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Enantioselectivity of polysaccharide-based chiral stationary phases in supercritical fluid chromatography using methanol-containing carbon dioxide mobile phases

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ABSTRACT

The enantioselectivity of twelve chiral stationary phases (CSPs) and four methanol-containing carbon dioxide mobile phases (MPs) is evaluated in supercritical fluid chromatography (SFC) with a test set of 59 chiral pharmaceutical compounds. Methanol (MeOH) is evaluated as modifier in carbon dioxide (CO_2) since it is commonly used in chiral SFC because of its favorable characteristics and proven successes. In addition to the MP of earlier defined generic screening conditions, new MPs, which contained both a basic (isopropylamine) and an acidic (trifluoroacetic acid) additive, were investigated and yielded broad enantioselectivities. The joint use of the additives impacts the enantioselectivity differently than the individual. Polysaccharide-based CSPs from different manufacturers were assessed, which showed that CSPs containing the same selector do not always display the same enantioselectivity. This work enabled not only to identify the individual chiral systems with the broadest enantioselectivity but also to determine their complementarity, resulting in a limited set of systems with the broadest enantioselectivity. As a result an updated, fast and efficient screening sequence was proposed.

1. Introduction

Chirality has a significant impact on the interaction between biologically active compounds and their receptor binding sites and therefore has been an extensively studied topic in the area of drug discovery [1–5].

Chiral chromatography remains the most important and cost-effective approach to resolve synthesized racemates into enantiopure test substances, particularly in early drug development processes [6–10]. While different chromatographic techniques, such as gas chromatography (GC) and capillary electrochromatography (CEC), can to a certain extend be used for these purposes, the different modes of high-pressure liquid chromatography (HPLC), such as normal-phase (NPLC), reversed-phase (RPLC) and polar organic solvents chromatography (POSC), remain the most applied techniques in the pharmaceutical industry [4,11–14]. This is mainly due to the easy applicability, advanced instrumentation and extensive knowledge of these techniques [9,10]. However, some drawbacks are also related to them, such as rather long analysis times that limit the throughput and a rather high consumption of organic (toxic and flammable) solvents. This stimulated the search for more efficient and environmental-friendly alternatives [15].

As a consequence, a renewed interest in supercritical fluid chromatography for the separation of drug enantiomers was seen over the past years. The major benefits of this technique result from the fact that supercritical fluids possess a higher diffusivity and lower viscosity. Hence, higher flow rates can be used, which reduce analysis- and equilibration times, and increase the throughput capacity, without compromising efficiency. The separated enantiomers can also be easily recovered as pure compounds by simply reducing the pressure and evaporating the supercritical mobile phase [16–19]. Carbon dioxide, having a relatively low critical pressure (73.8 bar) and temperature (31.1 °C), and being a non-toxic, non-flammable and naturally produced substance is by far the most used supercritical eluent in SFC [17]. Taking into account these favorable properties of the principal mobile phase component, SFC can be considered as a "green" technology [15].

Since CO_2 has a polarity comparable to that of n-hexane, polar organic modifiers are required in the mobile phase to adjust the elution strength. These modifiers affect the chromatographic results, mainly through an increase in mobile phase-polarity and -density, leading to an increased solvent strength [20–22]. Both modifier type and concentration influence the chiral separation. Methanol, 2-propanol, ethanol and acetonitrile are the most commonly used in SFC. Although 2-propanol generally provides a broader

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enantioselectivity, MeOH is often chosen as first-choice modifier, because of its lower viscosity, which is more preferred for a later upscaling [15].

Additionally, additives are frequently added to the mobile phase at concentrations of 0.1–2.0% [20,23]. To improve peak shapes charged additives (basic or acidic) suppress the ionization of analytes by ion-pair formation, which improves the interaction with neutral polysaccharide-based columns [24].

Although more than 1500 chiral stationary phases have been reported in the scientific literature, none possesses a universal chiral selector. Moreover, only a small number seem to offer the crucial advantages needed for high-throughput chiral screening, such as a broad enantioselectivity, easy accessibility and a good stability. Meeting these requirements, polysaccharide-based CSPs, developed by Okamoto et al. [25] and first commercialized by Daicel Chemical Industries, have taken a dominant position among the currently commercially available phases [7,24,26]. Unfortunately, the enantioselective behavior of a (novel) chiral molecule remains unpredictable. This can make the choice of the appropriate CSP, displaying the desired enantioselectivity towards a given compound, a very time-consuming step in chiral method development [7]. To enable faster chiral method development, generic screening conditions can be defined, which consist of a limited number of experiments. The aim of these screening experiments is to get a quick idea of the enantioselectivity of certain chromatographic systems in a given technique towards a racemate [27]. This implies that there are three main requisites for a screening: it should be fast, enantioselective and needs to have a broad, generic applicability in order to (partially) separate most of the racemates with at least one of the screened systems.

As mentioned above, SFC can offer several advantages, for these reasons, an update of an existing screening approach in SFC, defined by Maftouh et al. [15], is highly desirable in order to improve the efficiency and/or throughput capacity. The screening from [15] evaluates four Daicel CSPs containing the coated polysaccharide-based selectors: amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak® AD-H), cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel® OD-H), cellulose tris(4-methylbenzoate) (Chiralcel® OJ-H) and amylose tris((S)-α-methylbenzylcarbamate) (Chiralpak® AS-H). Since the definition of this screening approach, new polysaccharide-based CSPs have been commercialized. Some stationary phases contain chlorinated polysaccharide derivatives as chiral selectors. These selectors have shown a complementarity and/or broader enantioselectivity in POSC, NPLC and CEC compared to the non-chlorinated polysaccharide-based selectors. The broader enantioselectivity is induced by the introduction of chlorine, an electron-withdrawing group, in the selector which can enhance the enantioselective interactions [26].

Twelve CSPs, of which the four included in the initial screening, are screened with the aim of selecting the systems with the broadest enantioselectivity. A total of 48 chiral systems (twelve CSPs × four mobile phases) are tested in this study (Table 1). The results will enable concluding whether (i) the initial methanol-based carbon dioxide screening conditions can be successfully applied on the new CSPs, (ii) these screening conditions can be improved in order to achieve a broader enantioselectivity and/or a faster screening, and (iii) the new CSPs can be included in an updated screening approach for SFC with methanol-based carbon dioxide mobile phases.

2. Material and methods

2.1. Chiral test compounds

The 59 pharmaceutical racemates were already used in previous research and are therefore not specified in detail, they can be found in reference [28].

All solutions were prepared at a concentration of 0.5 mg/ml. They were dissolved in MeOH, with the exceptions of leucovorin, which was dissolved in water, and methotrexate for which MeOH + 0.5% TFA was used as solvent, because of solubility issues. Solutions were stored at 4 °C.

2.2. Chemicals

Carbon dioxide 2.7 (purity ≥ 99.7%) was obtained from Linde Gas (Grimbergen, Belgium), methanol (HPLC grade) from Fisher Chemical (Loughborough, Leicestershire, UK). IPA and TFA were from Aldrich.

2.3. Chiral stationary phases

Lux® Cellulose-1 (LC-1), Lux® Cellulose-2 (LC-2), Lux® Cellulose-3 (LC-3), Lux® Cellulose-4 (LC-4), Lux® Amylose-2 (LA-2) and Sepapak®-5 (SP-5), were purchased from Phenomenex® (Utrecht, The Netherlands). Chiralcel® OD-H (OD-H), Chiralcel® OJ-H (OJ-H), Chiralcel® OZ-H (OZ-H), Chiralpak® AD-H (AD-H), Chiralpak® AS-H (AS-H) and Chiralpak® AY-H (AY-H) were from

Table 1
Screened chromatographic systems.

Mobile phases (MPs)	
MP A[a]	90/10 (v/v) CO_2/(MeOH + 0.5% IPA) (*for basic, neutral and amphoteric compounds*) or 90/10 (v/v) CO_2/(MeOH + 0.5% TFA) (*for acidic compounds*)
MP B	80/20 (v/v) CO_2/(MeOH + 0.5% IPA) (*for basic, neutral and amphoteric compounds*) or 80/20 (v/v) CO_2/(MeOH + 0.5% TFA) (*for acidic compounds*)
MP C	90/10 (v/v) CO_2/(MeOH + 0.25% IPA + 0.25% TFA)
MP D	80/20 (v/v) CO_2/(MeOH + 0.10% IPA + 0.10% TFA)

Stationary phases (SPs)		
Chiral selector	Commercialized column names	
Amylose tris(3,5-dimethylphenylcarbamate)	Chiralpak® AD-H[a]	
Cellulose tris(3,5-dimethylphenylcarbamate)	Chiralcel® OD-H[a]	Lux® Cellulose-1
Cellulose tris(4-methylbenzoate)	Chiralcel® OJ-H[a]	Lux® Cellulose-3
Amylose tris((S)-α-methylbenzylcarbamate)	Chiralpak® AS-H[a]	
Cellulose tris(3-chloro-4-methylphenylcarbamate)	Chiralcel® OZ-H	Lux® Cellulose-2
Cellulose tris(4-chloro-3-methylphenylcarbamate)		Lux® Cellulose-4
Amylose tris(5-chloro-2-methylphenylcarbamate)	Chiralpak® AY-H	Lux® Amylose-2
Cellulose tris(3,5-dichlorophenylcarbamate)		Sepapak®-5

[a] Included in the initial screening.

Chiral Technologies® Europe (Illkrich-Cedex, France). All columns had dimensions 250 mm × 4.6 mm i.d. with 5 μm particle size.

2.4. SFC instrumentation

An analytical system from Waters® (Milford, Massachusetts, USA) was used, consisting of a Thar® SFC fluid delivery module (a liquid CO_2 pump and a modifier pump with a six solvent switching valve), a cooling bath of Thermo Scientific® type Neslab RTE7 controlled by a Digital One thermoregulator to cool pumpheads and CO_2-delivery tubings, a Thar® autosampler with a 48-vial plate, a Thar® SFC analytical-2-prep oven with a 10-column selection valve, a Thar® SFC automated backpressure regulator SuperPure Discovery Series and a Waters® 2998 photodiode array detector. The autosampler was equipped with a 5 μl injection loop. The instrument was controlled by Superchrom® software (TharSFC®, 2003–2009) and data were processed using Chromscope® software (TharSFC®, 2009) both programs from Thar Technologies® (Pittsburgh, PA, USA).

2.5. Chromatographic screening conditions

All experiments were performed at the following conditions: a total flow rate of 3.0 ml/min, a temperature of 30 °C, a backpressure of 150 bar and an analysis time of 30 min. All mobile-phase compositions are expressed in volume-ratios (v/v). For each enantioseparation, the resolution (R_s) was calculated using peak widths at half height.

Peaks with $R_s > 1.5$ are considered baseline separated, $0 < R_s < 1.5$ partially separated, and $R_s = 0$ not separated. Compounds that do not elute within the predefined analysis time of 30 min are evaluated as non-eluted (NE). Racemates were one enantiomer elutes within and the other outside the analysis time window, are defined as partially eluted (PE).

Compounds with two stereogenic centers, consisting of two enantiomeric pairs, are considered as (partially) separated when at least three peaks are observed, implying that at least one enantiomeric pair and one diastereomer are separated. This applies for labetalol and nadolol.

Retention factors of the first eluted peak (k_1) are calculated using the European Pharmacopoeia equation. The dead time was marked as the first disturbance of the baseline after injection, caused by the solvent peak.

3. Results and discussion

In this study, a test set of 59 pharmaceutical chiral compounds is used to evaluate the enantioselectivity of different chromatographic systems. The chiral test set was empirically composed with the intention to cover a broad range of chemically- and pharmacologically-different pharmaceutical compounds. Of the test set, 47 compounds have basic, amphoteric or neutral properties, while 12 display acidic properties.

In Maftouh's screening approach [15], MeOH was considered as first choice modifier and the chiral substances were split into two groups: the basic/amphoteric/neutral, and the acidic. The first group was screened with mobile phases containing isopropylamine (IPA) as additive, the second with mobile phases containing trifluoroacetic acid (TFA) [15]. This implies that a different additive is used depending on the compound's nature. Screening with combined basic and acidic additives in a single mobile phase has been reported in POSC [29]. It is claimed that memory effects on the CSP are reduced by using a mixture of additives, favoring a faster screening with different mobile phases [26,29]. For SFC, the combined use

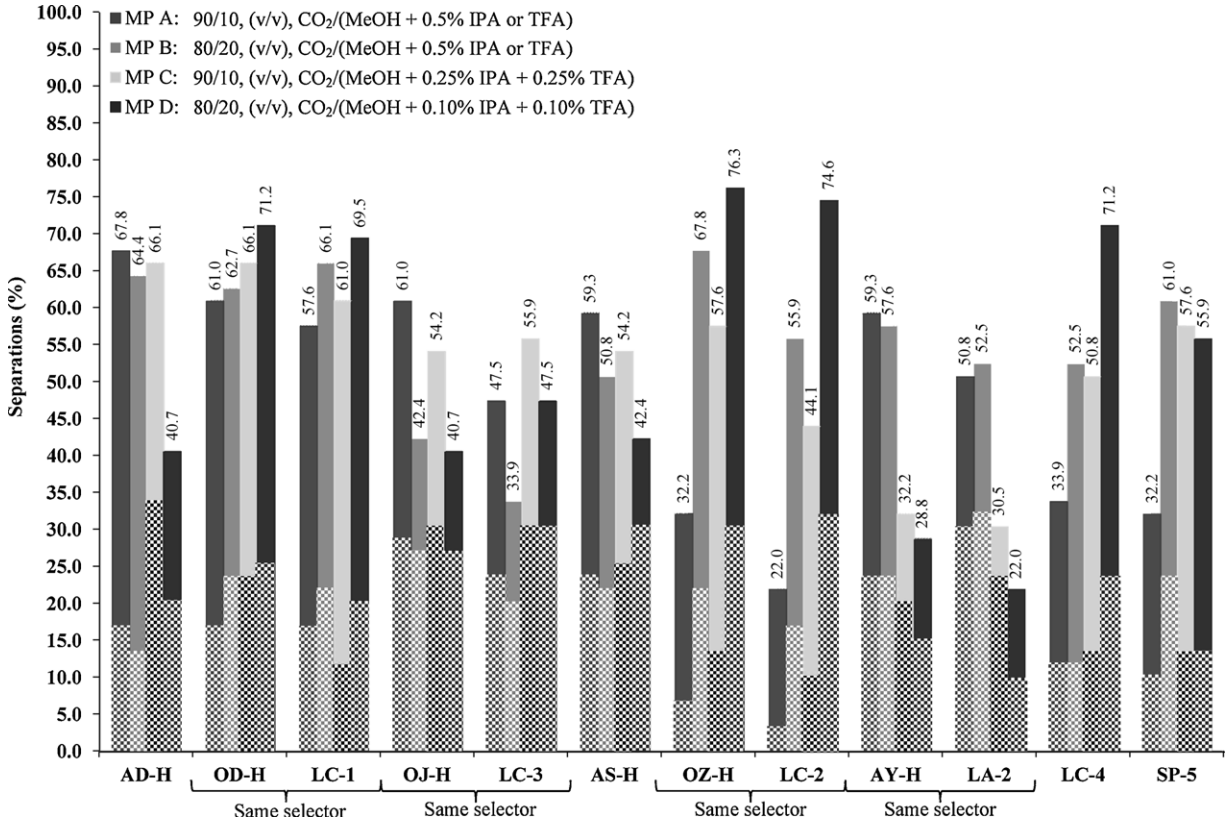

Fig. 1. Percentages of separated compounds (expressed relatively to 59 compounds) using mobile phase A–D. Full bars represent the baseline separations ($R_s > 1.5$) and dotted bars partial separations ($0 < R_s < 1.5$). Above each bar the total separation rate is shown.

of basic and acidic additives in the mobile phase for chiral screening has been only limitedly investigated and reported [28,30]. For this reason, additionally to the original methanol-based carbon dioxide mobile phase of Maftouh's screening step, two new mobile phases with a combination of IPA and TFA were evaluated.

Twelve CSPs, of which the four included in the initial screening, are screened with the aim of selecting the systems with the broadest enantioselectivity. The screened chromatographic systems are shown in Table 1.

3.1. Applying the initial screening conditions on the test set

The test set was screened under the initial conditions, *i.e.* mobile phase A, 90/10 (v/v) CO_2/(MeOH + 0.5% IPA or TFA) on OD-H, AD-H, OJ-H and AS-H. Using Chiralpak® AD-H, 40 compounds (67.8%) were separated of which 30 baseline (50.8%). Chiralcel® OD-H and Chiralcel® OJ-H performed almost equally well, yielding both 36 successful enantioseparations (61.0%), with 26 baseline separations (44.1%) for Chiralcel® OD-H and 19 (32.2%) for Chiralcel®

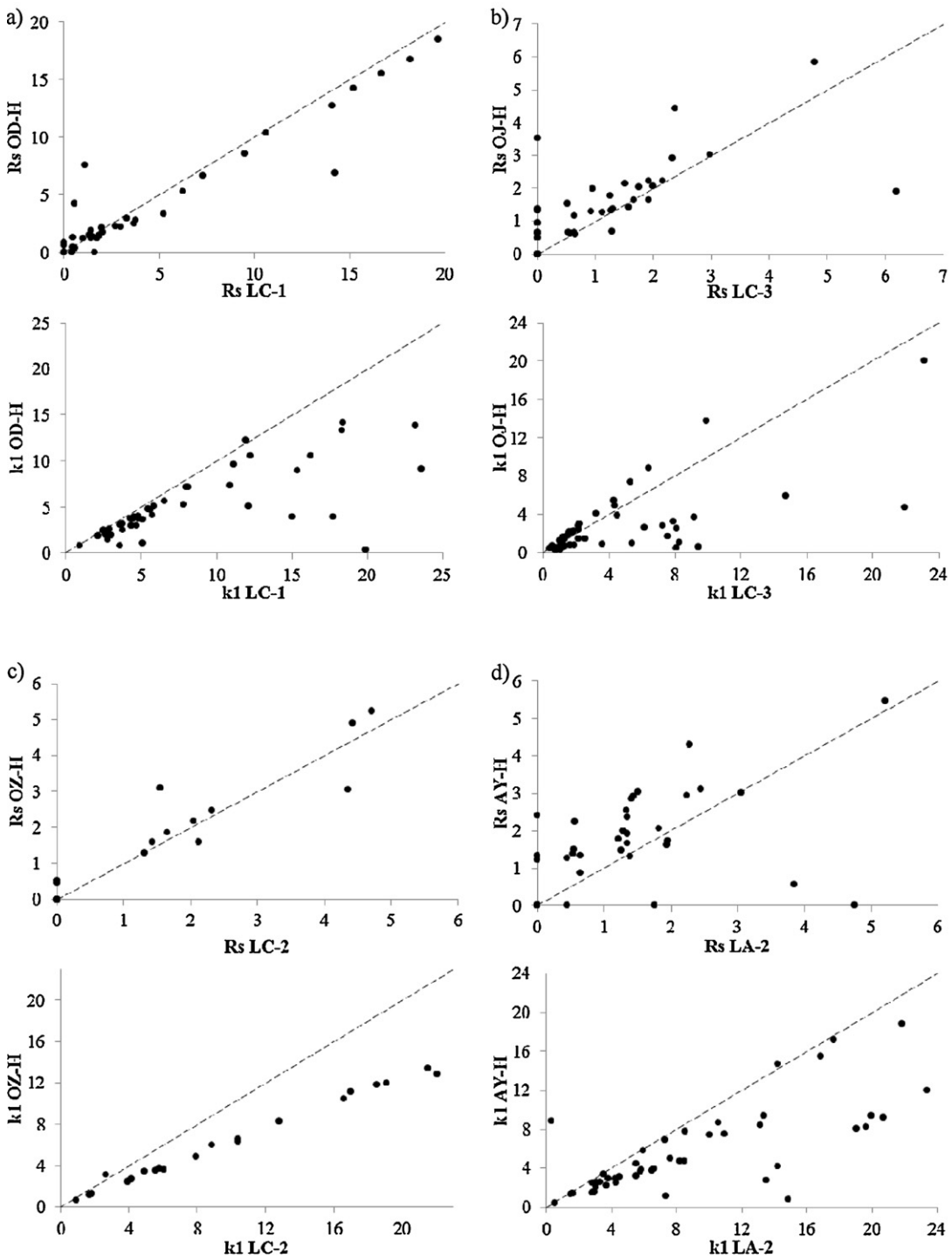

Fig. 2. Comparison of the resolutions (R_s) and retention factors of the first eluted peak (k_1) of a chiral compound using MP A 90/10 (v/v) CO_2/(MeOH + 0.5% IPA or TFA) on (a) LC-1 and OD-H, (b) LC-3 and OJ-H, (c) LC-2 and OZ-H, and (d) LA-2 and AY-H.

Table 2
Success rates from the screening experiments with the four tested mobile phases [MP A (90/10 (v/v) CO_2/(MeOH + 0.5% IPA or TFA)), B (80/20 (v/v) CO_2/(MeOH + 0.5% IPA or TFA)), C (90/10 (v/v) CO_2/(MeOH + 0.25% IPA + 0.25% TFA)) and D (80/20 (v/v) CO_2/(MeOH + 0.10% IPA + 0.10% TFA))] expressed in absolute numbers and in percentages (relative to 59 compounds).

Column		OD-H	AD-H	OJ-H	AS-H	OZ-H	AY-H	LC-1	LC-2	LC-3	LC-4	LA-2	SP-5
Baseline separations	MP A	26 (44.1%)	30 (50.8%)	19 (32.2%)	21 (35.6%)	15 (25.4%)	21 (35.6%)	24 (40.7%)	11 (18.6%)	14 (23.7%)	13 (22.0%)	12 (20.3%)	13 (22.0%)
$R_s > 1.5$	MP B	23 (39.0%)	30 (50.8%)	9 (15.2%)	17 (28.8%)	27 (45.8%)	20 (33.9%)	26 (44.1%)	23 (39.0%)	8 (13.6%)	23 (39.0%)	12 (20.3%)	22 (37.3%)
	MP C	26 (44.1%)	19 (32.2%)	14 (23.7%)	17 (28.8%)	26 (44.1%)	7 (11.9%)	28 (47.4%)	21 (35.6%)	15 (25.4%)	22 (37.3%)	4 (6.8%)	25 (42.4%)
	MP D	27 (45.8%)	12 (20.3%)	8 (13.6%)	7 (11.9%)	27 (45.8%)	9 (15.2%)	29 (49.2%)	25 (42.4%)	10 (16.9%)	28 (47.4%)	7 (11.9%)	25 (42.4%)
Partial separations	MP A	10 (16.9%)	10 (16.9%)	17 (28.8%)	14 (23.7%)	4 (6.8%)	14 (23.7%)	10 (16.9%)	2 (3.4%)	14 (23.7%)	7 (11.9%)	18 (30.5%)	6 (10.2%)
$0 < R_s < 1.5$	MP B	14 (23.7%)	8 (13.6%)	16 (27.1%)	13 (22.0%)	13 (22.0%)	14 (23.7%)	13 (22.0%)	10 (16.9%)	12 (20.3%)	8 (13.6%)	19 (32.2%)	14 (23.7%)
	MP C	13 (22.0%)	20 (33.9%)	18 (30.5%)	15 (25.4%)	8 (13.6%)	12 (20.3%)	8 (13.6%)	5 (8.5%)	18 (30.5%)	8 (13.6%)	14 (23.7%)	9 (15.3%)
	MP D	15 (25.4%)	12 (20.3%)	16 (27.1%)	18 (30.5%)	18 (30.5%)	8 (13.6%)	12 (20.3%)	19 (32.2%)	18 (30.5%)	14 (23.7%)	6 (10.2%)	8 (13.6%)
Not separated	MP A	16 (27.1%)	10 (16.9%)	20 (33.9%)	14 (23.7%)	11 (18.6%)	17 (28.8%)	12 (20.3%)	10 (16.9%)	26 (44.1%)	7 (11.9%)	17 (28.8%)	8 (13.6%)
$R_s = 0$	MP B	21 (35.6%)	19 (11.2%)	34 (57.6%)	27 (45.8%)	16 (27.1%)	20 (33.9%)	18 (30.5%)	17 (28.8%)	36 (61.0%)	16 (27.1%)	20 (33.9%)	18 (30.5%)
	MP C	10 (16.9%)	12 (20.3%)	21 (35.6%)	18 (30.5%)	7 (11.9%)	31 (52.5%)	9 (15.3%)	8 (13.6%)	23 (39.0%)	7 (11.9%)	31 (52.5%)	15 (25.4%)
	MP D	16 (27.1%)	32 (54.2%)	35 (59.3%)	32 (54.2%)	11 (18.6%)	41 (69.4%)	17 (28.8%)	14 (23.7%)	28 (47.4%)	17 (28.8%)	46 (78.0%)	24 (40.7%)
Partially observed in the	MP A	3 (5.1%)	0 (0.0%)	0 (0.0%)	4 (6.8%)	8 (13.6%)	1 (1.7%)	3 (5.1%)	1 (1.7%)	2 (3.4%)	2 (3.4%)	2 (3.4%)	5 (8.5%)
chromatogram (PO)	MP B	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	MP C	6 (10.2%)	3 (5.1%)	3 (5.1%)	5 (8.5%)	4 (6.8%)	5 (8.5%)	6 (10.2%)	1 (1.7%)	0 (0.0%)	7 (11.9%)	3 (5.1%)	2 (3.4%)
	MP D	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Not eluted (NE)	MP A	4 (6.8%)	9 (15.2%)	3 (5.1%)	6 (10.2%)	21 (35.6%)	6 (10.2%)	10 (16.9%)	35 (59.3%)	3 (5.1%)	30 (50.8%)	10 (16.9%)	27 (45.8%)
	MP B	1 (1.7%)	2 (3.4%)	0 (0.0%)	2 (3.4%)	3 (5.1%)	5 (8.5%)	2 (3.4%)	9 (15.2%)	3 (5.1%)	12 (20.3%)	8 (13.6%)	5 (8.5%)
	MP C	4 (6.8%)	5 (8.5%)	3 (5.1%)	4 (6.8%)	14 (23.7%)	4 (6.8%)	8 (13.6%)	24 (40.7%)	3 (5.1%)	15 (25.4%)	7 (11.9%)	8 (13.6%)
	MP D	1 (1.7%)	3 (5.1%)	0 (0.0%)	2 (3.4%)	3 (5.1%)	1 (1.7%)	1 (1.7%)	1 (1.7%)	3 (5.1%)	0 (0.0%)	0 (0.0%)	2 (3.4%)

OJ-H. Chiralpak® AS-H was able to generate 21 baseline separations (35.6%) on a total of 35 (59.3%) (Fig. 1 and Table 2).

To define a chiral screening approach it is important not only to select systems displaying a broad enantioselectivity but also to take into account the complementarity of the systems. Only then, the highest number of separated compounds can be obtained by screening the least chromatographic systems. To evaluate the complementarity of the systems, the cumulative success rate was determined as follows: the CSP yielding the highest number of enantioseparations is selected first, after which the CSP with most additionally separated compounds is considered, and so on. This sequence and the cumulative success rate at the initial screening conditions was determined to be: AD-H (40–30 baseline) → OJ-H (50–38 baseline) → AS-H (54–42 baseline) → OD-H (54–45 baseline). This implies that OJ-H is most complementary to AD-H, yielding ten additional enantioseparations. Chiralpak® AS-H yields four supplementary separations, bringing the total cumulative success rate to 91.5% or 54 separated compounds. No additional separations were obtained with Chiralcel® OD-H, although three partially separated racemates were baseline resolved with the latter system.

The most noticeable difference compared to the initial screening sequence of Maftouh et al. [15]: AD-H → OD-H → OJ-H → AS-H, is that OD-H was proposed to screen secondly, while our results only showed a more limited complementarity to the already selected systems. However, the Maftouh sequence was defined using two modifiers (methanol and 2-propanol), while our work only focuses on methanol-based carbon dioxide mobile phases. Additionally, for our screening sequence, the complementarity of the evaluated chromatographic systems is taken into account.

3.2. Screening all CSPs with the initial mobile phase

The test set was also analyzed with mobile phase A on eight other polysaccharide-based CSPs: LC-1, LC-2, LC-3, LC-4, LA-2, OZ-H, AY-H and SP-5 (Fig. 1 and Table 2).

AD-H, OD-H, and OJ-H gave the best overall performances. LC-2 is found the least successful system of the twelve evaluated CSPs. However, this low success rate should be interpreted cautiously, since 36 compounds (61.0% of the test set) failed to elute within 30 min (Table 2). This implies that compounds are too strongly retained on LC-2, and that a higher solvent strength is needed in order to obtain more enantioseparations within the fixed time frame. MP A is thus not the most appropriate for LC-2, which makes it impossible to draw proper conclusions about the enantioselectivity of this CSP under these conditions. The equivalent column with the same chiral selector from Daicel, OZ-H, showed a similar behavior, although less pronounced (49.2% or 29 racemates failed to elute within 30 min, see Table 2).

As mentioned above, some of the evaluated columns contain the same selector, but are manufactured by different suppliers, *e.g.* Chiralcel® OD-H and Lux® Cellulose-1, Chiralcel® OJ-H and Lux® Cellulose-3, Chiralcel® OZ-H and Lux® Cellulose-2; and Lux® Amylose-2 and Chiralpak® AY-H (Table 1). Comparing the performances of these columns, at the above conditions, the Daicel columns slightly outperform their Lux equivalents (Fig. 1, MP A). In Fig. 2, the R_s and k_1 on these equivalent columns are compared. For some compounds, R_s and k_1 could not be compared because one or both enantiomer(s) eluted after 30 min. The dashed lines describe equal values for the compared response. These graphs indicate that the resolutions obtained with the equivalent CSPs are generally rather similar. For OZ-H and LC-2, only 14 compounds (23.7%) showed a resolution on both columns, which is related to the significant difference in retention on these equivalent stationary phases.

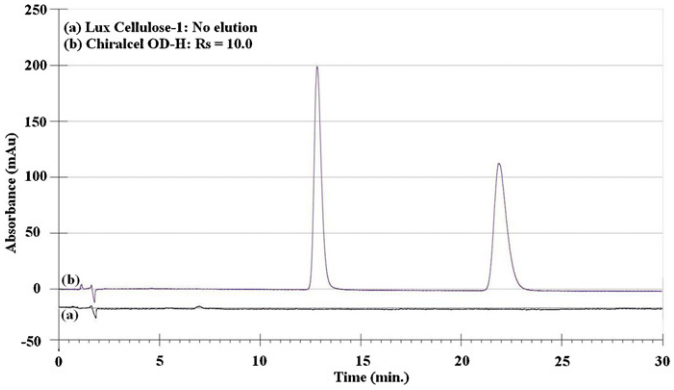

Fig. 3. Overlay of the chromatograms of propranolol on (a) Lux Cellulose-1 and (b) Chiralcel OD-H with mobile phase A over a 30 min time frame.

From Fig. 2, it is also observed that all Lux phases systematically show higher retention factors than the equivalent Daicel phases, a trend that is also confirmed from the number of non-eluted compounds, which is higher for the Lux phases. The difference in success rates between equivalent columns is thus partly due to the excessive retention on the Lux phases. For instance, propranolol is not eluted on LC-1 within 30 min, while a baseline separation (R_s = 10.0) is obtained on OD-H (Fig. 3).

However, retention differences do not fully explain the difference in success rates of equivalent columns. For instance, promethazine is not separated on LC-3 but a partial separation is obtained on OJ-H (R_s = 1.38), while their k_1 are similar (Fig. 4). Similar tendencies are observed for other compounds and other equivalent column pairs, *i.e.* LC-1 *vs.* OD-H, LC-2 *vs.* OZ-H and for LA-2 *vs.* AY-H. In these cases, the difference in enantioseparation

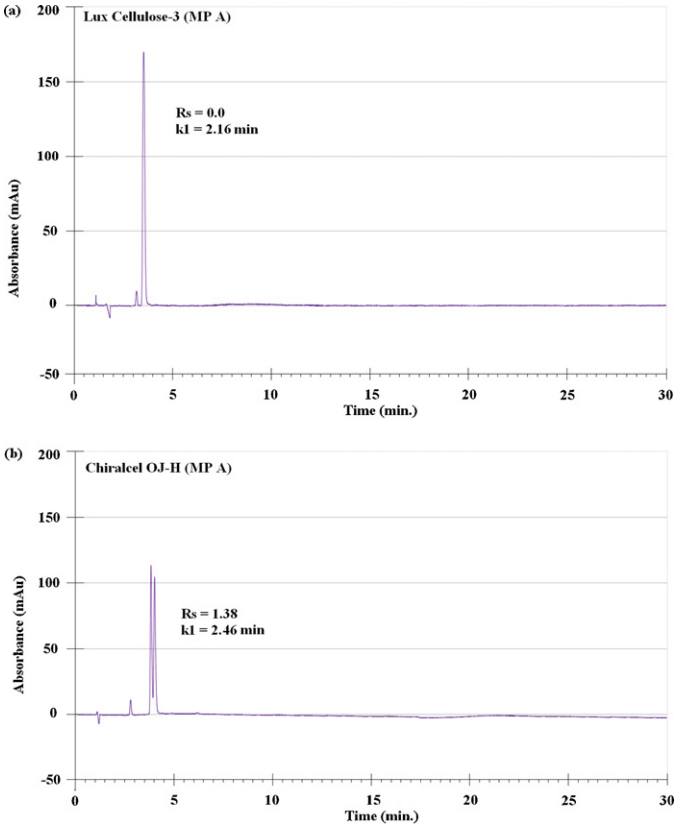

Fig. 4. Analysis of promethazine on (a) Lux Cellulose-3 and (b) Chiralcel OJ-H with mobile phase A over a 30 min time frame.

arises from a different enantioselective behavior exhibited by the CSPs.

Summarized it can be stated that the equivalent CSPs yielded different success rates, which were for some compounds due to retention differences, and for others to a different enantioselectivity. Differences in the raw materials and the procedures to manufacture the CSPs, might result in different three-dimensional structures of the chiral selector and consequently a different enantioselectivity. Since stereoselective inclusion in the helical structure of the polysaccharide chain is an important mechanism in enantiorecognition, this might contribute to the different enantioselectivity. However, it is clear that these equivalent CSPs do not generate identical results in terms of enantioselectivity and are therefore not readily interchangeable (applying the same MP).

To evaluate the complementarity of all twelve CSPs with MP A, the cumulative sequence was determined and found to be: AD-H (40–30 baseline) → OJ-H (50–38 baseline) → AS-H (54–42 baseline) → AY-H (55–44 baseline). The first three systems of this sequence are the same as for the initially defined screening. Since screening AY-H only yields one extra separation, one may also choose for a faster screening using only these first three systems.

However, an equivalent sequence in terms of total cumulative separation rate can also be defined as follows: OJ-H (36–19 baseline) → AS-H (50–31 baseline) → AY-H (55–41 baseline). AY-H could also be substituted with LA-2, LC-3, OZ-H or SP-5 but this yields less baseline separations. Under these screening conditions, OJ-H and AS-H were the only columns that generated unique enantioseparations. This illustrates the importance of including these CSPs in the screening approach with MP A. This screening is limited to three CSPs, which benefits the throughput.

3.3. Increasing the methanol-content in the mobile phase

Using gradients for screening, results in a less straightforward method development, since successful enantioseparation methods require additional steps to be transferred into isocratic conditions (because chiral separations usually do not resolve complex mixtures requiring gradient analysis). For this reason, isocratic screening approaches are used in this work. This makes it important to investigate the most appropriate solvent strength and thus modifier content.

Increasing the modifier content increases the elution strength and compounds will elute faster, favoring a faster screening. A higher modifier content can thus result in more successful enantioseparations due to the elution of more compounds within the time frame of 30 min, partially eluted compounds can then be entirely detected within one chromatogram. On the other hand, a higher modifier content decreases the resolution of a separation, potentially resulting in co-elution of enantiomers which before were only partially separated. Thus, the most appropriate modifier content depends on a compromise between elution time and resolution considering all compounds. To verify whether a higher methanol content successfully could be used, it is important to evaluate whether the number of non-retained compounds, which compromises the total number of enantioseparations, does not increase too much.

A new mobile phase (B), containing 20% methanol, was evaluated on the twelve previously tested columns (Table 1 and Fig. 1). The number of non-eluted compounds decreased (significantly) on all CSPs with exception of LC-3 where no change was observed (Table 2). After increasing the modifier content, no more racemates were eluted only partially within 30 min.

The number of successful separations was affected differently on different CSPs. Although partial separations were lost on LC-1, LC-2, LC-4, SP-5, LA-2, OZ-H and OD-H when switching from MP A to B, the gained number of separations led to a net increase of the success rate. This implies that, with the exception of LC-3, all Lux columns perform better using mobile phase B than mobile phase A. The resolution of the separations on these CSPs was, as expected, lower with MP B than A. This is related to the shorter retention with MP B, reflected in k_1. However, as the aim of a screening step is to determine the enantioselectivity towards a certain chromatographic system in a fast manner, this higher solvent strength is more appropriate for screening these CSPs.

The remaining CSPs, *i.e.* AD-H, OJ-H, LC-3, AS-H and AY-H, all showed a decrease of the total separation rate, implying that with exception of OD-H and OZ-H, all Daicel columns performed better with MP A. The loss of separations was due to a loss of retention when increasing the methanol content. This was reflected in k_1 which decreased significantly for these CSPs changing from MP A to MP B.

To quantify the success of screening under these conditions, the cumulative success rate was also determined using mobile phase B. The screening sequence, determined as above, was: OZ-H (40–27 baseline) → AY-H (51–37 baseline) → OJ-H (55–40 baseline) → OD-H (56–45 baseline).

Thus, a slight improvement (54 → 56 resolved racemates) could be made to the initial screening step by screening four CSPs with MP B. Both screening steps enable a baseline separation for 45 racemates. However, one may choose a faster screening, using only the first three columns and omitting OD-H. This CSP can then be tested only for compounds not (baseline) separated on the first three (in an optimization step further in the strategy). As discussed earlier, k_1 with MP B are shorter than with MP A, resulting in shorter analysis times, which is preferable.

When replacing the above Daicel phases OZ-H, AY-H, OJ-H, and OD-H with the equivalent Lux phases LC-2, LA-2, LC-3 and LC-1, respectively, the following sequence and success rate are obtained: LC-2 (33–23 baseline) → LA-2 (42–29% baseline) → LC-3 (47–32 baseline) → LC-1 (53 → 42 baseline). LC-4 and SP-5 generate a baseline separation for three compounds partially separated with the proposed sequence, but do not further increase the total cumulative separation rate.

3.4. Using a mixture of additives in the mobile phase

In the above screenings, there is still a need to divide the compounds into two groups according to their acid–base properties. This limits the generic applicability of the screening and reduces its throughput capacity. In POSC, the simultaneous use of a basic and an acidic additive showed to be advantageous and favored a faster screening by reducing memory effects on the stationary phase [29]. In an attempt to simplify the screening approach, a mixture of IPA and TFA in mobile phases A and B (called mobile phases C and D, respectively) was evaluated (Tables 1 and 2). Mobile phase C consisted thus of 90/10 CO_2/(MeOH + 0.25% IPA + 0.25% TFA) and mobile phase D of 80/20 CO_2/(MeOH + 0.10% IPA + 0.10% TFA). The additive concentrations in MP D are lower compared to MP C, since higher percentages in 20% methanol resulted in precipitation problems, due to the formation of insoluble ion-complexes between both additives [28].

The impact of the simultaneous use of IPA and TFA in the mobile phase (MP C and D) relative to the separate use (MP A and B) was not the same for all CSPs (Fig. 1). On a given CSP we generally noted a trend of lower k_1 using MP C or D instead of MP A or B, thus shorter retention times are displayed and less compounds do not elute or elute only partially within 30 min (Table 2). This can be explained by the influence of the additives. IPA suppresses the non-stereospecific interactions between analytes and the silica matrix of the stationary phase, thereby enhancing the enantioselective interactions, but also decreasing the analytes' retention [31]. TFA on the other hand, is believed to deactivate free silanol groups by forming hydrogen

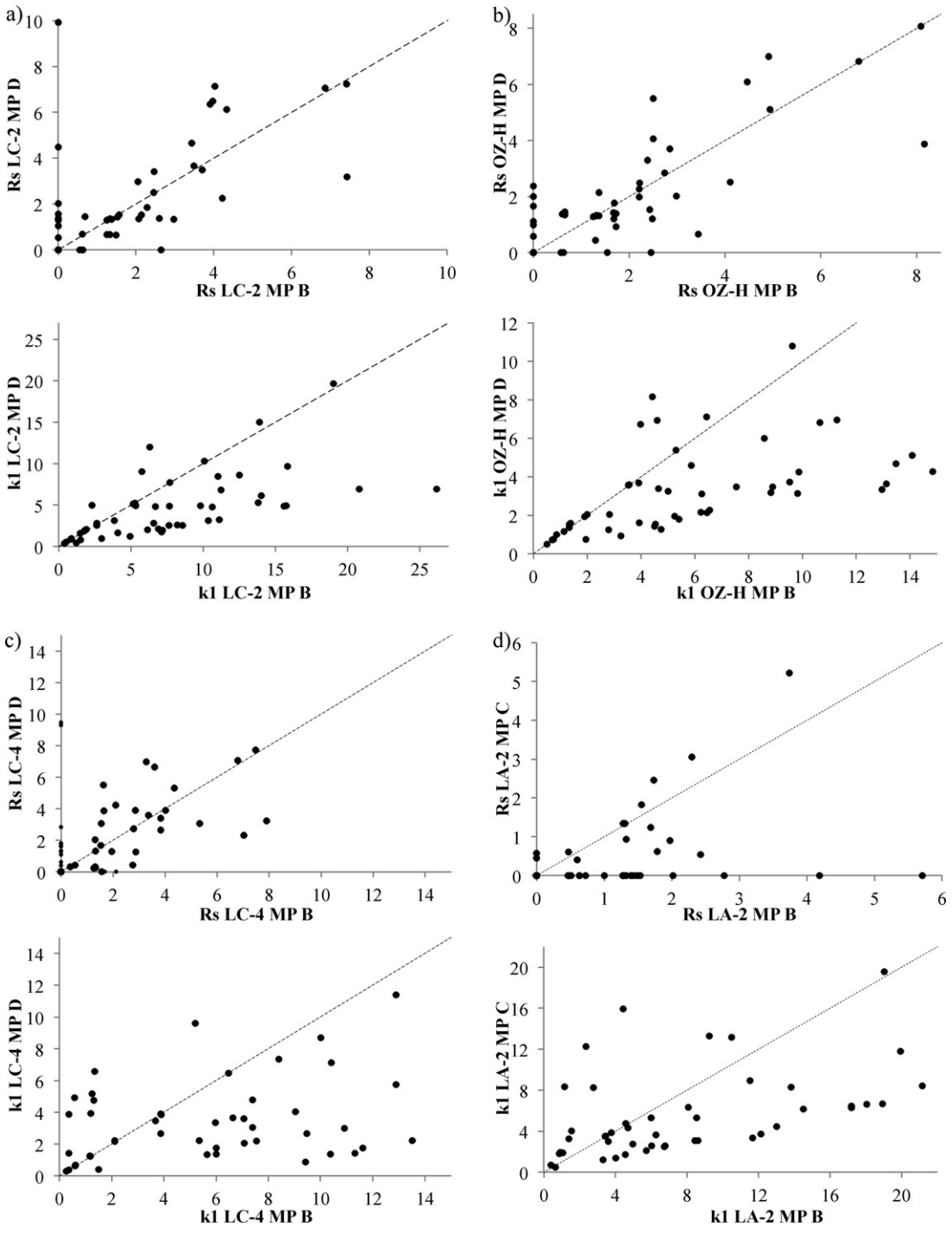

Fig. 5. Comparison of the resolutions (R_s) and retention factors of the first eluted peak (k_1) with MP A 90/10 (v/v) CO_2/(MeOH + 0.5% IPA or TFA) or B 80/20 (v/v) CO_2/(MeOH + 0.5% IPA or TFA) and C 90/10 (v/v) CO_2/(MeOH + 0.25% IPA + 0.25% TFA) or D 80/20 (v/v) CO_2/(MeOH + 0.1% IPA + 0.1% TFA) on (a) LC-2, (b) LC-4, (c) OZ-H and (d) LA-2.

bonds. Moreover the addition of an acidic additive lowers the pH, partially protonating and charging basic functional groups and thus also shortening the retention time [21].

Using MP C and D seven and six columns, respectively, displayed a higher success rate than with MP A and B. Chiralcel® OJ-H and all amylose-based CSPs (AD-H, AS-H, AY-H and LA-2) performed worse with combined additives in the mobile phase.

Of the 48 chromatographic systems screened, the highest number of separations was achieved using mobile phase D and OZ-H (45 separations/76.3%), LC-2 (44 separations/74.6%), and OD-H or LC-4 (42 separations/71.2%).

Resolutions obtained on these CSPs with the most successful MPs with separate additives or joint additives are compared in Fig. 5. The different success rates when using a mixture of IPA and TFA in a CO_2/MeOH mobile phase can partly be explained by the change in k_1, *i.e.* excessive retention or unretained behavior. However, this does not provide a complete explanation for the observed differences in separation success. For some compounds, k_1 were relatively similar with both MPs, while they clearly yielded different separation results. As an example the chromatogram of mianserin is presented in Fig. 6. With separate additives in a CO_2/methanol-based mobile phase this compound could not be

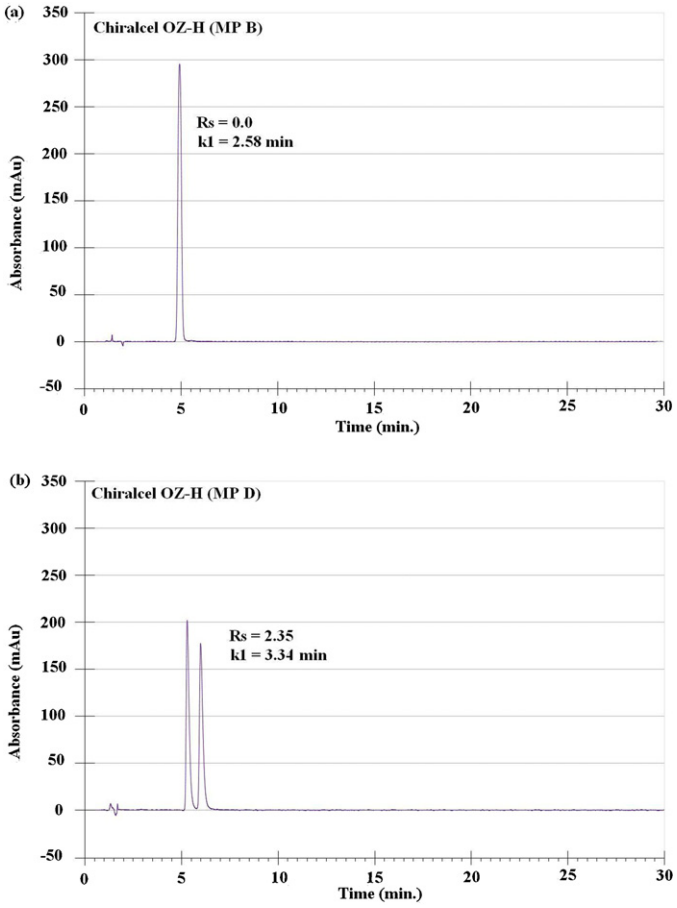

Fig. 6. Analysis of mianserin on Chiralcel OZ-H with (a) MP B and (b) MP D over a 30 min time frame.

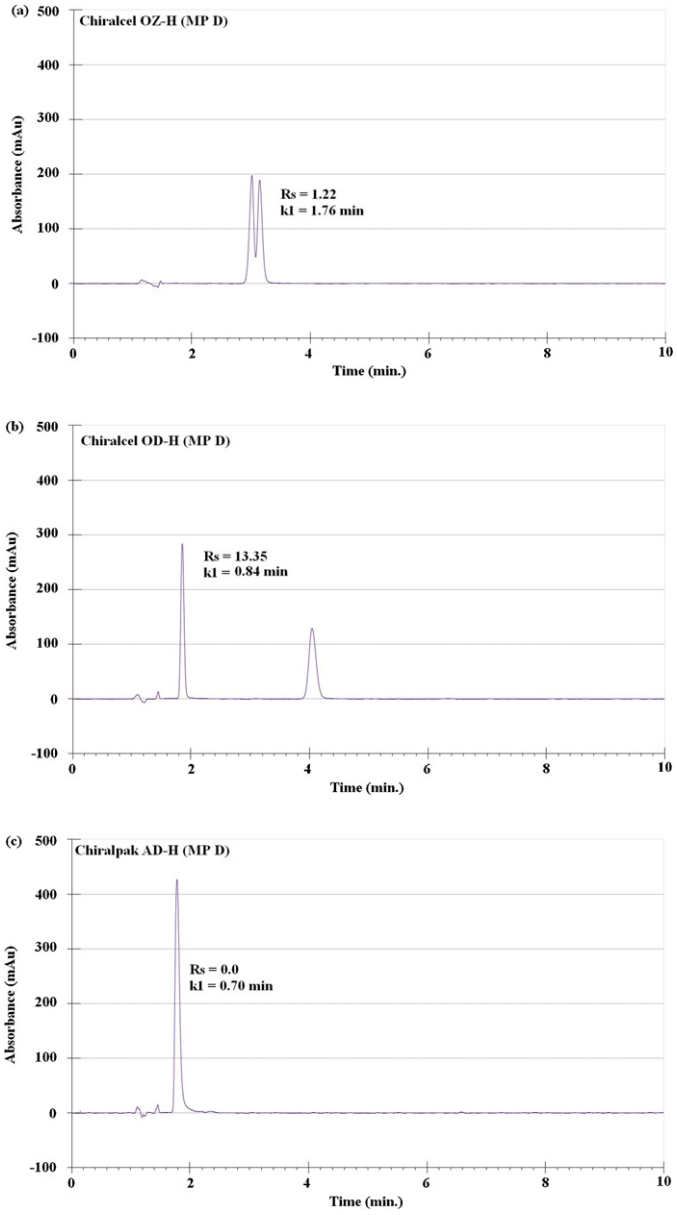

Fig. 7. Chromatograms of the screening experiments with MP D for betaxolol on (a) Chiralcel OZ-H (b) Chiralcel OD-H and (c) Chiralpak AD-H.

separated on OZ-H, while the joint use of IPA and TFA results in a baseline separation. This difference can be attributed to a different enantioselective behavior when combining IPA and TFA.

Since enantioselectivity remains unpredictable and additives exert their effect through different mechanisms, the cause of the behavior observed with combined additives is tedious to determine. All CSPs consist of neutral polysaccharide derivatives, therefore they interact best with uncharged compounds. Possibly soluble ion-complexes are formed between TFA and basic compounds, or IPA and acidic compounds. These complexes might then be separated chiraly in SFC and thus generate a different stereoselective behavior compared to the uncomplexed enantiomers [21,31]. In addition, complexes are formed between IPA and TFA, which also potentially affect the enantioselective interactions [28]. The geometric structure of compounds can thus be affected and an influence can be exerted on the enantioselective interactions. Our experiments showed a beneficial effect from combining IPA and TFA on most CSPs, though the exact mechanisms of this effect remain unclear. Results showed that a combination of IPA and TFA in the MP is detrimental for all amylose-based CSPs. The helical structures of amylose and cellulose differ fundamentally, which might result in different interactions with the additives in the mobile phase, consequently influencing the enantioselectivities of amylose- and cellulose-based CSPs differently. Another hypothesis is that the complexes formed between the additives and the analyzed compounds are too large to allow stereoinclusion in the denser structures of the amylose chains and thus a loss of enantioseparations is seen [32]. However, as the enantioselective interaction mechanisms on polysaccharide-based CSPs are complex and to date not fully elucidated, we have no straightforward explanation for the observed behavior.

The cumulative success rate determined for mobile phase C is: LC-1 (36–29 baseline) → LC-3 (51–39 baseline) → AD-H (54–45 baseline) → LC-2 (55–50 baseline) → AS-H (56–51 baseline). Screening these five CSPs results in the highest number of total and baseline separations for a screening with MP C. The gain of screening AS-H as last column, is only one additional and one baseline separation. For this reason, omitting this CSP and reaching a cumulative separation rate of 55 compounds (50 baseline resolved), would be more advisable.

In relation to the screening steps with mobile phases A or B (using three CSPs), a higher rate of successful baseline separations is obtained with MP C, while the need for compound classification and the use of two mobile phases is eliminated. This speeds up and simplifies the screening approach. In relation to the initially defined screening step one extra partial separation and five extra baseline separations are obtained screening four CSPs.

The cumulative sequence determined with mobile phase D is: OZ-H (45–27 baseline) → OD-H (52–40 baseline) → AD-H (56–44 baseline) → AS-H (57–45 baseline). From a time-saving point of view, it would again be advisable to omit AS-H from the screening step, the rate of enantioseparations would then reach 94.9%. Compared to the proposed screening sequence with MP C, one extra separation is obtained using one CSP less.

To illustrate the complementarity of the systems in the proposed screening step, the chromatograms of the screening experiments with MP D for betaxolol are shown in Fig. 7. Chiralcel OZ-H yields a partial separation, OD-H a baseline separation within 5 min with $R_s > 13$ and AD-H yields no separation.

In this screening only Daicel phases were selected. Replacing OZ-H and OD-H with the equivalent Lux phases, the resulting cumulative separation rate would be almost similar, *i.e.* LC-2 (44–25 baseline) → LC-1 (50–39 baseline) → AD-H (55–43 baseline) → AS-H (56–44 baseline).

4. Conclusions

The enantioselectivity of twelve polysaccharide-based chiral stationary phases and four methanol-containing CO_2 mobile phases was investigated in SFC using a test set of 59 drug compounds. The broadest enantioselectivity was displayed when 80/20 (v/v) CO_2/(MeOH + 0.1% IPA + 0.1% TFA) was used as a mobile phase. This MP can be used for all compounds regardless their chemical properties. This benefit not only simplifies, but also speeds up the screening approach. The joint use of IPA and TFA resulted in a significant improvement of the performance of a CSP in terms of successful separations.

For incorporation in a screening approach, the complementarity of the CSPs is an important requisite. Therefore, cumulative success rates were determined. The most efficient screening sequence was determined to be: Chiralcel® OZ-H → Chiralcel® OD-H → Chiralpak® AD-H, using the above-mentioned mobile phase. It allows separating 56 compounds or 94.9% of the test set, of which 44 racemates are baseline separated. Compared to the initial screening, compound classification depending on acid-basic properties is no longer necessary making the screening simpler with one less CSP to be screened and two more separations obtained.

Conflict of interest

Authors declared no conflict of interest.

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