, after executing this screening, one might not have achieved the desired separation yet. In that context, further method optimization steps can be defined. These aim to optimize resolution, selectivity, analysis time, and in relevant

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cases also the peak shape. A first part of this paper focuses on the influence of different parameters on a chiral SFC separation. Based on this information, appropriate optimization steps are derived to complete the entire generic separation strategy. To evaluate the performance of this strategy, a novel 20-racemates set is tested.

To catch up with the state-of-the-art technology found in the field of HPLC, SFC equipment is becoming better adapted, more robust and more reliable to achieve chromatographic separations with acceptable repeatability and reproducibility. In particular, the mobile phase density can be controlled much stricter, which is a crucial aspect in SFC since the density has a direct impact on the mobile-phase strength. Following the trend in HPLC, SFC is undergoing an evolution to ultra-high performance SFC (UHP-SFC) [15,16]. With minimal void volumes and maximal sensitivity, fast separations can be achieved with high efficiencies. Because certain parameters are different between the different systems, (enantio)separations might be impacted when transferred. A second part of this research therefore focuses on the method transfer from conventional SFC to UHP-SFC.

2. Experimental

2.1. Chromatographic equipment

The analytical SFC method station from Thar® (Pittsburgh, PA, USA, a Waters® company) equipped with a Waters® 2998-DAD detector (Milford, MA, USA) was used for the first part of the experiments (definition of the separation strategy). The autosampler was equipped with a 10 μl loop. For all analyses partial loop injections of 5 μl were done. Data acquisition and processing were performed using Chromscope® V1.10 software (2011) from Waters®.

For the strategy evaluation and method transfer to UHP-SFC, an Acquity UltraPerformance Convergence Chromatography (UPC^2) from Waters® was used. The system was equipped with a binary solvent manager, a sample manager with a fixed loop of 10 μl, a convergence manager, an external Acquity column oven and a PDA detector. For all analyses partial loop injections of 5 μl were done. Empower® 3 V7.10 software (2010, Waters®, Milford, MA, USA) was used for data acquisition and processing.

The chromatographic conditions were different for the analyses performed during the optimization process. For this reason they are specified further.

2.2. Materials

The columns Chiralpak® AD-H and Chiralcel® OD-H, OJ-H and OZ-H were purchased from Chiral Technologies (West Chester, PA, USA). Lux® Cellulose-1, -2, and -4 were purchased from Phenomenex (Utrecht, The Netherlands). To allow a fair comparison, all columns had dimensions of 250 mm × 4.6 mm i.d. with 5 μm particle size.

2.3. Chemicals

Methanol (MeOH), ethanol (EtOH) and 2-propanol (2PrOH) were HPLC grade and purchased from Fisher Chemicals (Loughborough, UK). Isopropylamine (IPA) and trifluoroacetic acid (TFA) were from Aldrich (Steinheim, Germany). CO_2 was used as advised by the manufacturers of the individual SFC instruments. For the Thar® equipment this was quality 2.7 (purity ≥99.7%) from Linde Gas (Grimbergen, Belgium); for the UPC^2® equipment quality 4.5 (purity ≥99.995%) from Messer (Sint-Pieters-Leeuw, Belgium).

All percentages expressed in the context of mobile-phase composition are volume percentages.

2.4. Chiral test set

For the definition of the optimization steps and separation strategy, a generic chiral test set of 56 pharmaceuticals was used. Test solutions of these 56 racemates with a concentration of 0.5 mg/ml were made in methanol. The solutions were kept at 4 °C when not used. The test set was composed of racemates with diverse structural, chemical, and pharmacological properties. Because it was used in earlier research, we refer to these papers for detailed information [17,18]. To evaluate the proposed separation strategy, a novel test set composed of 20 pharmaceutical racemates is used (Table 1). These racemates were also dissolved in MeOH at a concentration of 0.5 mg/ml and kept at 4 °C.

2.5. Data processing

For all enantioseparations, the resolution (Rs) is calculated using the European Pharmacopoeia equations applying peak widths at half heights [19]. Separations obtained with a resolution higher than 1.5 are considered as baseline separated. When the resolution is between 0 and 1.5 the separations are designated as partial. The selectivity (α) is calculated as the ratio of the retention factors of the last and first eluting enantiomers of a pair [19]. The void time was marked as the first disturbance of the baseline after injection of solvent. The retention time of the last eluting peak is taken as the analysis time.

Microsoft® Excel (Microsoft® Corporation, 2010) was used for constructing the plots and graphs and for the statistical interpretation of the data (Student *t*-test and ANOVA).

3. Results and discussion

3.1. Screening step

A generic chiral screening approach was derived from the evaluation of 12 polysaccharide-based chiral stationary phases in combination with eight mobile phases (MP) (total of 96 chromatographic systems). The performance in terms of successful enantioseparations, and the complementarity of the latter systems were taken into account, to define a screening sequence (Fig. 1) [13]. The screening entails four experiments, evaluating four complementary polysaccharide-based stationary phases. This approach allowed the separation of all compounds from the 56-compound test set. However, not every separation is optimal, *e.g.* Rs < 1.5 (partial separations) or excessive analysis time can be obtained. In these cases further optimization imposes itself in order to obtain the desired enantioseparation. Because a number of factors influence enantioseparation in SFC, *e.g.* organic modifier, flow rate, pressure, temperature, *etc.*, the optimization is not always evident. In a first part of this work, attention will be paid to these factors impacting enantioseparation. The obtained information will be used to define specific optimization steps in the context of a generic chiral separation strategy.

3.2. Factors influencing enantioseparations in SFC

3.2.1. Organic modifier type

In most cases, pure CO_2 is not adequate to elute (pharmaceutical) compounds. Most pharmaceutical compounds possess a structure with hydrophobic, hydrogen-bonding donor and -acceptor sites. This requires the addition of an organic modifier to the mobile phase to increase the solvent strength, allowing elution and analysis of these relatively polar compounds [4,5].

It is well-known that the organic modifier type in the mobile phase alters the enantioselectivity of a CSP towards certain racemates. The lipophilicity, polarity, basicity, *i.e.* the properties of the

Table 1
Test-set compounds used to evaluate the separation strategy.

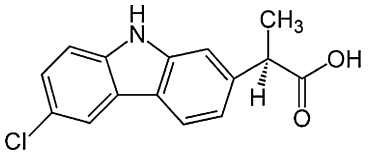
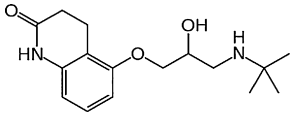
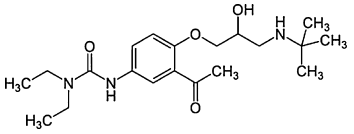
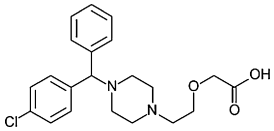
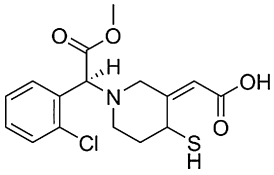
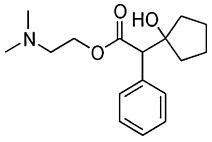
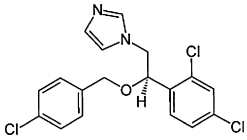
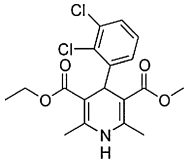
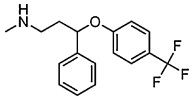
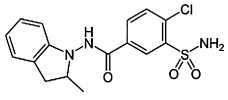
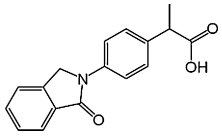
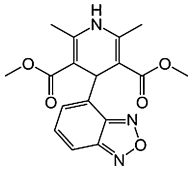
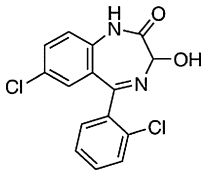
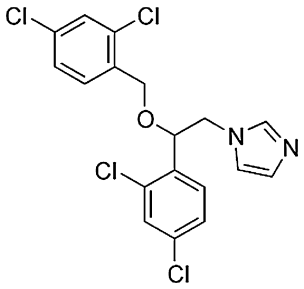
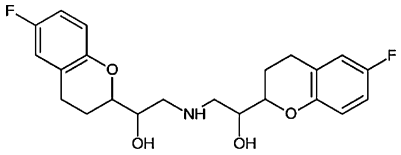
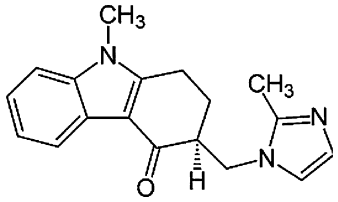
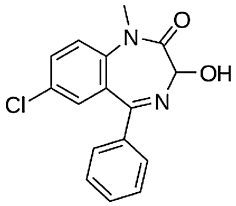
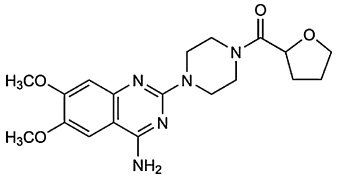
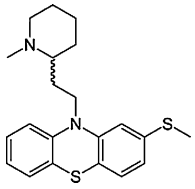
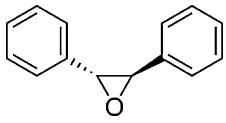
Racemate	Structure	Origin
Carprofen		Sigma–Aldrich, Steinheim, Germany
Carteolol		Madaus AG, Köln, Germany
Celiprolol		Origin unknown
Ceterizine		Sigma–Aldrich, Steinheim, Germany
Clopidogrel		Origin unknown
Cyclopentolate		Gift from Phenomenex
Econazol		Janssen research foundation, Beerse, Belgium
Felodipine		Hassle (Astra), Sweden
Fluoxetine		Sigma–Aldrich, Steinheim, Germany
Indapamide		Sigma–Aldrich, Steinheim, Germany
Indoprofen		Sigma–Aldrich, Steinheim, Germany
Isradipine		Origin unknown

Table 1 (*Continued*)

Racemate	Structure	Origin
Lorazepam		Wyeth, NY, USA
Miconazol		Janssen research foundation, Beerse, Belgium
D/L-Nebivolol		Janssen research foundation, Beerse, Belgium
Ondansetron		Glaxo Wellcome, Belgium
Temazepam		Origin unknown
Terazosine		Sigma–Aldrich, Steinheim, Germany
Thioridazine		Origin unknown
trans-Stilbene oxide		Origin unknown

organic modifier affect the interactions between the solute and stationary phase [20]. Consequently, by changing the organic solvent in the mobile phase, different enantioseparations can be achieved on the same CSP. In chiral SFC, methanol, 2-propanol and ethanol are most often used as modifiers [6,12,7,21–23]. In our experience, MeOH is slightly more successful on the polysaccharide-based CSPs, followed by 2PrOH and EtOH. MeOH offers the additional advantage that its boiling point is lower than that of 2PrOH and EtOH, making solvent evaporation after analysis easier. The viscosity of MeOH is also lower and its use poses thus less stress on the CSPs.

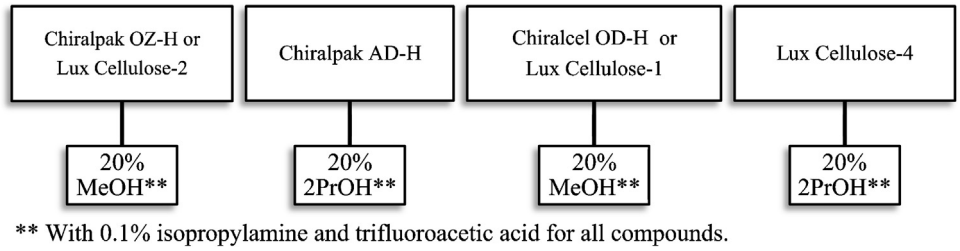


Fig. 1. Scheme of the screening step as defined in [13]. In the top row the chiral stationary phases are presented, while the second row represents the used modifier concentration in the carbon-dioxide based mobile phase.

In earlier research, 12 polysaccharide-based chiral stationary phases were evaluated with eight MeOH- or 2PrOH-containing mobile phases [13]. On eight of these twelve CSPs, a MeOH-containing mobile phase provided the highest success rate. For this reason we slightly favour MeOH over 2PrOH.

As far as enantioselectivity is concerned, it is impossible to predict which solvent will provide the most favourable separation conditions for a given racemate. Earlier we selected four successful and complementary chromatographic systems, using a generic compound test set. We included these systems in a screening approach [13]. For most compounds, executing this screening should deliver appropriate selectivity to achieve the desired enantioseparation. We were able to separate (baseline or partially) the entire 56-compound test set using MeOH in combination with OZ-H (or LC-2) and OD-H (or LC-1); and using 2PrOH with AD-H and LC-4.

However, in case no (satisfying) separation is obtained after this screening, it is advisable to screen the same CSPs with the alternative modifier, *i.e.* 2PrOH (for OZ-H/LC-2 and OD-H/LC-1) or MeOH (for AD-H and LC-4), since this broadens the enantioselective range. Different enantioselectivities, were observed when considering both modifiers. In most cases MeOH yields more separations (Table 2). AD-H seems an exception to this trend, since 2PrOH is much more successful than MeOH on this CSP. Nevertheless, on each CSP, a number of unique separations is provided by both modifiers. This explains the second step in our screening strategy, which proposes to screen the selected CSPs with an alternative modifier.

We also noticed a unique enantioselectivity of some stationary phases in combination with EtOH. In case no enantioselectivity is obtained after screening with MeOH or 2PrOH, EtOH can therefore be tested as alternative modifier. However, given the lower general success rate of EtOH, it would be less advisable to include this modifier in a first screening attempt.

3.2.2. Concentration of the organic modifier

In low concentrations (<2–5%), the organic modifier competes with the analytes for interaction with residual silanol groups on the stationary phase. By surrounding the active silanol sites, the stationary phase becomes more uniform in terms of polarity and consequently peak shapes become more symmetrical. Hence, in the lower concentration range, an increase in modifier content is advantageous for the resolution of the separations. Once all silanol sites are covered by modifier molecules, a further increase in modifier concentration negatively influences the resolution by impacting the solvent strength of the mobile phase [24]. The separation efficiency also tends to deteriorate, since the analyte diffusion through the column is inhibited by the increasing mobile phase viscosity [25].

These trends are clearly seen in the separation of clopidogrel on Chiralpak® AD-H (Fig. 2). When the modifier content is increased from 5 to 10% the resolution increases from 3.8 to 4.6. Increasing the modifier content above 10%, decreases the resolution. On the other hand, the analysis time is impacted by the mobile phase strength. A decrease from 7.98 to 2.75 min occurs when the modifier increases from 5 to 20%. Further increasing the modifier in the mobile phase to 40% decreases the analysis time to 1.72 min. However, the relation between the analysis time and modifier content is not linear and the observed decrease in analysis time is higher in the lower concentration range (5–20%).

Conclusively it can be stated that the most appropriate modifier content in the mobile should be a compromise between analysis time and resolution. In our strategy we propose to increase the modifier content when shorter analysis times are desired. If higher

Table 2
Number of separations obtained with the 56-compound test set using 20% methanol (MeOH) or 2-propanol (2PrOH) in the mobile phase (with 0.1% isopropylamine and 0.1% trifluoroacetic acid added to the modifier). The separations that are only obtained with one modifier on a given stationary phase are considered as unique separations.

	Baseline separations		Partial separations		Unique separations	
	MeOH	2PrOH	MeOH	2PrOH	MeOH	2PrOH
OZ-H	27	22	18	10	15	2
AD-H	12	25	11	12	3	17
OD-H	27	25	14	10	7	2
LC-4	28	24	14	12	9	4

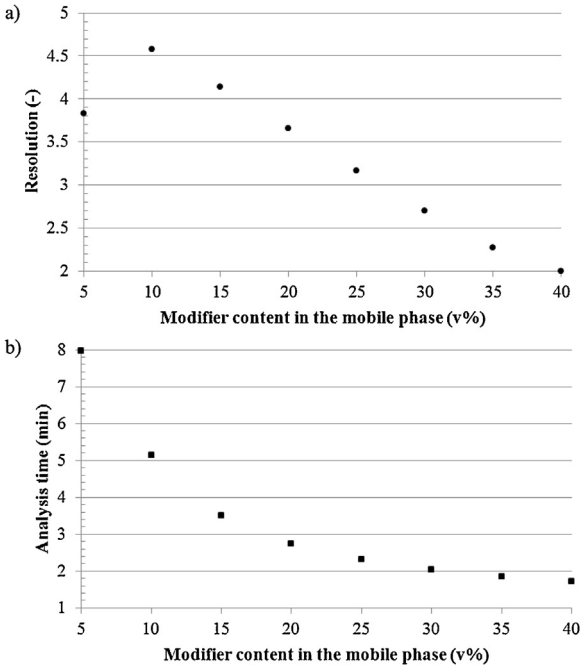


Fig. 2. Separation results of clopidogrel on Chiralpak® AD-H with (2PrOH + 0.1%TFA + 0.1%IPA) in the mobile phase in varying concentrations. (a) Obtained resolutions and (b) the total analysis time in function of the percentage modifier content.

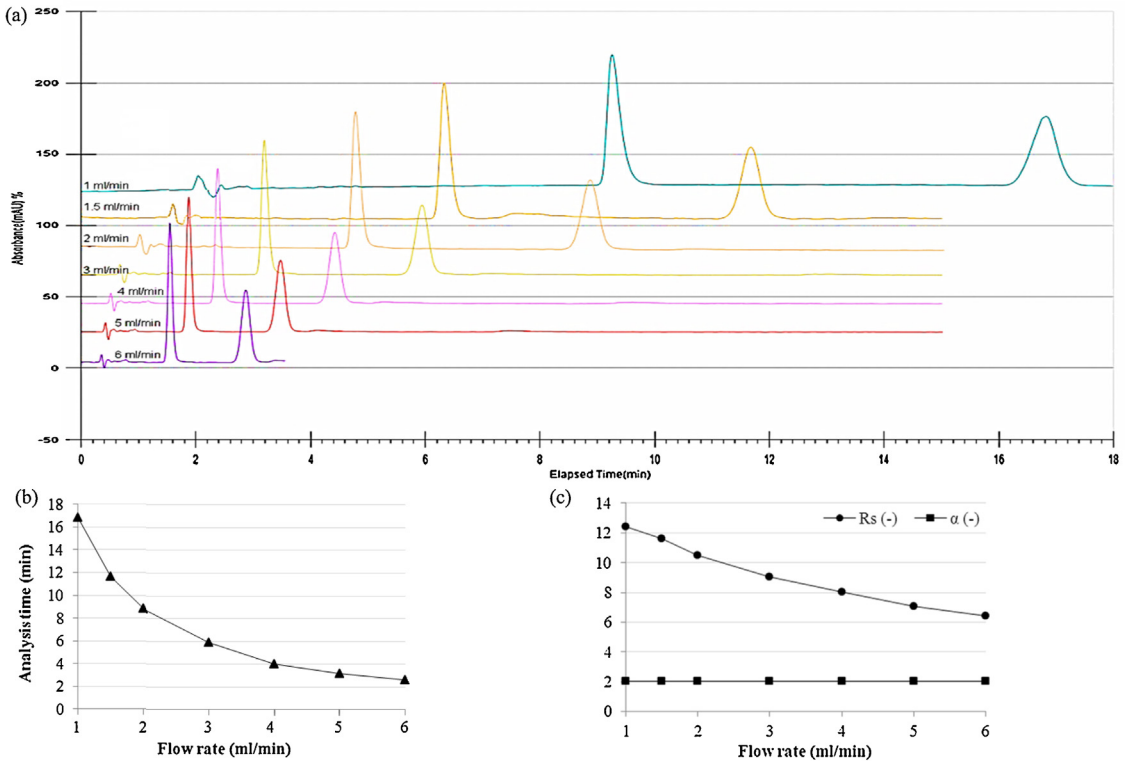


Fig. 3. Results of the enantioseparation of cetirizine on Chiralpak AD-H with 20% (2PrOH + 0.1%IPA + 0.1%TFA) in the mobile phase as a function of the flow rate. (a) Overlay of the obtained chromatograms; (b) analysis time; (c) resolution and selectivity as a function of the flow rate.

resolutions are desired we advise the opposite. As a compromise 20% modifier is used in the screening.

3.2.3. Flow rate

Supercritical fluid chromatography is suitable for fast analyses. Because the sub- or supercritical mobile phase has a low viscosity and high diffusivity, higher flow rates can be used compared to HPLC. Flow rates up to 5.0 ml per min are no exception in analytical SFC. Increasing the flow rate will fasten an analysis significantly, without compromising the separation efficiency too drastically.

For example, when the flow rate for the enantioseparation of cetirizine is increased from 1 to 6 ml/min, the analysis time reduces with 84% (from 16.8 to 2.6 min), the Rs decreases less than 50% (from 12.42 to 6.41), while α remains almost unchanged (Fig. 3). The separation at 6 ml/min is still largely acceptable, and requires 14 min less than that at flow rate 1 ml/min. Increasing the flow rate above 6 ml/min was not possible due to pressure limitations of the CSP.

Above an example is shown which actually is valid for all chiral SFC-separations. This is explained by the flatter profile of the Van Deemter curve in SFC compared to HPLC, allowing analyses at higher mobile phase velocities without a substantial loss in efficiency [5]. Hence, when optimizing analysis times in SFC, it is advisable to increase the flow rate, since the impact on the resolution remains rather limited. The limiting factors in this approach are the pressure restrictions imposed by the equipment and the chromatographic column.

3.2.4. Back pressure

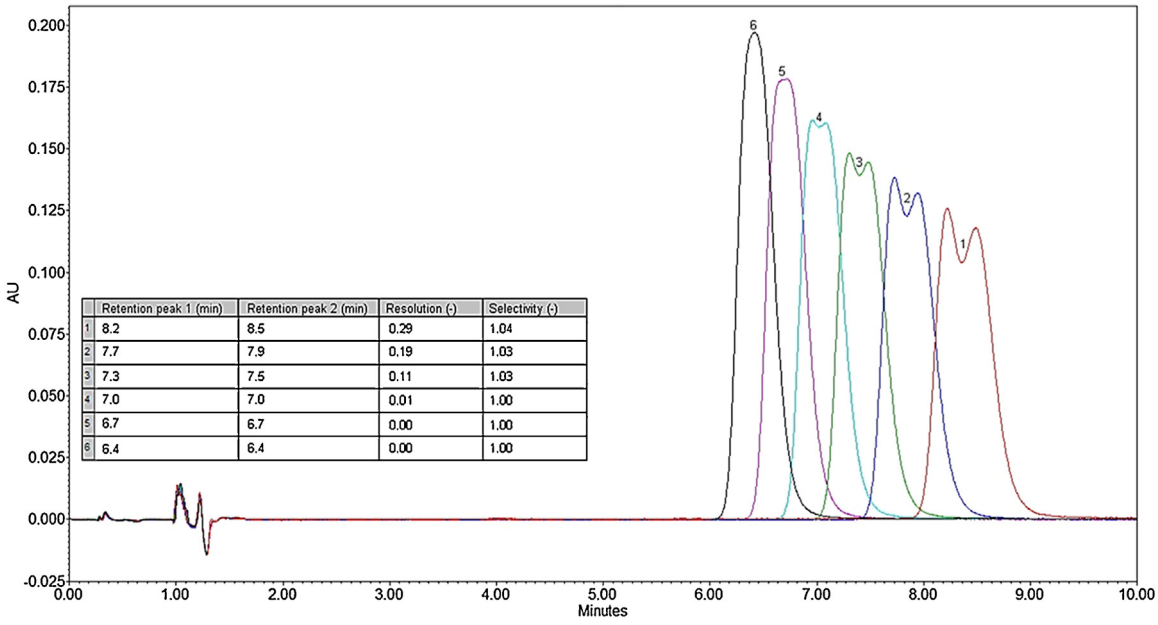
To guarantee a constant mobile-phase density, a back-pressure regulator is employed in SFC controlling the pressure. The mobile-phase density has a direct impact on the mobile-phase strength, thus on the (enantio)selectivity and retention. A higher back pressure means a higher mobile-phase density, and -strength, and shorter retention times. As a consequence, the selectivity might also decrease.

However, when exploring a pressure range in the search for optimal separation conditions, a user is restricted by the limitations of the polysaccharide-based column and the equipment. In practice, back pressures between 125 and 250 bar are commonly used for chiral SFC separations. Using lower pressures harms the chromatographic results significantly since the sub-critical state of the mobile phase is no longer guaranteed [26].

In this pressure range (125–250 bar), the actual impact of the back pressure on the retention and selectivity is rather limited and considerably lower than that of the organic modifier content. In other words, when a large change in retention or selectivity is desired, the first step should be to adopt the modifier content in the MP. When fine-tuning a separation, the back pressure can be changed. For shorter retention/analysis times the back pressure should be increased, while decreasing is advisable when the selectivity should be improved.

For the separation of econazole, a doubling of the back pressure from 125 to 250 bar decreases the retention of the last eluting peak from 8.5 to 6.4 min (Fig. 4). As a consequence, the partial resolution is lost when the back pressure is elevated above 200 bar.

For screening purposes, it is proposed to set the back pressure at 150 bar as a compromise between retention time and enantioselectivity. Consequently, reducing the back pressure to the lower limit of 125 bar would only result in a minimal gain in enantioselectivity. Therefore this step is not included in the partial separation branch of the strategy (see further). On the other hand, to speed up the analysis, it is more effective to increase the flow rate and/or modifier content than the back pressure. Therefore, an increase in back pressure is only recommended as a third choice to reduce the analysis time of baseline separations (see further).



	Retention peak 1 (min)	Retention peak 2 (min)	Resolution (-)	Selectivity (-)
1	8.2	8.5	0.29	1.04
2	7.7	7.9	0.19	1.03
3	7.3	7.5	0.11	1.03
4	7.0	7.0	0.01	1.00
5	6.7	6.7	0.00	1.00
6	6.4	6.4	0.00	1.00

Fig. 4. Overlay of the chromatograms of econazole on Chiralcel® OZ-H with 20% (MeOH:IPA:TFA, 100:0.1:0.1, v/v/v) in the mobile phase. A flow rate of 3 ml/min and temperature of 30 °C was used. The backpressures were (1) 125 bar; (2) 150 bar; (3) 175 bar; (4) 200 bar; (5) 225 bar; and (6) 250 bar. (Results generated with the UPC^2 system.)

3.2.5. Temperature

Temperature also influences the mobile-phase density. An increase results in a decrease of the mobile-phase density and has the above-mentioned consequences. It is important to realize that by reducing the temperature, the chromatographic conditions deviate further from the super- into the subcritical region. This does not create practical issues until the subcritical state turns into a two-phase state, which would deteriorate the chromatographic results significantly and prevents proper analyses. The vapour–liquid curve of the pressure–temperature phase diagram separates the two-phase region from the subcritical region. For (chiral) SFC separations it is thus important to remain above that vapour–liquid curve, but there are no further restrictions to the chosen conditions. SFC separations can thus also be performed below 31 °C, *i.e.* the critical temperature of pure carbon dioxide [26].

For polysaccharide-based columns, the temperature range is limited from 5 to 40–50 °C, varying by column-manufacturer info. The actual impact of the temperature on the retention and selectivity in this workable range is rather limited. When the temperature is increased from 10 to 45 °C (a 350% increase), the retention of the last eluting peak of carprofen only decreases from 2.70 to 2.56 min (a decrease of 5%) (Fig. 5). The resolution and selectivity of the separation are hardly affected by this temperature change.

Summarized, it can be stated that although the temperature has an important impact on SFC separations, the workable temperature range with polysaccharide-CSPs is too limited to have a significant gain in analysis time or selectivity. For this reason, a temperature optimization is not included in the final separation strategy (see further). The temperature was therefore set at 30 °C for all experiments, based on the study of Maftouh et al. [12].

3.2.6. Additives

In the screening, 0.1% isopropylamine (IPA) and 0.1% trifluoroacetic acid (TFA) are added to the modifier, of which only 20% in used in the mobile phase. Hence, the final concentration in the MP is 0.02% IPA and TFA. Nevertheless, their addition, even in these low concentrations, affects the interactions between the analytes and the stationary phase. Without the presence of additives in the MP, chromatographic results tend to deteriorate significantly. IPA and other basic amine-additives shield silanol sites on the stationary phase, decreasing the non-specific retention of analytes. They also compete with the basic functional groups of analytes for interactions with specific sites on the stationary phase. These additives also neutralize charged groups of basic analytes, which is

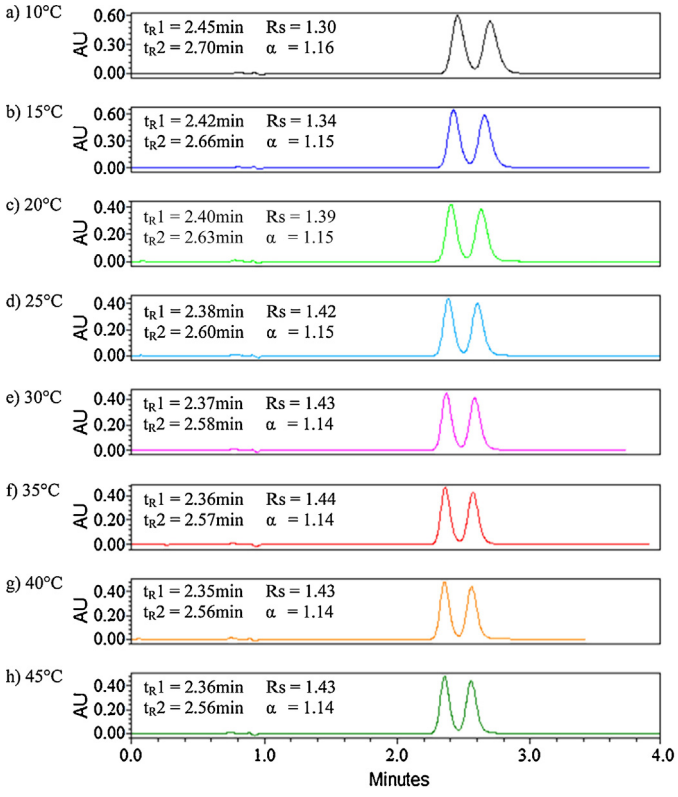


Fig. 5. Chromatograms of the enantioseparation of carprofen on Chiralcel® OZ-H with 20% (2PrOH:IPA:TFA, 100:0.1:0.1, v/v/v) in the mobile phase. A total flow rate of 4 ml/min and back pressure of 150 bar was used. The temperatures were (a) 10 °C; (b) 15 °C; (c) 20 °C; (d) 25 °C; (e) 30 °C; (f) 35 °C; (g) 40 °C; and (h) 45 °C.

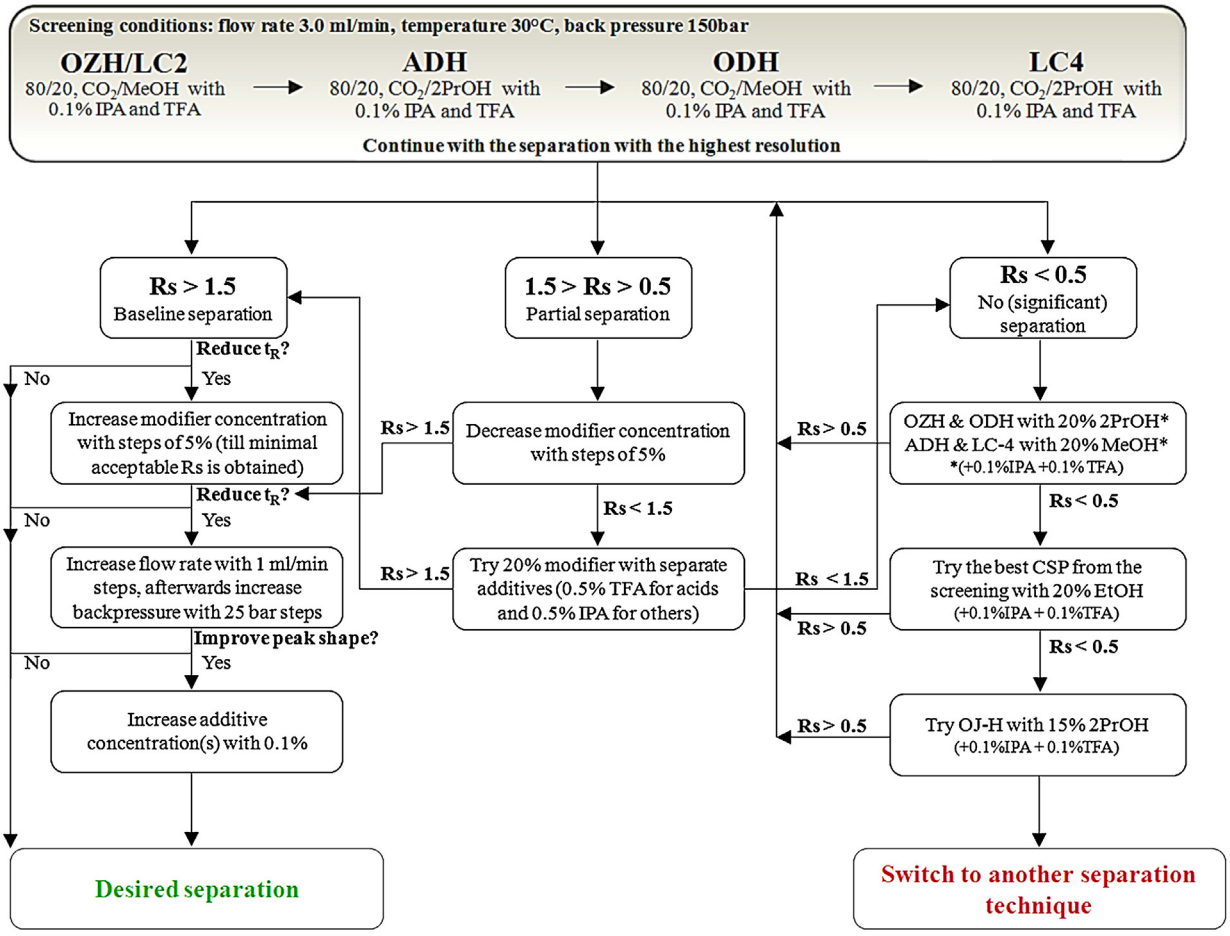


Fig. 6. Chiral separation strategy for polysaccharide-based columns in SFC.

important for the interactions with neutral chiral selectors, such as polysaccharide-derivatives [18,27]. Acidic additives, such as TFA, suppress the ionization of acidic analytes.

For polysaccharide-based chiral columns, these effects do not seem directly related to the concentration of the additives in the MP [28]. We investigated different additive concentrations in a range from 0.1 to 0.25% and saw only a minor impact on the retention or resolution. Peak shapes tend to be slightly sharper with increasing additive concentrations. On the other hand, adding less than 0.1% to the modifier was not sufficient to induce the desired effect; peak

Table 3
For separation strategy: separation results and optimal separation conditions for the 20 compounds from the test set.

	Separation results			Selected optimal separation conditions			
	Rs	α	AT (min)	CSP	Flow rate (ml/min)	Modifier (%)	Modifier type
Carprofen	1.6	1.2	2.6	OZ-H	4.0	20	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Carteolol	2.6	6.5	1.3	OD-H	4.0	30	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Celiprolol	1.5	1.3	3.8	AD-H	4.0	15	EtOH:IPA:TFA, 100:0.1:0.1, v:v:v
Ceterizine	5.5	2.4	2.2	AD-H	4.0	35	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Clopidogrel	2.5	1.5	1.4	AD-H	4.0	35	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Cyclopentolate	4.8	1.7	2.8	AD-H	3.0	20	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Econazole	1.6	1.1	5.3	OZ-H	4.0	20	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Felodipine	2.0	1.2	4.9	AD-H	4.0	10	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Fluoxetine	1.3	1.1	15.0	OZ-H	2.0	5	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Indapamide	1.5	1.3	3.6	OD-H	4.0	30	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Indoprofen	2.7	1.2	4.5	AD-H	4.0	35	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Isradipine	1.6	1.1	7.2	LC-4	3.0	10	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
Lorazepam	3.0	1.4	2.9	OZ-H	4.0	35	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Miconazol	2.0	1.2	5.4	AD-H	4.0	15	2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v
D/L-Nebivolol	2.2	1.5	1.9	OZ-H	4.0	25	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Ondansetron	3.4	1.4	3.0	OD-H	4.0	40	MeOH:IPA, 100:0.1, v:v
Temazepam	2.0	1.2	4.2	OZ-H	4.0	35	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
Terazosine	1.7	1.2	3.7	AD-H	4.0	30	MeOH:IPA, 100:0.1, v:v
Thioridazine	1.8	1.2	3.4	OZ-H	4.0	35	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v
trans-Stilbene oxide	4.4	1.6	1.9	OZ-H	3.0	20	MeOH:IPA:TFA, 100:0.1:0.1, v:v:v

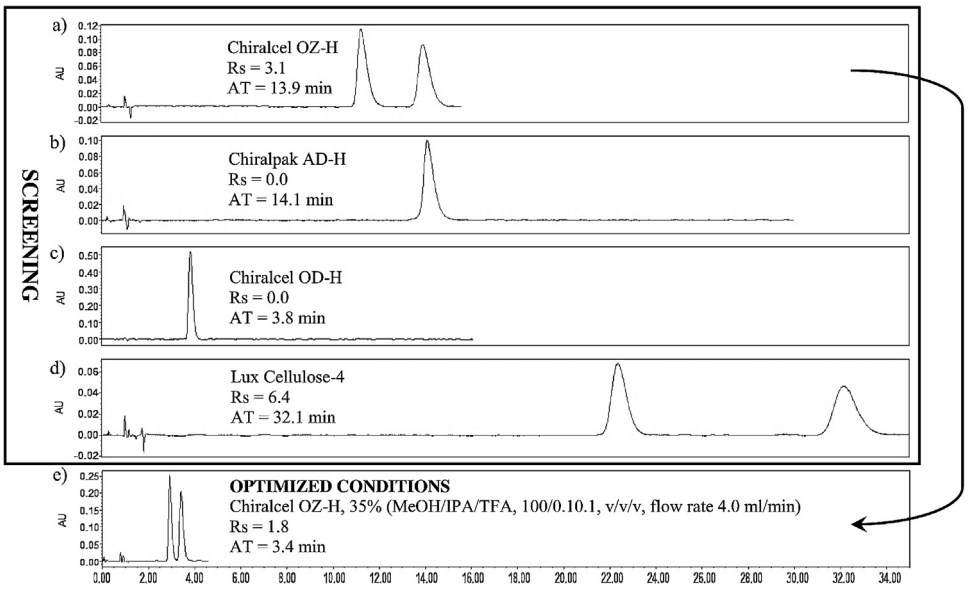


Fig. 7. Separation strategy applied on the racemate thioridazine. Chromatograms a–d: experiments from the screening step, e: optimized conditions.

shapes and chromatographic results were unacceptable. Hence, in the screening, the additive concentration is set at 0.1% IPA and TFA in the modifier.

Earlier, we observed a significant difference in enantioselectivity between the simultaneous use of IPA and TFA and the individual use of these additives [18]. In the latter case, TFA is used for acidic compounds and IPA for all other compounds. Since the success rate tended to be higher when combining the additives, we advise using this approach in a screening stage [18]. Moreover, the benefit is that the screening conditions are the same for all compounds, independent of their chemical properties. However, in case the desired enantioseparation is not achieved, it can be useful to try only one single additive in the modifier. This is therefore recommended in the partial separation branch of the strategy (see further).

3.3. Separation strategy

Based on the above information and earlier experience, a separation strategy was defined (Fig. 6). This strategy was evaluated with a novel test set of 20 pharmaceutical racemates (Table 1). After executing the screening experiments, 18/20 compounds were separated. After applying the entire strategy, all compounds were baseline separated, with the exception of fluoxetine, which was partially separated (Rs = 1.3) (Table 3).

Analysis time for these optimized separations was in 16/20 cases below 5 min, for 19/20 below 10 min and for fluoxetine 15 min.

The separation strategy applied on two racemates, *i.e.* thioridazine and clopidogrel is presented in Figs. 7 and 8, respectively. The chromatograms (a–d) clearly show the complementarity of the

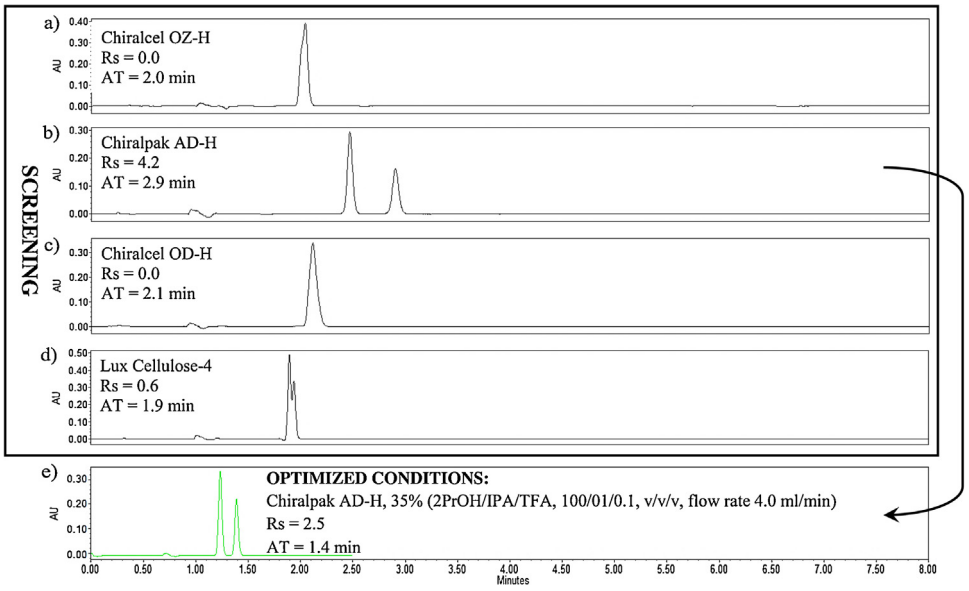


Fig. 8. Separation strategy applied on clopidogrel racemate. Chromatograms a–d are the results from the screening step, e is the result after optimization.

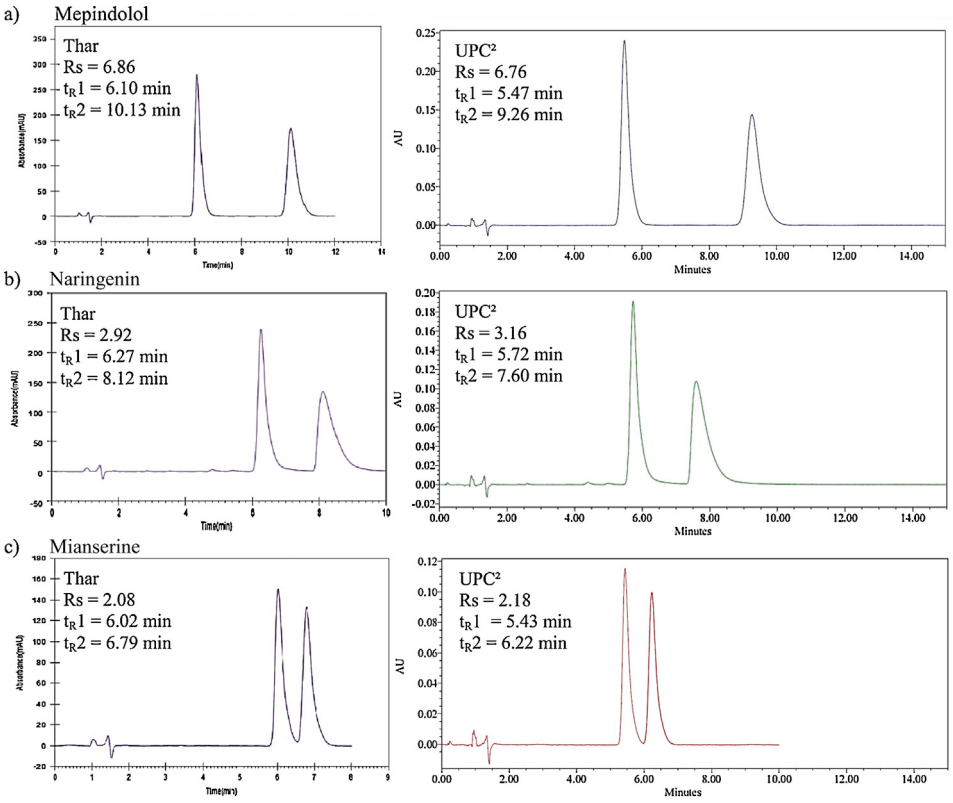


Fig. 9. Transfer of the chromatographic conditions from conventional SFC (Thar equipment) to ultraperformance SFC (UPC^2 equipment). The separations are obtained on Lux Cellulose-2, with 20% (MeOH:IPA:TFA, 100:0.1:0.1, v:v:v) in the mobile phase, flow rate 3.0 ml/min, 30 °C, detection at 220 nm, and a back pressure of 150 bar. (a) Mepindolol, (b) naringenin, (c) mianserine.

chromatographic systems included in the screening step. After the optimization steps, good baseline separations with satisfying peak shapes and short analysis times are obtained.

3.4. Method transfer from conventional SFC to UHP-SFC

The screening conditions from the separation strategy were transferred from a conventional SFC to an ultra-performance (UPC^2) SFC equipment. To evaluate the transfer, the 56-compound test set used as for the definition of the screening was applied. We refer to these earlier papers for more information on its composition [13,17,18]. The four chromatographic systems from the screening were evaluated, *i.e.* OZ-H and OD-H, with 20% (MeOH:IPA:TFA, 100:0.1:0.1, v:v:v), and AD-H and LC-4, with 20% (2PrOH:IPA:TFA, 100:0.1:0.1, v:v:v) in the MP. The same columns and conditions were used on both instruments.

Generally the method transfer from conventional to ultra-performance SFC seems rather straightforward. Usually similar separation results are achieved when applying the same chromatographic conditions in conventional and ultra-performance SFC (Fig. 9).

However, the success rates on all chromatographic systems obtained with the ultra-performance system are slightly lower (Fig. 10). In this context, it is important to analyze the results further since the difference in success rate may originate from small differences in resolution. A partial separation is any separation with a resolution higher than zero, while baseline separations have Rs > 1.5. Hence, in case a separation with resolution 0.2 is obtained on one instrument, a small decrease in Rs on the other may result in a loss of the separation.

We thus compared the resolutions and analysis times (AT) of the 56 compounds. The obtained Rs and analysis times are similar but tend to be slightly lower on the UPC^2 than on the conventional equipment (Fig. 11). These lower resolutions are reflected in the lower success rates on the UPC^2. To assess the significance of the difference in Rs and AT between both instruments, a two-tailed paired Student *t*-test was performed.

Table 4 summarizes the results in terms of the calculated *t*- and *p*-values. For two chromatographic systems, *i.e.* AD-H and LC-4 with 2-propanol in the mobile phase, the resolutions were not significantly different on the conventional Thar SFC and UPC^2. For OD-H and OZ-H with methanol, the difference was determined to be significant.

The analysis times were slightly lower on the UPC^2 than on the conventional Thar instrument. This difference was determined to be significant for all chromatographic systems, with the exception of OD-H with methanol in the mobile phase.

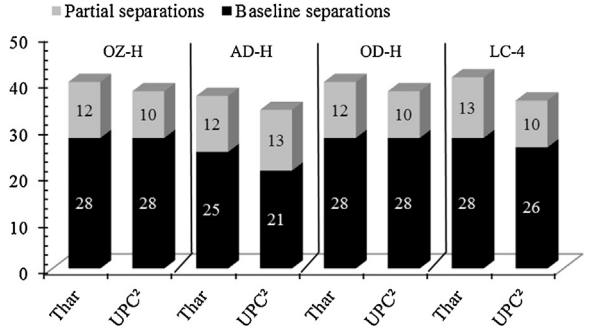


Fig. 10. Number of baseline (Rs > 1.5) and partial (0 < Rs < 1.5) separations achieved with the Thar SFC and UPC^2 systems on the four chromatographic systems of the screening.

Table 4
Two-tailed paired Student *t*-test applied on the data obtained for the 56 compounds (58 enantiomer pairs) on the Thar and UPC^2.

		Chiralcel OZ-H 20% MeOH:IPA:TFA	Chiralpak AD-H 20% 2PrOH:IPA:TFA	Chiralcel OD-H 20% MeOH:IPA:TFA	Lux Cellulose-4 20% 2PrOH:IPA:TFA
Rs	*t*-Value	**3.11**	1.66	**3.10**	0.41
	p-Value	$\mathbf{1.46 \times 10^{-3}}$	5.15×10^{-2}	$\mathbf{1.50 \times 10^{-3}}$	3.42×10^{-1}
AT	*t*-Value	**2.06**	**1.71**	1.50	**4.43**
	p-Value	$\mathbf{2.20 \times 10^{-2}}$	$\mathbf{4.62 \times 10^{-2}}$	6.93×10^{-2}	$\mathbf{2.14 \times 10^{-5}}$

With Rs the resolution and AT the analysis time as responses. Null hypothesis $H_0 : X_{\text{Thar}} = X_{\text{UPC}^2}$, with X a given response (Rs or AT). $t_{57,\alpha=0.05} = 1.67$. Significant *t*- and *p*-values are marked in bold.

Hence, to conclude it can be stated that, in general, the analysis times on the UPC^2 are shorter than on the conventional SFC system. This can be related to the minimization of the void volume in this equipment, resulting in a lower void time. However, this is not translated into separations with higher resolutions. In most cases, the resolutions were slightly lower on the ultra-performance SFC system, on two of the four systems, this decrease was significant. Thus, the resolutions are rather comparable between the two systems, while a gain in analysis time is obtained with the ultra-performance system.

However, the maximal potential of the UHP-SFC system might not be achieved with the 5 μm particle columns used in this study. Reducing the particle size to sub-2 μm dimensions, would possibly increase the separation efficiency significantly [15,16]. So far, no sub-3 μm chiral polysaccharide-based stationary phases are commercially available. The coating of the polysaccharide-based selector on the silica and the uniform and reproducible packing of these smaller particles appears to be very tedious. Hence, more potential lies in UHP-SFC for chiral separations provided that adapted CSP become available.

For this study, where the same columns were used, the method transfer from the conventional to the ultra-performance system was very easy and straightforward.

3.5. Precision study: conventional SFC vs UPC^2

To evaluate the precision of experiments on both systems, six enantioseparations; bopindolol, mepindolol, methadone, mianserine, naringenin, and sotalol, were selected and repeated twice over six consecutive days. The same chromatographic conditions were used on both systems. Lux Cellulose-2 was used as stationary phase, with 20% MeOH:IPA:TFA (100:0.1:0.1) in the mobile phase. The total flow rate was 3.0 ml/min, the temperature 30 °C and back pressure 150 bar. Detection was done at 220 nm. The sample loop was 10 μl and partial injections of 5 μl were done for each sample.

The inter- and intra-day variabilities and the intermediate precision (expressed in variance) were estimated for each separation using ANOVA. Table 5 shows the results for all separations on both systems. Two responses were considered: the resolution and the analysis time (AT). The variances obtained with both systems were compared with an *F*-test.

The intra-day variance on the Rs was not significantly different between the UPC^2 and Thar for three compounds. For methadone and sotalol the variance was smaller on the Thar than on the UPC^2, for mepindolol the opposite was seen. The inter-day variability was not significantly different for three compounds, while for mepindolol, mianserine, and sotalol it was lower on the UPC^2. The intermediate precision was significantly different for two separations: the variance for mepindolol was lower on the UPC^2 and for methadone on the Thar system. These results indicate that there is no distinct benefit of one system over the other concerning the repeatability of experiments when considering the resolution as response.

Next, we considered the analysis time as response. Three separations yielded a significantly different intra-day variability, *i.e.* bopindolol, methadone and naringenin. The first two separations showed a lower variability on the Thar system, while the opposite situation was seen for the last. The inter-day variability was significantly lower on the Thar system for bopindolol and methadone and on the UPC^2 for mianserine, sotalol and naringenin. The intermediate precision on the AT, was lower for bopindolol and methadone on the Thar system and for naringenin on the UPC^2.

Table 5
Results of the six precision studies of two sample injections on six consecutive days on the UPC^2 and Thar systems, expressed in variances.

	Intra-day variability		Inter-day variability		Intermediate precision	
	UPC^2	Thar	UPC^2	Thar	UPC^2	Thar
Bopindolol						
Rs	3.86×10^{-2}	2.03×10^{-2}	1.14×10^{-1}	5.85×10^{-2}	1.53×10^{-1}	7.88×10^{-2}
AT	2.47×10^{-2}	$\mathbf{6.23 \times 10^{-3}}$	4.19×10^{-3}	$\mathbf{6.58 \times 10^{-4}}$	2.89×10^{-2}	$\mathbf{6.88 \times 10^{-3}}$
Mepindolol						
Rs	$\mathbf{1.57 \times 10^{-3}}$	1.44×10^{-2}	$\mathbf{4.98 \times 10^{-3}}$	1.78×10^{-1}	$\mathbf{6.56 \times 10^{-3}}$	1.93×10^{-1}
AT	2.06×10^{-2}	7.67×10^{-3}	1.18×10^{-2}	1.89×10^{-3}	3.24×10^{-2}	9.56×10^{-3}
Methadone						
Rs	9.9×10^{-4}	$\mathbf{1.89 \times 10^{-5}}$	2.99×10^{-4}	4.06×10^{-4}	1.29×10^{-3}	$\mathbf{4.25 \times 10^{-4}}$
AT	2.06×10^{-2}	$\mathbf{3.87 \times 10^{-3}}$	1.18×10^{-2}	$\mathbf{9.17 \times 10^{-5}}$	3.24×10^{-2}	$\mathbf{3.96 \times 10^{-3}}$
Mianserine						
Rs	1.88×10^{-3}	8.06×10^{-4}	$\mathbf{7.03 \times 10^{-5}}$	1.39×10^{-3}	1.95×10^{-3}	2.20×10^{-3}
AT	8.78×10^{-4}	1.08×10^{-3}	$\mathbf{6.33 \times 10^{-6}}$	2.50×10^{-5}	8.84×10^{-4}	1.11×10^{-3}
Sotalol						
Rs	2.03×10^{-3}	$\mathbf{3.14 \times 10^{-4}}$	$\mathbf{5.30 \times 10^{-5}}$	7.43×10^{-4}	2.08×10^{-3}	1.06×10^{-3}
AT	3.46×10^{-3}	2.80×10^{-3}	$\mathbf{7.17 \times 10^{-6}}$	4.17×10^{-5}	3.46×10^{-3}	2.84×10^{-3}
Naringenin						
Rs	2.48×10^{-3}	7.13×10^{-2}	2.03×10^{-4}	1.33×10^{-3}	2.68×10^{-3}	7.26×10^{-2}
AT	$\mathbf{8.87 \times 10^{-4}}$	4.15×10^{-2}	$\mathbf{3.75 \times 10^{-6}}$	2.65×10^{-4}	$\mathbf{8.91 \times 10^{-4}}$	4.18×10^{-2}

With Rs the resolution and AT the analysis time. The results obtained on the UPC^2 and Thar are compared with an *F*-test, the smallest variance of both is marked in bold if the difference is calculated to be significant, $F_{11,11;\alpha=0.05} = 2.82$.

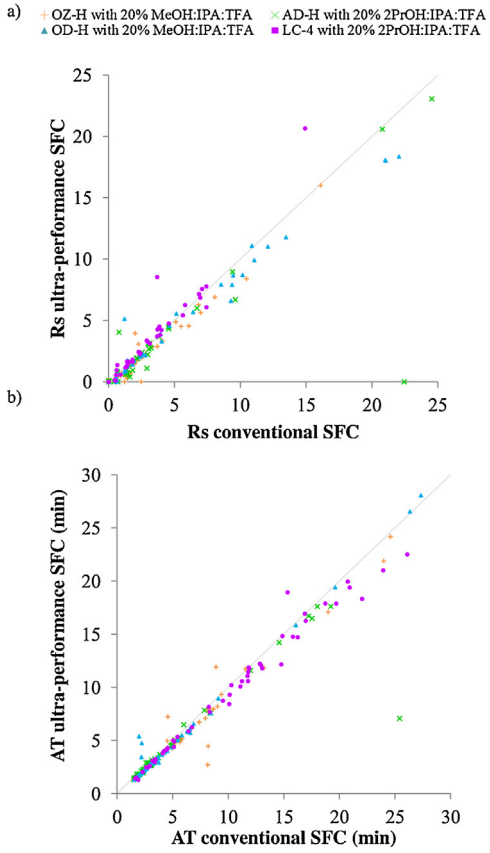


Fig. 11. Comparison of the screening results of the 56-compound test set on the ultra-performance and conventional SFC equipment. (a) Resolutions (Rs), (b) analysis times. Straight line = line of equality.

Hence, the intra- and inter-day variability and intermediate precision of the analysis times between both systems are comparable, and no distinct advantage of one system over the other was seen.

Conclusively, these experiments showed that in terms of precision the performance of both systems were similar.

4. Conclusions

To define a generic separation strategy, the impact of different parameters on chiral SFC separations was investigated. The influence of organic modifier type and – concentration, flow rate, back pressure, temperature and additives, were considered.

When dissimilar enantioselectivity is sought, it is advisable to screen different modifiers in the mobile phase. Methanol was favoured over 2-propanol and ethanol, since this modifier tended to generate higher success rates on the polysaccharide-based CSPs, although a broad complementarity exists between MeOH and 2PrOH. To extend the enantioselective recognition, it is thus advisable to screen a CSP with both modifiers.

When higher resolutions are desired, the modifier concentration can be decreased. When aiming to decrease the analysis time, the flow rate can be increased without compromising the efficiency much. The back pressure and temperature only exert minor influences on the resolution or analysis time of chiral SFC separations on polysaccharide-derivatives. The latter information was used to define a separation strategy, which applicability was evaluated with a novel test set of 20 pharmaceutical racemates. All racemates could be baseline separated, with the exception of fluoxetine. Analysis times were below 10 min for all separated compounds.

The developed approach was transferred from a conventional to an ultra-performance SFC system. Similar separation results in terms of Rs were generated by both systems, while the analysis times were slightly lower on the ultra-performance system. The method transfer thus proved to be very easy and straightforward.

A precision study was performed for six separations on the Thar and UPC^2 system. Results showed no distinct advantage of one system over the other concerning the intra-, and inter-day variabilities or the intermediate precision of the resolution and analysis time of the separations.

More efficient separations could potentially be achieved using sub-2 μm columns. However, so far, no CSPs are commercially available with these particle dimensions. Undoubtedly, there still remains a whole unexplored domain in this context for chiral separations.

Conflict of interest

The authors declared no conflict of interest.

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