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Updating a generic screening approach in sub- or supercritical fluid chromatography for the enantioresolution of pharmaceuticals

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ABSTRACT

This work focusses on the update of a generic chiral screening approach in sub- or supercritical fluid chromatography (SFC). Newly generated data with 2-propanol-containing mobile phases on 12 polysaccharide-based stationary phases was combined with previous data obtained using methanol-containing mobile phases. As modifiers 2-propanol and methanol were selected for their earlier performance. An evaluation of the most appropriate solvent strength is made and the enantioselective behaviour of the chromatographic systems is discussed. A comparison of the systems is made and their complementarity investigated by analyzing the data by different means, *e.g.* principal component analysis. The generic screening sequence is proposed by selecting the most enantioselective and complementary systems. This allows updating an existing screening strategy. With the novel screening, all compounds of a 57-compounds test set were separated (48 baseline), on at least one of four selected systems, within an analysis time of 30 min. The applicability and performance of the updated screening was demonstrated with a compound from the test set, *i.e.* alprenolol.

1. Introduction

Because of the prevalence of chiral compounds in pharmaceutical ingredients, agrochemicals, food additives, *etc.*, the importance and necessity of enantioseparations has been highlighted numerous times in the literature and consequentially chiral analysis continues to be an extensively studied topic [1–4]. European Medicines Agency, and Food and Drug Administration regulations state that chiral drug compounds need to be tested as pure enantiomers and as racemate during pharmacological and toxicological tests. This necessitates the development of chiral separations methods in early-drug-discovery and in further stages of drug development, such as formulations and biological safety testing [5,6].

Two approaches can be used to obtain pure enantiomers: asymmetric enantiopure synthesis or resolution of the racemic mixture into the constituent enantiomers. Lacking time- and cost efficiency, asymmetric synthesis is not applied at a discovery stage, where only small quantities of a large and diverse set of molecules are synthesized. Enantioresolution of racemates is there the more preferred approach and offers the additional benefit that both enantiomers, needed for biological safety tests, are separated. This is mainly achieved by chromatographic resolution on chiral stationary phases [7–9].

Enantioseparations performed by high-pressure liquid chromatography (HPLC) represent the major part of all reported articles in the field of chiral chromatography, although alternative techniques, such as gas chromatography, capillary electrochromatography and simulated moving bed chromatography, are also used [7–10]. In addition, chiral sub- or supercritical fluid chromatography has found an increased use over the past years and allows high-performance enantioseparations with short analysis- and equilibration times [8,11–15].

To enable fast and efficient chiral method development, screening strategies are proposed (often as part of a larger method-development strategy). These generic screenings consist of a limited number of complementary chromatographic experiments which can be applied successfully on diverse racemates. Further optimization steps allow then obtaining the desired enantioseparation. Thus, the screening step aims to quickly evaluate the enantioselectivity of certain (complementary) chromatographic systems for a given compound, rather than obtaining final optimal separation conditions [16,17].

A screening step in SFC defined earlier by Maftouh et al. [14] was used as a starting point for this study. Their screening step consists

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of eight experiments in which the Chiralpak® AD-H, Chiralcel® OD-H, Chiralcel® OJ-H and Chiralcel® AS-H columns are screened with two mobile phases (MPs), containing besides CO_2 10(v)% methanol (MeOH) or 20(v)% 2-propanol (2PrOH). To improve the peak shapes, 0.5(v)% isopropylamine (IPA) is added to the mobile phase when screening basic, neutral or amphoteric compounds, while 0.5(v)% trifluoroacetic acid (TFA) is used for acids.

In a first study, the methanol-containing MP of Maftouh's screening step was applied on eight more recently introduced polysaccharide-based chiral stationary phases (CSPs) [18]. This type of CSPs was chosen because of their widespread use and broad enantioselectivity [10,19]. Other methanol-containing mobile phases were also tested, aiming to select the most generic conditions. Resulting from that work, a new and more efficient screening sequence was proposed testing cellulose tris(3-chloro-4-methylphenylcarbamate) → cellulose tris(3,5-dimethylphenylcarbamate) → amylose tris(3,5-dimethylphenylcarbamate) → amylose tris((S)-α-methylbenzylcarbamate) with a carbon-dioxide based mobile phase containing 20(v)% MeOH and 0.1(v)% IPA and TFA. This mobile phase showed a broad enantioselectivity and applicability for all compounds, regardless their chemical properties. Moreover, the higher methanol content increases the analysis speed and therefore the screening throughput. A performance difference in terms of successful separations was seen between equivalent columns with the same selector from different manufacturers. The new screening step required only four experiments and allowed separating 56 compounds (of which 44 baseline separations) from a test set of 57, corresponding to a success rate of 98% (77% baseline separations).

In this paper, experiments with mobile phases containing 2-propanol are conducted, aiming to extend the applicability and further improve the success rate and/or throughput of the screening by searching for complementary chromatographic systems. The enantioselectivity of 12 polysaccharide-based CSPs was evaluated using four 2-propanol-containing mobile phases. Combined with the previous results from the methanol-containing mobile phases [18] a selection of the most complementary systems was then made based, performing an exploratory data analysis.

2. Materials and methods

2.1. Chiral test compounds

The same test set of pharmaceutical racemates was used as in [18,20] with the exception of leucovorin and naproxen, thus for this work a 57-compounds test set was used. They are listed in Table 1. All solutions were prepared at a concentration of 0.5 mg/ml. They were dissolved in MeOH since this tended to give better chromatographic results than in 2PrOH. Methotrexate was dissolved in MeOH/TFA, 100/0.5 (v/v) because of solubility issues. Solutions were stored at 4 °C.

2.2. Chemicals

CO_2 2.7 (purity ≥99.7%) was obtained from Linde Gas (Grimbergen, Belgium) and 2PrOH (HPLC grade) from Fisher Chemical (Loughborough, Leicestershire, UK). IPA and TFA were from Aldrich (Steinheim, Germany).

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 Chiral Technologies Europe (Illkrich-Cedex, France). All columns had dimensions 250 mm × 4.6 mm i.d. with 5 μm particle size (Table 2).

Table 1
Commercial racemates used in this study.

Compound	Manufacturer	Compound	Manufacturer
Acebutolol	Sigma–Aldrich, Steinheim, Germany	Methadone	Federa, Brussels, Belgium
Acenocoumarol	Novartis, Basel, Switzerland	Methotrexate	Cyanamid Benelux, Brussels, Belgium
Alprenolol	Sigma–Aldrich, Steinheim, Germany	Metoprolol	Astra Hassle AB, Lund, Sweden
Ambucetamide	Janssen Pharmaceutica, Beerse, Belgium	Mianserine	Diosynth & Organon, Brussels, Belgium
Atenolol	Sigma–Aldrich, Steinheim, Germany	Nadolol	Sigma–Aldrich, Steinheim, Germany
Atropine	Sigma–Aldrich, Steinheim, Germany	Naringenin	Sigma–Aldrich, Steinheim, Germany
Betaxolol	Sigma–Aldrich, Steinheim, Germany	Nicardipine	UCB, Brussels, Belgium
Bisoprolol	Origin unknown	Nimodipine	Bayer, Leverkusen, Germany
Bopindolol	Sandoz, Holskirchen, Germany	Nisoldipine	Bayer, Leverkusen, Germany
Bupranolol	Schwarz Pharma, Monheim, Germany	Nitrendipine	Bayer, Leverkusen, Germany
Carazolol	Astellas Pharma, Munchen, Germany	Oxazepam	Sigma–Aldrich, Steinheim, Germany
Carbinoxamine	Origin unknown	Oxprenolol	Cynamid Benelux, Brussels, Belgium
Carvedilol	Boehringer, Mannheim, Germany	Pindolol	Sigma–Aldrich, Steinheim, Germany
Chlorphenamine	Sigma–Aldrich, Steinheim, Germany	Praziquantel	Sigma–Aldrich, Steinheim, Germany
Chlorthalidone	Sigma–Aldrich, Steinheim, Germany	Procyclidine	Sigma–Aldrich, Steinheim, Germany
Dimethindene	Novartis, Basel, Switzerland	Promethazine	Sigma–Aldrich, Steinheim, Germany
Ephedrine	Sigma–Aldrich, Steinheim, Germany	Propiomazine	Origin unknown
Esmolol	Du Pont de Nemours, Saconnex, Switzerland	Propranolol	Fluka, Neu-Ulm, Switzerland
Fenoprofen	Sigma–Aldrich, Steinheim, Germany	Salbutamol	Glaxo Wellcome, Genval, Belgium
Flurbiprofen	ICN Biomedicals, Ohio, USA	Salmeterol	Glaxo Wellcome, Genval, Belgium
Hexobarbital	Origin unknown	Sotalol	Merck, Darmstadt, Germany
Ibuprofen	Sigma–Aldrich, Steinheim, Germany	Sulpiride	Sigma–Aldrich, Steinheim, Germany
Isothipendyl	Origin unknown	Suprofen	Sigma–Aldrich, Steinheim, Germany
Ketoprofen	Sigma–Aldrich, Steinheim, Germany	Terbutaline	Astra-Draco, Lund, Sweden
Labetalol	Sigma–Aldrich, Steinheim, Germany	Tertatolol	Servier Technology, Suresnes, France
Mandelic acid	Sigma–Aldrich, Steinheim, Germany	Tetramisole	Sigma–Aldrich, Steinheim, Germany
Mebeverine	Duphar, Amsterdam, The Netherlands	Verapamil	Fluka, Neu-Ulm, Switzerland
Mepindolol	Origin unknown	Warfarine	Sigma–Aldrich, Steinheim, Germany
Meptazinol	Origin unknown		

Table 2
Mobile- and stationary phases applied in screening.

2-Propanol-containing Mobile Phases (MP)	
MP A 90/10 (v/v) CO_2/(2PrOH + 0.5% TFA) *(acidic compounds)* or 90/10 (v/v) CO_2/(2PrOH + 0.5% IPA) *(all other compounds)*	
MP B 80/20 (v/v) CO_2/(2PrOH + 0.5% TFA) *(acidic compounds)* or 80/20 (v/v) CO_2/(2PrOH + 0.5% IPA) *(all other compounds)*	
MP C 90/10 (v/v) CO_2/(2PrOH + 0.25% IPA + 0.25% TFA)	
MP D 80/20 (v/v) CO_2/(2PrOH + 0.10% IPA + 0.10% TFA)	
Stationary Phase (SP)	Chiral selector
Chiralpak® AD-H	Amylose tris(3,5-dimethylphenylcarbamate)
Chiralcel® OD-H/Lux® Cellulose-1	Cellulose tris(3,5-dimethylphenylcarbamate)
Chiralcel® OZ-H/Lux® Cellulose-2	Cellulose tris(3-chloro-4-methylphenylcarbamate)
Chiralcel® OJ-H/Lux® Cellulose-3	Cellulose tris(4-methylbenzoate)
Lux® Cellulose-4	Cellulose tris(4-chloro-3-methylphenylcarbamate)
Chiralpak® AS-H	Amylose tris((S)-α-methylbenzylcarbamate)
Chiralpak® AY-H/Lux® Amylose-2	Amylose tris(5-chloro-2-methylphenylcarbamate)
Sepapak®-5	Cellulose tris(3,5-dichlorophenylcarbamate)

2.4. SFC instrumentation

An analytical system from Waters® (Milford, MA, 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® (Thar, 2003–2009, Pittsburgh, PV, USA) or Chromscope® Instrument Edition V1.10 software (Water Corporation, 2011, Milford, CT, USA) and data were processed using the Chromscope® (TharSFC®, 2009) or Chromscope® Instrument Edition V1.10 software.

2.5. Chromatographic screening conditions

For this study it is important to apply the same conditions for all test set compounds. This enables a fair comparison of the chiral stationary phases. The tested conditions are not necessarily supercritical, they may be subcritical but were taken from earlier research on chiral screenings in SFC by Maftouh et al. [14] and were found to give good results. The following conditions are prescribed as starting conditions: a total flow rate of 3.0 ml/min, a detection wavelength of 220 nm, a temperature of 30 °C, a back pressure of 150 bar and an analysis time of 30 min. Further, all mobile phase compositions are expressed in volume-ratios (v/v). The screened chromatographic systems are presented in Table 2. The mobile phases are lettered from A to D in analogy with [18], but the MPs in this study contain 2PrOH as modifier instead of MeOH.

2.6. Data processing

For each enantioseparation, the resolution (R_s) was calculated using the European Pharmacopoeia [21] equation, containing peak widths at half heights. Peaks with $R_S > 1.5$ are considered baseline separated, $0 < R_S < 1.5$ partially separated, and $R_S = 0$ not separated. This parameter is our first choice to quantify the separation quality since it takes into account peak shape and peak separation and since a clear limit value ($Rs = 1.5$) can be defined for baseline-separated compounds. The resolution was compared to the selectivity (α). Since this parameter does not take into account peak shape, it is not possible to define a limit for baseline separations.

Compounds that do not elute within the predefined analysis time of 30 min are indicated as non-eluted (NE). Racemates where one enantiomer is eluted within and the other outside this analysis-time window are indicated as partially eluted (PE).

Compounds with two chiral centres, 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.

3. Results and discussion

The screening results for the test set on the different chromatographic systems are shown in Fig. 1. The three most successful columns with each MP are underlined. Overall AD-H is the most successful CSP, since it is ranked in the top three with each MP. The highest baseline-separation rate was obtained on AD-H with MP B. AS-H separates generally least compounds (always less than half of the test set) regardless the evaluated MP and performs overall worst. All other CSPs enabled separating (baseline or partial) at least half of the test set for two or more MPs, except for LA-2 this is seen for one MP only.

3.1. Impact of the solvent strength on the success rate

In the screening step of [14], 20% 2PrOH (with 0.5% IPA or TFA) was used as organic modifier. When a lower 2PrOH concentration is used, the mobile-phase solvent strength decreases, which increases retention times. Using 10% 2PrOH thus might increase the success rate if the analysis time does not become too long. It is important to find an acceptable compromise between resolution and analysis time. In this stage, a gradient elution is not considered, since this requires additional steps to transfer later to isocratic mode when optimizing a method for an individual compound.

MP B (with 20% 2PrOH) generally generates a higher number of separations than MP A (with 10% 2PrOH) (Fig. 1). This is linked to a higher number of non-eluted or partially eluted racemates for MP A. Exception to this trend is OJ-H which generates more baseline and partial separations using MP A.

The same trend is observed when comparing MP C and -D (Fig. 1). The higher success rate of MP D is again linked to the lower number of non-eluted compounds when using a higher percentage of 2PrOH. Thus, for both MPs, with individual and joint additives, it can be concluded that 20% 2PrOH is more appropriate for screening purposes, yielding more elutions/separations within the predefined analysis time of 30 min.

On most CSPs, at least 17 extra compounds elute with MP B than with MP A. On OJ-H and AS-H remarkably less compounds are excessively retained both with MP A and B. Retention factors on these CSPs were generally lower, while their average success rates were not different from the others. This might indicate that on OJ-H

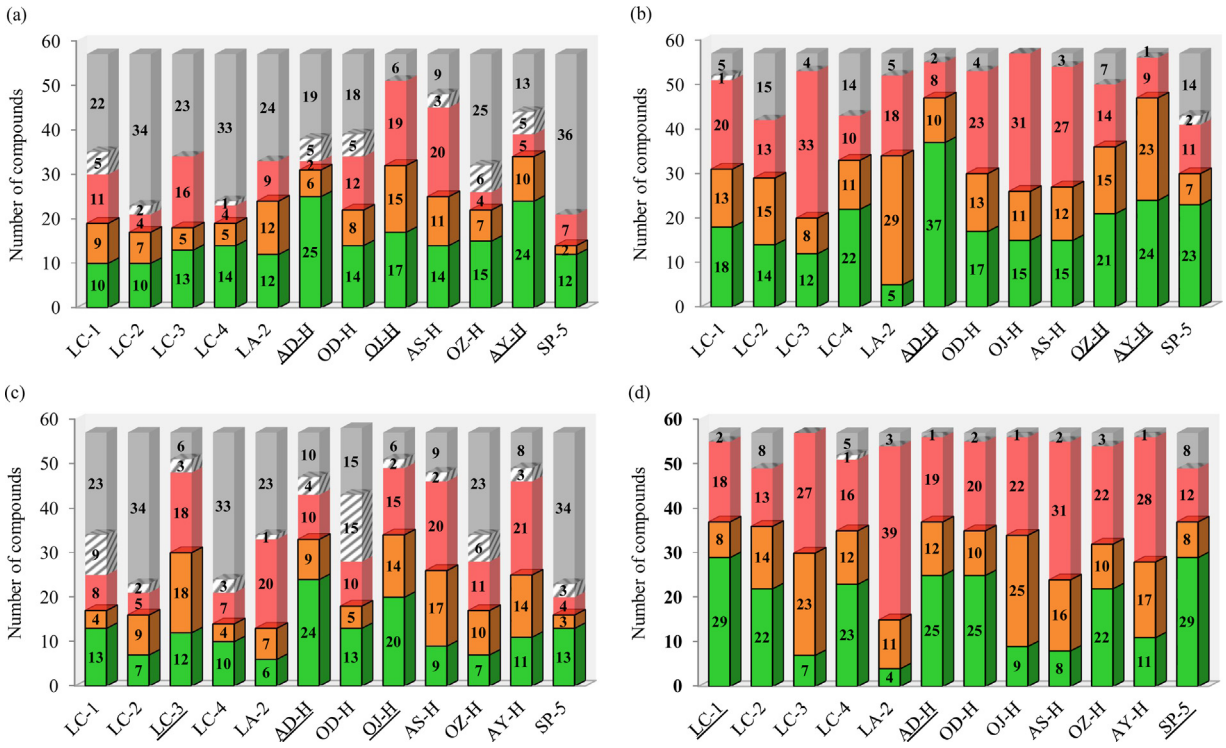


Fig. 1. Screening results, expressed in absolute numbers, achieved on the evaluated chiral stationary phases with 2-propanol-containing mobile phases: (a)–(d) mobile phases A–D (composition: see Table 2). The three most successful columns with each mobile phase are underlined.

and AS-H non-stereospecific interactions play a less important role in the retention mechanism than on the other CSPs.

The difference in separation rate between mobile phases -C and -D, is rather limited (<5 separations) on LA-2, AD-H, AS-H, and AY-H. A considerable number of initially non-eluted or partially eluted compounds elute using a higher solvent strength, but then do not show enantioresolution anymore on these CSPs. This is reflected in the rather limited increase in separations when comparing MP C with -D.

On LC-3 and OJ-H, MP D resulted in the additional elution of nine and seven extra compounds, respectively, but this did not increase the separation rate since the higher solvent strength also results in the loss of some separations. A net loss of two separations was noted for AS-H, switching from MP C to -D. For all other CSPs, MP D yielded more separations. Therefore, MP D was selected as more appropriate for screening purposes than MP C.

Remarkable is that while OJ-H and LC-3 contain an identical selector, there is a difference in the number of non-eluted compounds when using MP A (Fig. 1). On LC-3, 23 compounds were not eluted *versus* only six on OJ-H. Generally, lower retention factors are obtained with the CSPs from Chiral Technologies (OD-H, OZ-H, OJ-H, and AY-H) than with their equivalents from Phenomenex (LC-1, LC-2, LC-3, and LA-2, respectively). This explains the lower number of non-eluting compounds on the Daicel CSPs. The same was noted for the other mobile phases, although less pronounced.

On polysaccharide-based CSPs, enantioselective recognition arises from different interactions of which hydrogen bondings are essential since they are polysaccharide-configuration dependent. Polar moieties of chiral analytes form hydrogen bondings with the carbamate- or benzoate groups of the selector, hereby facilitating other interactions. Aromatic groups of the analyte and CSP form π–π bondings. Furthermore dipole–dipole interactions and stereospecific inclusion into the polysaccharide helix also influence the enantiorecognition [22]. As all of these retention mechanisms are highly configuration dependent, differences in raw material, in the synthesis- and/or packing process may account for the different retention observed between equivalent CSPs. Achiral interactions with the stationary phase will be impacted by this. In addition, the degree of substitution on the polysaccharide chain can largely affect the enantioselectivity.

3.2. Effect of additives on the enantioselectivity

The most appropriate organic modifier content in the mobile phase was determined to be 20% 2PrOH, corresponding to Maftouh's [14] screening step. In the latter screening, 0.5% TFA was used as mobile phase additive for acidic compounds and 0.5% IPA for all other compounds. In an attempt to simplify the screening and to eliminate the need to divide the compounds according to their chemical properties, other mobile phases were evaluated.

Preliminary experiments showed that without additive, the chromatographic results deteriorated. Equally poor results were obtained when using IPA for acidic compounds or TFA for the others. Consequently, these mobile phases were not further considered. MPs with both IPA and TFA were examined next. For methanol-containing mobile phases, combination of IPA and TFA proved generating a unique and broad enantioselectivity compared to their individual use [18,20].

Seven of the CSPs displayed the broadest enantioselectivity using mobile phases with combined additives, *i.e.* LC-1, LC-2, LC-3, LC-4, OD-H, OJ-H, and SP-5 (Fig. 1). For these CSPs, MP D (with 20% 2PrOH) yielded similar or higher success rates than MP C. The other five CSPs, *i.e.* LA-2, AD-H, AS-H, OZ-H and AY-H, performed better with MPs containing only one additive. Remarkable is that this latter list includes almost exclusively amylose-based CSPs. This might be explained by the possible working mechanisms of combined IPA and TFA: ion complexes are formed between IPA and acidic compounds on the one hand, and between TFA and basic compounds on the other. These neutral complexes might interact better with the neutral polysaccharide-based selectors than the non-complexed

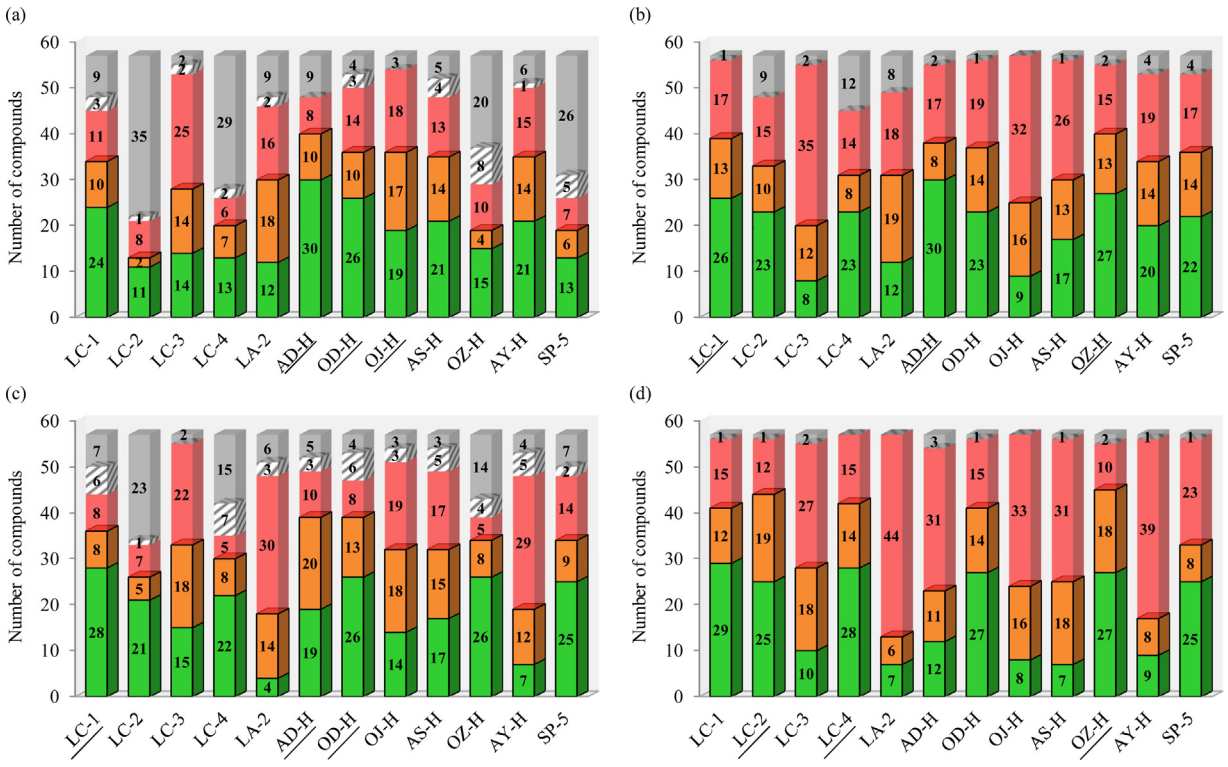


Fig. 2. Screening results, expressed in absolute numbers, achieved on the evaluated chiral stationary with methanol-containing mobile phases: (a)–(d) mobile phases A–D (composition: see Table 2). The three most successful columns with each mobile phase are underlined.

compound and generate in that way a unique selectivity [23]. In the alternative approach where TFA is used for acidic and IPA for all other compounds no analyte-additive complex formation occurs.

Amylose and cellulose are both glucose polymers. In amylose, the (1 → 4) glycosidic bonds are in α-position (axial) and in cellulose in β-position (equatorial). This results in a helical structure for amylose chains, while cellulose is more linear. Although the helical structure of amylose carbamate derivatives has not yet been determined by X-ray studies, it is obvious that the geometrical structure differs from that of cellulose derivatives [22,24]. This has an impact on the stereoselective inclusion in the polysaccharide helix of amylose-derivatives. As this inclusion is an important mechanism in enantiorecognition, this might account for the poorer results using combined additives with amylose-based CSPs [23,25].

The most successful chromatographic systems are obtained using MP B on AD-H or AY-H. These systems achieve a separation rate of 47 compounds (82%), of which 79% are baseline separated on AD-H and 51% on AY-H. These chromatographic systems, however, have the disadvantage that compound classification prior to the screening is necessary. The second most successful systems using MP D are LC-1, AD-H and SP-5, where success rates of 65% (37 separations) were achieved.

3.3. Impact of the organic modifier: 2PrOH vs. MeOH

The type of organic modifier affects the enantioselectivity by interacting with the stationary phase and analyte [15]. By changing the modifier type, different success rates can be achieved on the same stationary phase. Methanol is extensively used in chiral SFC because it generates high-efficient separations. However, it does not always yield the highest enantioselectivity [12]. The results from methanol-containing MPs, obtained in an earlier study [18], are summarized in Fig. 2. For eight CSPs (LC-1, LC-2, LC-3, LC-4, OZ-H, OJ-H, OD-H, and AS-H) high separation rates are achieved for a methanol-containing mobile phase. However, the highest rates were achieved with 2-propanol-containing MPs, *i.e.* AD-H/MP B and AY-H/MP B both separating 47 racemates (82%). For the latter stationary phases the 2-propanol-containing MPs generate significantly more separations.

To conclude which modifier yields most separations, a summary of all acquired data is given in Table 3. In this table the results from the four MeOH- and 2PrOH-containing MPs are considered, enabling to evaluate the overall performance of each modifier. The results are expressed either in terms of unique separations, *i.e.* for compounds resolved only when using either MeOH or 2PrOH in the MP, or of unresolved racemates using MeOH, 2PrOH or either modifier. Overall, the MeOH-containing MPs appear most successful: eight CSPs show a lower number of unresolved racemates, which is in accordance to the individual separation rates of the chromatographic systems, evaluated earlier.

Table 3
Overview of the number of unique separations per CSP and modifier, *i.e.* separations solely achieved with either MeOH or 2PrOH, and of the number of racemates that remained unseparated using MeOH, 2PrOH or either of both modifiers, in the MP.

	Unique separations		Not separated		
	MeOH	2PrOH	MeOH	2PrOH	MeOH or 2PrOH
LC-1	8	3	10	15	7
LC-2	7	3	8	12	5
LC-3	3	5	19	17	14
LC-4	10	4	7	13	3
LA-2	7	6	17	18	11
SP-5	5	3	11	13	8
AD-H	1	5	8	4	3
OD-H	9	3	9	15	6
OJ-H	1	6	13	9	7
AS-H	9	3	11	17	8
OZ-H	7	3	6	10	3
AY-H	3	12	16	7	4

Of the four other CSPs, AD-H and AY-H showed to be most successful with 2PrOH, while LC-3 and OJ-H only display a small difference in unseparated compounds, thus each modifier is rather equally successful.

A certain degree of complementarity can be achieved using different mobile phases with the same CSP (Table 3). MeOH and 2PrOH show the highest complementarity on AY-H and LC-4, with 15 and 14 unique separations, respectively, and only a respective 4 and 3 racemates remain unresolved. Thus, using several organic modifiers indeed leads to different enantioselectivities, potentially increasing the success rate.

Preliminary studies with ethanol showed that this modifier generates less separations than MeOH or 2PrOH, although unique separations were obtained for some compounds on given CSPs. However, given the high success rates with MeOH and 2PrOH it seemed more appropriate to reserve EtOH for specific optimization cases rather than to include in the screening.

3.4. Selection of the most complementary systems

When defining a screening approach it is also important to take into account the complementarity of chromatographic systems. In a first instance, the same mobile phase was considered for all CSPs. The advantage then is that only one mobile phase has to be prepared. The cumulative success rate was determined for MP D with 2PrOH as modifier (Fig. 3), because it yielded a high success rate and required no compound classification prior to screening. The cumulative success rate results from a sequence starting with the system that generates the highest number of separations. Next, the system generating the most additional separations (not achieved with the previous system) is added to the sequence and so on. The resulting sequence allows achieving the highest success rate by screening the fewest systems. CSPs that do not increase the success rate are not included in the proposed screening. For completeness, the number of baseline separated compounds is shown between brackets in the figure.

Using 2PrOH-D, the proposed screening sequence, presented in Fig. 3 is: LC-1 (37 or 65%) → SP-5 (51 or 89%) → AD-H (53 or 93%) → LC-3 (55 or 96%) → LC-4 (56 or 98%). AD-H, LC-3 and LC-4 yield the unique separation of labetalol, methotrexate and nitrendipine, respectively. This combination of chromatographic systems yields a baseline separation for 47 compounds.

As the nature of the polar organic modifier has an important influence on enantioselectivity, it is important to evaluate different modifiers [11,26]. The highest success rate for methanol-containing mobile phases was achieved when 80/20 (v/v) CO_2/(MeOH + 0.1% IPA + 0.1% TFA), *i.e.* MeOH-D, was used. Using this MP, the proposed screening sequence achieves a cumulative separation rate of 56 compounds (98%) (Fig. 3). To evaluate the complementarity of both the methanol- and 2-propanol-containing MPs, MeOH-D and 2PrOH-D were compared.

Using these two MPs, OZ-H in combination with MeOH-D generated the broadest enantioselectivity: 45 separations, of which 27 baseline (Fig. 3). Most complementary to this system is AD-H with 2PrOH-D, which generates eight additional separations. The OD-H with MeOH-D and LC-4 with 2PrOH-D systems are then selected, generating three and one additional separations, respectively. Thus this screening sequence separates all test set compounds at least partially, with 48 baseline separations. The last added system uniquely separates nitrendipine. From a practical point of view it might be more advisable to switch AD-H and OD-H in the sequence, which allows screening the first two systems with the same MP. None of the columns in the proposed sequence can be substituted without a loss in the cumulative success rate. This screening sequence generates one extra separation and three extra baseline separations, then when using only MeOH-D. When only 2PrOH-D and four CSPs are used, in total two extra and one additional baseline separation is generated. Therefore, the combined sequence was chosen to include in our screening strategy. However, the use of only one modifier thus can be considered as an alternative, leading to a slightly lower success rate.

The CSPs can be substituted by equivalent CSPs with the same selector but from other manufacturers, *i.e.* OZ-H with LC-2 and OD-H with LC-1. In this case the cumulative success rate would be identical: LC-2/MeOH-D (44 or 77%) → AD-H/2PrOH-D (52 or 91%) → LC-1/MeOH-D (55 or 96%) → LC-4/2PrOH-D (57 or 100%).

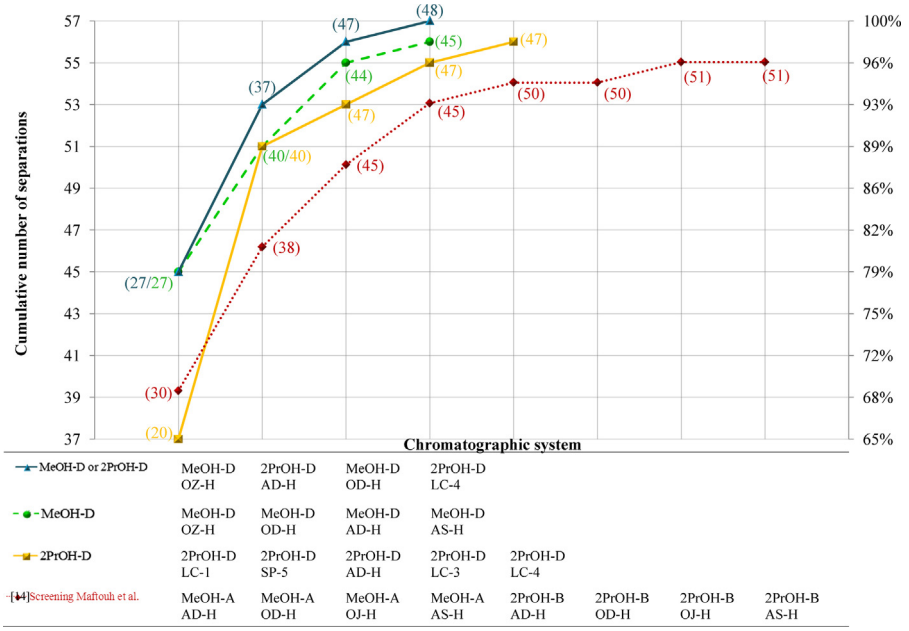
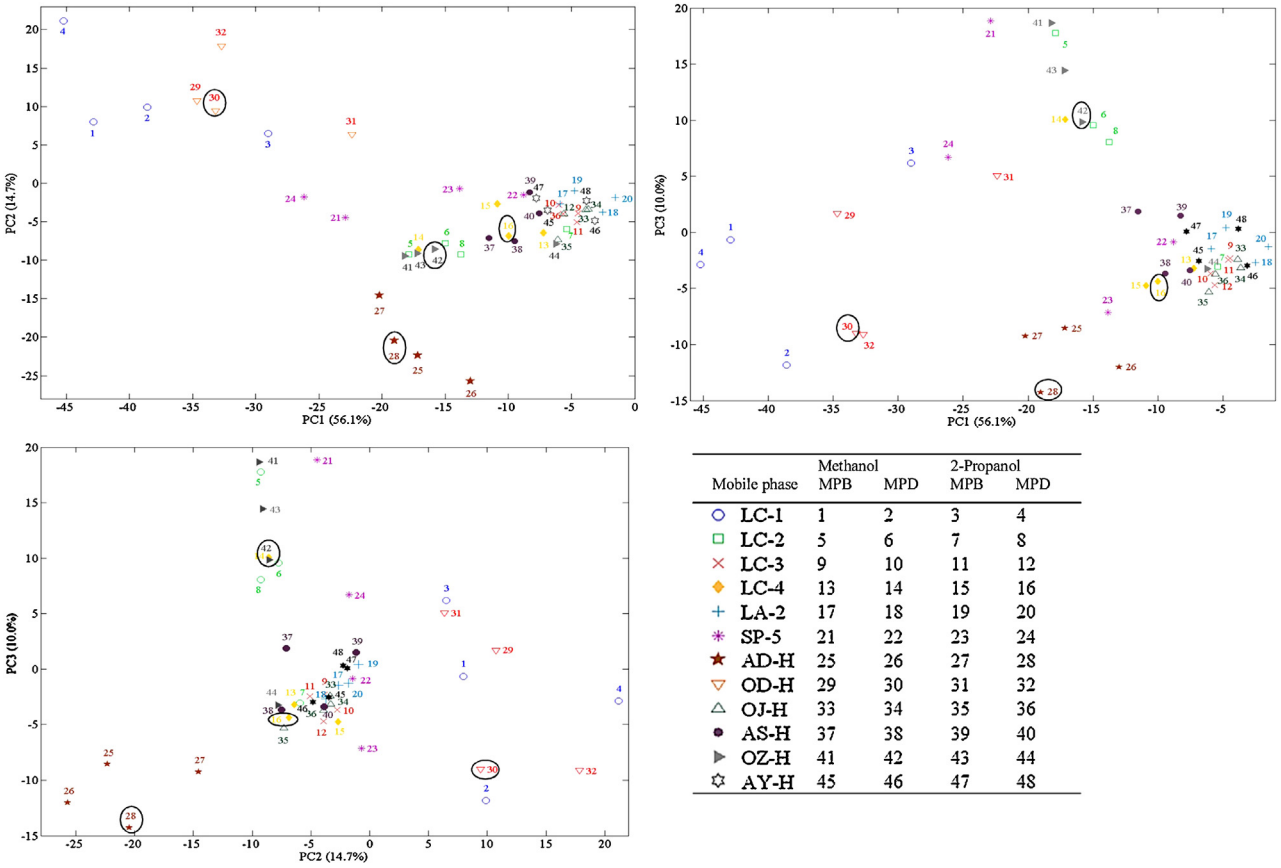


Fig. 3. Cumulative success rates (expressed in absolute numbers) achieved using the screening sequence as proposed by Maftouh et al. [14] (red curve), MeOH-D (green curve), 2PrOH-D (orange curve), and MeOH-D on 2PrOH-D (blue curve). The cumulative number of baseline separated compounds are given between brackets. Beneath the graph, the screened chromatographic systems are specified. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Mobile phase	Methanol MPB	Methanol MPD	2-Propanol MPB	2-Propanol MPD
LC-1	1	2	3	4
LC-2	5	6	7	8
LC-3	9	10	11	12
LC-4	13	14	15	16
LA-2	17	18	19	20
SP-5	21	22	23	24
AD-H	25	26	27	28
OD-H	29	30	31	32
OJ-H	33	34	35	36
AS-H	37	38	39	40
OZ-H	41	42	43	44
AY-H	45	46	47	48

Fig. 4. Score plots (PC1, PC2, PC3) of the principal component analysis (PCA) on 48 chromatographic systems. The legend summarizes the analyzed systems.

3.5. *Evaluation of enantioselectivity through an exploratory analysis*

For screening purposes, the mobile phases with a higher modifier content (20%) and with both additives were preferred. The cumulative success rate was determined with these MPs and the most efficient screening sequences were selected. However, to highlight the intrinsic complementarity and (dis)similarity of individual systems, an exploratory analysis of the data was performed. A principal component analysis (PCA) was made with the 48 systems using 20% modifier in the MP (MeOH-B, -D, and 2PrOH-B and–D) (Fig. 4). Systems that use MPs with 10% co-solvent were not included in the analysis, since they tend to result in unacceptably long analysis times.

To construct the PCA score plots, the resolution of 29 racemates on 48 systems was used (alprenolol, ambucetamide, betaxolol, bisoprolol, bupranolol, carbinoxamine, chlorphenamine, esmolol, methadone, metoprolol, mianserin, nimodipine, nisoldipine, nitrendipine, oxazepam, oxprenolol, pindolol, promethazine, propiomazine, propranolol, verapamil, fenoprofen, flurbiprofen, hexobarbital, ibuprofen, ketoprofen, naringenin, and suprofen). The reason for not being able to use the complete test set is that for certain racemates no resolution was obtained on given systems due to a too late elution. Thus, the data matrix would contain missing results and would not be processable. Therefore compounds that did not elute with in the analysis time were excluded from the data matrix, while for the partially eluted compounds experiments were repeated with a prolonged analysis time.

The data was pre-processed prior to the principal component analysis. In fact, three outcomes are possible for screening experiments *i.e.* not separated ($R_s = 0$), partially separated ($0 < R_s < 1.5$) or baseline separated ($R_s > 1.5$). Although high resolutions, *e.g.* $R_s > 10$, give information about the potency of the enantioselective interactions of the system towards a compound, these results should not over-influence the PCA results. To overcome the influence of large R_s ranges, all results were transformed by autoscaling:

$$R_{s_t} = \frac{(R_s - \bar{R}_s)}{s(R_s)}$$

with R_{s_t} the autoscaled resolution, $\bar{R}_s$ the average resolution and $s(R_s)$ the standard deviation of the resolutions on one system. After autoscaling, the average and the range of all the resolutions on all systems are similar.

PC1 accounts for 56.1% of the variability between the systems, PC2 for 14.7% and PC3 for 10.0%. The total variability is thus explained for 80.0% by three principal components. Given the reduced number of compounds that could be included in the PCA matrix, general conclusions from the PC-plots, have to be made with some reserve. They could confirm earlier made observations or formulated hypotheses, even though they, in a first instance, are made to observe general tendencies. The PC1–PC2 score plot, for instance, shows a large central group of systems next to some systems behaving rather differently, *i.e.* having different resolution profiles. The LC-1/OD-H systems (1–4; 29–32), containing the same selector, behave differently and with a rather large variability among the different systems. Further the AD-H based systems behave differently from the major group.

Chromatographic systems using the same CSP but different MPs tend to be grouped in close proximity in the PCA plots on Fig. 4. This observation is logic since the CSP has the largest influence on the enantioselectivity, while the MP

has less. In addition, equivalent CSPs with the same selector, but from different manufacturers, used under the same conditions are found to be relatively grouped, *e.g.* (LC-2/OZ-H) (5,6,7,8/41,42,43,44); (LC-3/OJ-H) (9,10,11,12/33,34,35,36); and (LA-2/AY-H) (17,18,19,20/45,46,47,48) and to a lesser extent the already mentioned (LC-1/OD-H).

As discussed higher, the CSPs with an identical selector do not always generate similar enantioselectivity. For instance LA-2 and AY-H contain the same chiral selector but tend to generate different separation results. Indeed, using 2PrOH-B nine more compounds are resolved on AY-H than on LA-2 (19/47) and using 2PrOH-D (20/48) even eleven (Fig. 1). Thus, it seems that these systems display a significant difference in enantioselectivity. However, on the PC plots these systems are close to each other. A possible explanation is that of the above mentioned compounds some are not included in the PCA matrix. For instance, of the 15 racemates (26%) that show a different resolution on AY-H and LA-2 using 2PrOH-B, only four (13.8%) are included in the 29-compounds subset. The fact that the systems are in each other's proximity indicates that the separation profiles, of the compounds are similar on the different systems. Thus the possibility exist that, while the profiles are similar, the absolute separations are systematically less good on one CSP relative to the other, which leads to the above conclusions when comparing individual systems.

Another observation made for the periferic systems in PC1–PC2 is that their B and D mobile phases for a given modifier sometimes are rather far away, indicating a different enantioselectivity for mobile phases with either one or two additives. This difference in enantioselectivity was already discussed above and is thus confirmed by the PCA plots.

Systems that are distant are expected to have a different enantioselectivity pattern for the 29 compounds. For instance, systems 4 and 20, LC-1/2PrOH-D and LA-2/2PrOH-D respectively are located far apart in the PC1–PC2 plot. On the latter systems 34 compounds of the total test set (60%) and 19 compounds of the subset (66%) are resolved on one system but not on the other systems. Systems with the most different enantioselectivities are thus expected at largest distances from each other, at the outside of the data cloud.

Systems with the most different enantioselectivities will be situated at the borders of the data cloud. The most dissimilar systems could easily be selected by, for instance, the Kennard and Stone or the duplex algorithms. However, this selection will not be the one with the highest cumulative success rate since both the (broad) enantioselectivity and the complementarity of the systems are ignored in this approach.

The systems (16, 28, 30 and 42) included in the proposed screening sequence with MeOH-D and 2PrOH-D are scattered in the plots. Systems 28, 30 and 42 are situated rather extreme in the different PC plots, while system 16 is situated in the larger, central cluster of systems. In Fig. 3 we see that system 16 (LC-4 with 2PrOH-D) was selected last. This means that it has some complementary to the previously selected and this is the reason for its inclusion. Fig. 3 does not provide information about the enantioselectivity difference/similarity with other systems, while the PC plots do. Systems 16 and 42, (2PrOH-4/LC-4) and (MeOH-D/OZ-H), respectively are located relatively close in the PC1/PC2 plot. Only six compounds of the 29-compound subset (21%) are resolved with one systems but not with the other. However looking at the complete 57-compounds test set, 18 compounds (32%) are separated on only one of both systems.

PCA-plots based on the selectivity α of the enantioseparations were also drawn (data not shown). These plots confirmed the trends discussed for the PCA-plots based on R_s. Furthermore using α, PC1 to PC3 account only for 52.5% of the total variability in the data set, while it is 80.8% when considering R_s.

Summarized, PCA visualizes the entire data set, it shows the systems with similar and different separation profiles, but does not allow selecting the systems with the highest cumulative success rate. Many observations seen from a classic comparison of systems, as was done higher, are confirmed, but also placed in the broader context of all systems.

3.6. Update of the generic screening approach

In Fig. 3, the cumulative success rate for the initial screening step defined by Maftouh [14], applied on our test set, is shown. The newly proposed screening has a higher separation rate than the initial from the first screening. Screening four CSPs, the new screening separates three more compounds and resolves three more compounds baseline. The last systems in the initial approach mainly improve the number of baseline separations. The update of the screening step is shown in Fig. 5.

In Fig. 6 the results of the screening experiments for a model compound, alprenolol, are presented as an example. All experiments had short analysis times, below 3.0 min. Performing the separation with MP MeOH-D on OZ-H yields a partial separation

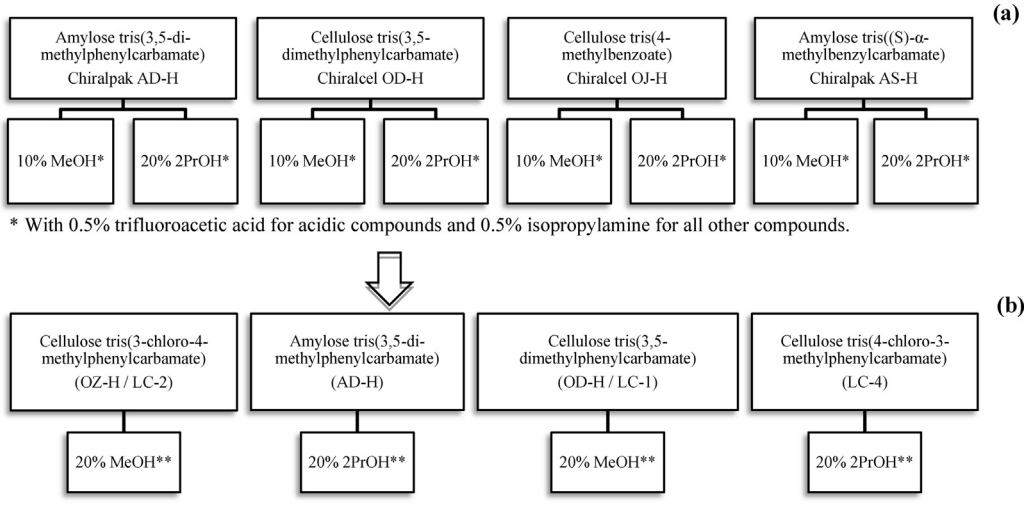


Fig. 5. Scheme of (a) the initial screening step as defined by Maftouh et al. [14] and (b) the updated version of the screening step. In the top row the CSPs are presented while the second row represent the used modifier in the carbon-dioxide based mobile phases.

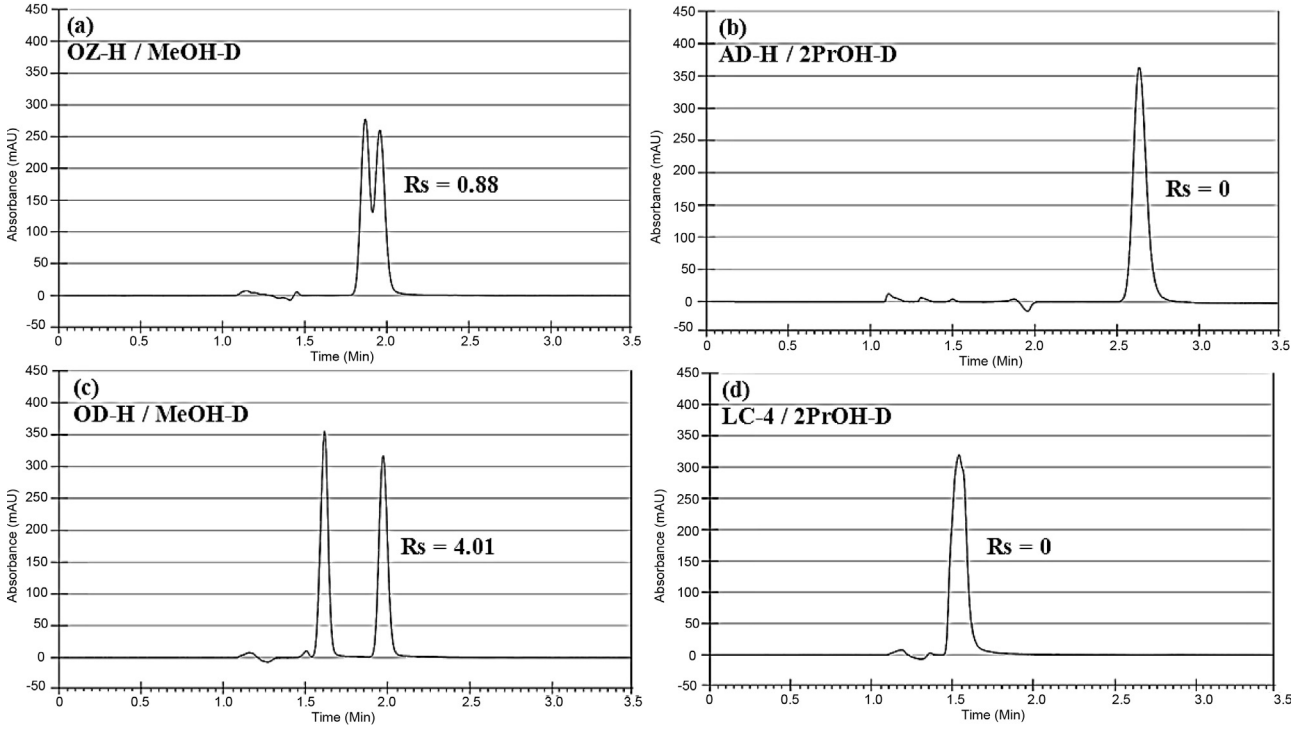


Fig. 6. Results of the screening experiments for alprenolol. Screening conditions were: mobile phase CO_2/(methanol + 0.10% isopropylamine + 0.10% trifluoroacetic acid) 80/20 (v/v) (a and c) and CO_2/(2-propanol + 0.1% isopropylamine + 0.1% trifluoroacetic acid) 80/20 (v/v) (b and d), total flow of 3.0 ml/min, temperature of 30 °C, detection at 220 nm, and back pressure of 150 bar.

of the racemate, while OD-H yields a baseline separation with resolution 4.0. The 2-propanol-based systems do not generate a separation of the alprenolol enantiomers. The screening provides different enantioselectivities, *i.e.* twice no separation, one partial- and one baseline separation. This emphasizes the complementarity of the selected systems. For this compound, the desired separation with satisfying resolution and analysis time was already achieved in the screening step (with OD-H as CSP). The peak shapes were also acceptable for this separation, (tailing factors were 0.99 and 1.12 for the first and second peak, respectively).

4. Conclusion

In this study, 48 chromatographic systems, composed from 12 polysaccharide-based stationary phases and four 2-propanol-containing MPs, were screened with 57 pharmaceutical racemates. It was possible to separate 82% of the test set (47/57 compounds) in one single experiment, *i.e.* using Chiralpak® AD-H or AY-H with CO_2/(2PrOH + 0.5% IPA or TFA), 80/20 (v/v). However other parameters than only the number of successful separations can be taken into account when defining a screening, *e.g.* its analysis time and simplicity. In this context, MPs with both IPA and TFA were preferred, because it eliminates the need for compound classification prior to the screening. The most efficient screening was defined taking into account the data generated in this study and earlier data obtained with methanol-containing MPs. Using the systems sequence: cellulose tris(3-chloro-4-methylphenylcarbamate) (OZ-H/LC-2)/MeOH-D > AD-H/2PrOH-D > cellulose tris(3,5-dimethylphenylcarbamate) (OD-H/LC-1)/MeOH-D > LC-4/2PrOH-D, a cumulative success rate of 100% (57/57 compounds) was achieved with analysis times below 30 min. Principal component analysis of the data confirmed the selectivity difference of the included systems. Moreover, the PCA showed that often the CSP type has a larger impact on the enantioselectivity than the MP.

Further optimization steps for compounds either fully separated after the screening step (to reduce analysis times or improve peak shapes), not separated within an acceptable time frame or partially separated, can be added to the screening strategy.

Conflict of interest

Authors declared no conflict of interest.

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