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Pharmaceutical-enantiomers resolution using immobilized polysaccharide-based chiral stationary phases in supercritical fluid chromatography☆

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ABSTRACT

Since their introduction on the market the applicability of immobilized polysaccharide-based chiral stationary phases in high-performance liquid chromatography has been thoroughly investigated. These immobilized phases have the benefit to be applicable with a wide range of modifiers, potentially extending the application range of the polysaccharide-based stationary phases. Because an increasing number of stationary phases are being introduced in the field of chiral chromatography it is important to evaluate their enantioselectivity in different techniques in order to get an idea about their applicability. In this study, three immobilized chiral polysaccharide-based stationary phases (Chiralpak IA, IB, and IC) are evaluated in supercritical fluid chromatography (SFC) with a test set of pharmaceutical racemates. This is done in a three-fold manner: their performance is evaluated (1) using traditional modifiers, (2) using mixtures of atypical modifiers, and (3) the results were compared to those on coated stationary phases with an equivalent chiral selector. To get a visual overview of the enantioselective patterns of the different chromatographic systems (mobile and stationary phase combinations), a Principal Component Analysis is performed, which allows determining the (dis)similarity between individual systems. To assess the complementarity cumulative success rates are determined. The immobilized chiral stationary phases prove to yield high cumulative success rates.

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1. Introduction

In direct chiral chromatography, polysaccharide derivatives are extensively used in chiral stationary phases (CSPs). There are multiple reasons, such as broad enantioresolving ability, easy availability, and high loadability under preparative conditions, that favour their use [1,2]. Commercially available CSPs of this type contain derivatised polysaccharides, usually coated onto a silica matrix. However, combined with given solvents this coating swells and/or dissolves, destroying the enantioselective capacity of the phase. The use of certain organic modifiers, such as chloroform, dichloromethane, acetone, ethyl acetate and tetrahydrofurane, in the mobile phase is therefore prohibited. To overcome these limitations, new stationary phases were developed, containing polysaccharide derivatives covalently bonded (to the silica) matrix [1–12]. At present, six immobilized polysaccharide-based CSPs are commercially available, *i.e.* Chiralpak® IA, IB, IC, ID, IE and IF. The chemical structures of these selectors, as reported by the manufacturer, are given in Table 1. Coated versions, with the same selector as Chiralpak® IA, IB, IC and IF, also exist, from the same manufacturer as well as from others.

Different approaches were reported to fix the selectors [5,13–20]. However, chemically linking the polysaccharide derivatives to the silica matrix potentially changes the higher-order structure of the selector, which is considered to be a prerequisite for enantioselective recognition. Consequently, the enantioselective recognition abilities of polysaccharide CSPs may be different when immobilized or coated [4,21].

Immobilized CSPs are robust and can be used with a broader variety of solvents, as mentioned above. This extends the application range of these selectors in terms of enantioselectivity, but also in terms of mobile-phase solubility of compounds, offering benefits at both analytical and preparative scales [7].

Although supercritical fluid chromatography (SFC) has repeatedly proven itself as a performant chromatographic technique for chiral separations, HPLC remains dominant in this field. This is likely due to the limited range of available SFC-equipments and the fact

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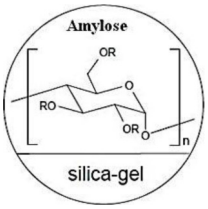
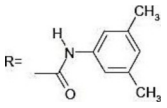
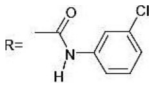
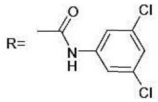
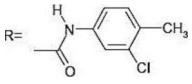
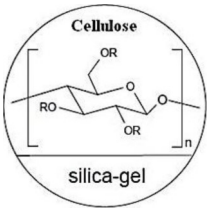
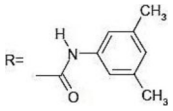
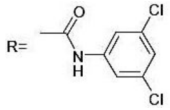
Table 1
A non-exhaustive overview of commercially available immobilized polysaccharide-based stationary phases and equivalent coated phases.

Chiral selector	Structure chiral selector	Commercial name		Manufacturer
		Immobilized	Coated	
Amylose tris(3,5-dimethylphenylcarbamate)	 	Chiralpak® IA	Chiralpak® AD Amycoat® RegisPack®	Chiral Technologies Kromasil Regis Technologies Inc.
Amylose tris(3-chlorophenylcarbamate)		Chiralpak® ID		Chiral Technologies
Amylose tris(3,5-dichlorophenylcarbamate)		Chiralpak® IE		Chiral Technologies
Amylose tris(3-chloro-4-methylphenylcarbamate)		Chiralpak® IF	Chiralpak® AZ	Chiral Technologies
Cellulose tris(3,5-dimethylphenylcarbamate)	 	Chiralpak® IB	Chiralcel® OD Lux® Cellulose-1 Cellucoat® Astec® Cellulose DMP RegisCell®	Chiral Technologies Phenomenex Kromasil Sigma–Aldrich Regis Technologies Inc.
Cellulose tris(3,5-dichlorophenylcarbamate)		Chiralpak® IC	Sepapak®-5	Chiral Technologies Sepaserve

that operators are less familiar with SFC compared to HPLC. Nevertheless, the number of reported chiral SFC applications is increasing [22]. Chiral SFC separations can be achieved in a short time span, with high efficiencies and minimal organic solvent consumption. SFC also has benefits in the context of upscaling to preparative levels, since returning to ambient conditions after analysis evaporates the main eluent (CO_2) from the mobile phase. For these reasons, interest remains in SFC for enantioseparations [23].

Till now, the literature on the performance of immobilized polysaccharide-based CSPs in supercritical fluid chromatography is rather limited. Kather et al. [21] compared Chiralpak® IB (immobilized cellulose tris(3,5-dimethylphenylcarbamate) to stationary phases containing the coated selector. Franco and Zhang [6] studied chiral method development on these immobilized polysaccharide-based phases. Their focus is on the use in HPLC, although a brief paragraph also discusses the applicability in SFC. Miller [7] investigated atypical modifiers in combination with Chiralpak® IA, IB and IC in SFC. The aim was to improve the productivity of samples with poor methanol solubility by using the atypical modifiers tetrahydrofurane and dichloromethane on a preparative scale. The author found that these atypical modifiers can provide higher productivities than MeOH, but drastic changes in enantioselectivities may occur.

Our study focuses on the performance of three immobilized polysaccharide-based CSPs, *i.e.* Chiralpak® IA, IB and IC, in supercritical fluid chromatography. Initially their use in combination with traditional modifiers (methanol and 2-propanol) is evaluated. This choice in modifiers was based on earlier experience with chiral separations using coated polysaccharide-based stationary phases. In previous investigations we evaluated methanol, 2-propanol, ethanol and acetonitrile as modifiers. We saw very high success rates with the first two modifiers. Ethanol and acetonitrile delivered unique separations in certain cases, but yielded much lower separation rates. For this reason we do not apply the latter two modifiers in a first screening attempt on polysaccharide-based CSPs [24–26].

The experiments are performed in a screening context. This means that the emphasis is put on general separation conditions that apply for a broad range of compounds. These conditions are by preference as simple as possible. Without addition of the additives to the mobile phase, peak shapes deteriorated significantly. The basic compounds showed excessive peak tailing due to aspecific interactions with the silica of the stationary phase. Compounds with acidic functional groups, failed to elute or eluted with distorted peak shapes [27]. This is due to the excessive retention caused by the interaction between carboxylic functional groups and the silica support of the stationary phase [28].

For these reasons, earlier we investigated the combined use of acidic and basic additives for the analysis of all compounds, regardless of their chemical nature instead of an acidic additive for acidic compounds, and a basic one for basic compounds. We found that the evaluated polysaccharide-based columns expressed a broader enantiorecognition ability when isopropylamine and trifluoroacetic acid were combined [27]. Hence, based on the success rate and the simpler approach (all compounds can be analyzed with the same mobile phase) we preferred the approach with

combined additives for screening purposes. Since a comparison will be made between coated and their equivalent immobilized stationary phases, it is important to use the same conditions and mobile phases to evaluate both types of stationary phases. It is important to notice that the use of two additives in the mobile phase might be less suitable when upscaling for preparative purification purposes.

The performance of the three immobilized CSPs, in terms of enantioselective resolving capacities and analysis times, are compared to those of coated CSPs, with a similar selector, *i.e.* Chiralpak® IA (IA) was compared to Chiralpak® AD-H (AD-H) and Kromasil® Amycoat (amycoat); Chiralpak® IB (IB) to Chiralcel® OD-H (OD-H), Lux Cellulose-1 (LC-1) and Kromasil® Cellucoat (cellucoat); and Chiralpak® IC (IC) to Sepapak®-5 (SP-5).

In a next phase of the study, atypical modifiers, *i.e.* tetrahydrofurane (THF), ethyl acetate (EtOAc) and dichloromethane (CH_2Cl_2), are investigated with the immobilized stationary phases, aiming to enlarge their enantiorecognition ability. The solvents choice was based on studies of the immobilized columns under SFC and NPLC conditions [6,7,12]. It is important to take into account the solvent strength of each modifier. The traditional modifiers, methanol and 2-propanol have much higher solvent strengths than THF, EtOAc or CH_2Cl_2. Higher fractions of these atypical modifiers will thus be needed to increase the elution strength of pure carbon dioxide and allow the elution of compounds within a reasonable time.

2. Experimental

2.1. Equipment

An analytical SFC method station from Thar® (a Waters company, Pittsburgh, PA, USA) equipped with a Waters® 2998-DAD detector (Milford, MA, USA) was used. Data acquisition and processing were performed using Chromscope® V1.10 software (2011) from Waters®.

2.2. Materials

The columns Chiralpak® AD-H, IA, IB, and IC and Chiralcel® OD-H were purchased from Chiral Technologies (West Chester, PA, USA). Lux® Cellulose-1 and Sepapak®-5 were from Phenomenex (Utrecht, The Netherlands). Kromasil® Amycoat and Cellucoat were kind gifts from Akzonobel (Brewster, NY, USA). All columns had 250 mm × 4.6 mm i.d. dimensions with 5 μm particle sizes.

2.3. Chemicals

2-Propanol (2PrOH), methanol (MeOH), EtOAc, THF, and CH_2Cl_2 were all HPLC grade and purchased from Fisher Chemicals (Loughborough, UK). Isopropylamine (IPA) and trifluoroacetic acid (TFA) were from Aldrich (Steinheim, Germany). The carbon dioxide (CO_2) advised by the manufacturer of the SFC equipment was used, *i.e.* quality 2.7 (purity >99.7%) (Linde Gas, Grimbergen, Belgium).

2.4. Methods

The chromatographic conditions are summarized in Table 2. All percentages expressed in the context of mobile phase compositions are volume percentages. To all modifiers and modifier mixtures 0.1% of both isopropylamine and trifluoroacetic acid were added. The modifier/additive mixtures were mixed with the carbon dioxide in the appropriate ratio by the equipment. The total flow rate of the mobile phase was always 3.0 ml/min. Depending on the ratio modifier/CO_2 the equipment adapted the individual flow rates of the modifier and CO_2 to obtain this final flow rate. The 56 commercial test racemates used in this study are summarized in Table 3.

Table 2
Chromatographic conditions used in the study.

Total flow rate	3.0 ml/min
Temperature	30 °C
Detection	220 nm
Backpressure	150 bar
CO_2/modifier ratio in the MP	80/20 (v/v), for modifier compositions (1) and (2) 75/25 (v/v), for modifier compositions (3)–(8)
Modifier composition[a]	(1) Methanol (2) 2-Propanol (3) 4/1 (v/v), methanol/ethyl acetate (4) 4/1 (v/v), methanol/dichloromethane (5) 4/1 (v/v), methanol/tetrahydrofurane (6) 1/2 (v/v), methanol/ethyl acetate (7) 1/2 (v/v), methanol/dichloromethane (8) 1/2 (v/v), methanol/tetrahydrofurane

[a] To each modifier mixture 0.1% (v/v) of both isopropylamine and trifluoroacetic acid were added.

All test solutions were prepared in a concentration of 0.5 mg/ml in methanol.

2.5. Responses

For all enantioseparations, the resolution (R_S) is calculated using peak widths at half heights. 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 defined as partial.

3. Results and discussion

3.1. Immobilized column performance with traditional modifiers

In a first stage, the immobilized CSPs were evaluated with 20% traditional modifier (MeOH and 2PrOH) in the mobile phase (modifier compositions (1) and (2) in Table 2). These modifiers were selected based on their success in earlier research with coated polysaccharide-based CSPs [24,25].

For IA, 2-propanol yields slightly more baseline and partial enantioseparations than methanol (Fig. 1). For IB, the opposite situation is seen, *i.e.* methanol yields somewhat more baseline and partial separations. For IC, methanol and 2-propanol yield a similar number of separations, considerably higher than on IA or IB.

It is important to keep in mind that these conditions are defined for screening purposes. This means that generic conditions yielding (some) enantioselectivity for a high number of compounds are sought, rather than conditions delivering high resolutions for a given individual compound. In such context, the chromatographic system providing the highest success rate (partial and baseline separated compounds) is preferred. Optimizing the obtained separation can be done in subsequent optimization steps, following the screening step. Taking this into account, no general preference for either modifier in combination with these immobilized stationary phases can be made.

Complementary enantioselectivity is obtained considering the MeOH and 2PrOH results, regardless the success rates of these two modifiers. In Table 4 the number of unique separations achieved when comparing two modifier(s) (mixtures) is shown. A separation is considered unique if it is achieved with one modifier but not with the other. For instance, the result in row 1 column 2 indicates the number of compounds separated using 2PrOH modifier (column 2) but not with the MeOH modifier (row 1). Similarly, the result in row 8 column 1 indicates the number of compounds separated with MeOH as modifier (column 1) but not with MeOH/THF, 1/2 (v/v).

Table 3
56 Racemates used in this study.

	Compound	Manufacturer		Compound	Manufacturer
1	Acebutolol	Sigma Aldrich, Steinheim, Germany	29	Meptazinol	Origin unknown
2	Acenocoumarol	Novartis, Basel, Switzerland	30	Methadone	Federa, Brussels, Belgium
3	Alprenolol	Sigma Aldrich, Steinheim, Germany	31	Metoprolol	Astra Hassle AB, Lund, Sweden
4	Ambucetamide	Janssen Pharmaceutica, Beerse, Belgium	32	Mianserine	Diosynth & Organon, Brussels, Belgium
5	Atenolol	Sigma Aldrich, Steinheim, Germany	33	Nadolol	Sigma Aldrich, Steinheim, Germany
6	Atropine	Sigma Aldrich, Steinheim, Germany	34	Naringenin	Sigma Aldrich, Steinheim, Germany
7	Betaxolol	Sigma Aldrich, Steinheim, Germany	35	Nicardipine	UCB, Brussels, Belgium
8	Bisoprolol	Origin unknown	36	Nimodipine	Bayer, Leverkusen, Germany
9	Bopindolol	Sandoz, Holskirchen, Germany	37	Nisoldipine	Bayer, Leverkusen, Germany
10	Bupranolol	Schwarz Pharma, Monheim, Germany	38	Nitrendipine	Bayer, Leverkusen, Germany
11	Carazolol	Astellas Pharma, Munchen, Germany	39	Oxazepam	Sigma Aldrich, Steinheim, Germany
12	Carbinoxamine	Origin unknown	40	Oxprenolol	Cynamid Benelux, Brussels, Belgium
13	Carvedilol	Boehringer, Mannheim, Germany	41	Pindolol	Sigma Aldrich, Steinheim, Germany
14	Chlorphenamine	Sigma Aldrich, Steinheim, Germany	42	Praziquantel	Sigma Aldrich, Steinheim, Germany
15	Chlorthalidone	Sigma Aldrich, Steinheim, Germany	43	Procyclidine	Sigma Aldrich, Steinheim, Germany
16	Dimethindene	Novartis, Basel, Switzerland	44	Promethazine	Sigma Aldrich, Steinheim, Germany
17	Ephedrine	Sigma Aldrich, Steinheim, Germany	45	Propiomazine	Origin unknown
18	Esmolol	Du Pont de Nemours, Saconnex, Switzerland	46	Propranolol	Fluka, Neu-Ulm, Switzerland
19	Fenoprofen	Sigma Aldrich, Steinheim, Germany	47	Salbutamol	Glaxo Wellcome, Genval, Belgium
20	Flurbiprofen	ICN Biomedicals, Ohio, USA	48	Salmeterol	Glaxo Wellcome, Genval, Belgium
21	Hexobarbital	Origin unknown	49	Sotalol	Merck, Darmstadt, Germany
22	Ibuprofen	Sigma Aldrich, Steinheim, Germany	50	Sulpiride	Sigma Aldrich, Steinheim, Germany
23	Isothipendyl	Origin unknown	51	Suprofen	Sigma Aldrich, Steinheim, Germany
24	Ketoprofen	Sigma Aldrich, Steinheim, Germany	52	Terbutaline	Astra-Draco, Lund, Sweden
25	Labetalol	Sigma Aldrich, Steinheim, Germany	53	Tertatolol	Servier Technology, Suresnes, France
26	Mandelic acid	Sigma Aldrich, Steinheim, Germany	54	Tetramisole	Sigma Aldrich, Steinheim, Germany
27	Mebeverine	Duphar, Amsterdam, The Netherlands	55	Verapamil	Fluka, Neu-Ulm, Switzerland
28	Mepindolol	Origin unknown	56	Warfarine	Sigma Aldrich, Steinheim, Germany

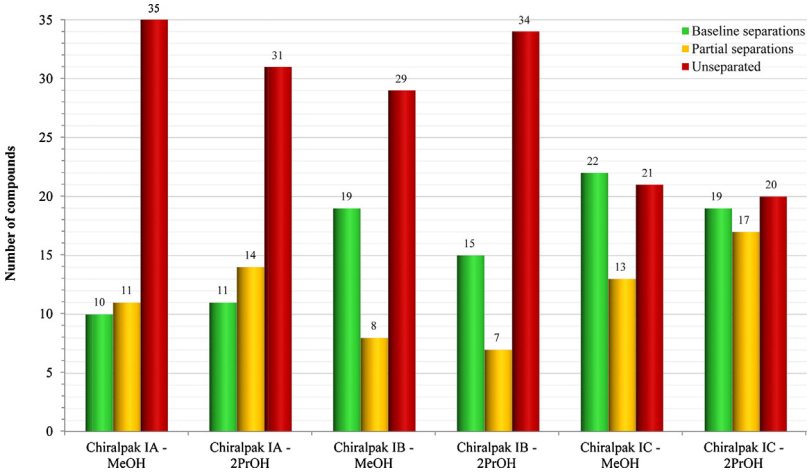


Fig. 1. Global results of the isocratic evaluation of Chiralpak IA, IB and IC with 20% MeOH/2PrOH + 0.1% IPA and TFA in the mobile phase.

Table 4
The number of compounds separated with one mobile phase but not with another on (a) Chiralpak IA, (b) Chiralpak IB and (c) Chiralpak IC. The table should be read as follows. Separations are compared for two mobile phases. The result in row 1 column 2 indicates the number of compounds only separated using 2PrOH modifier (column 2) which were not with the MeOH modifier (row 1). Similarly, the result in row 8 column 1 indicates the number of compounds only separated with MeOH as modifier (column 1) in comparison with MeOH/THF, ½ as modifier.

Systems that yield no separation	Systems that yield a separation							
	MeOH	2PrOH	MeOH/EtOAc, 4/1	MeOH/CH_2Cl_2, 4/1	MeOH/THF, 4/1	MeOH/EtOAc, 1/2	MeOH/CH_2Cl_2, 1/2	MeOH/THF, 1/2
Chiralpak IA								
MeOH	–	13	4	4	4	4	6	7
2PrOH	8	–	9	9	9	6	7	10
MeOH/EtOAc, 4/1	4	14	–	6	4	2	4	6
MeOH/CH_2Cl_2, 4/1	1	11	3	–	2	3	4	5
MeOH/THF, 4/1	1	11	1	2	–	2	3	5
MeOH/EtOAc, 1/2	2	18	9	13	12	–	6	8
MeOH/CH_2Cl_2, 1/2	10	16	8	9	10	3	–	6
MeOH/THF, 1/2	5	16	7	9	9	2	3	–
Chiralpak IB								
MeOH	–	2	5	1	3	4	7	7
2PrOH	7	–	9	8	10	9	13	13
MeOH/EtOAc, 4/1	2	1	–	2	3	4	5	6
MeOH/CH_2Cl_2, 4/1	1	2	4	–	2	4	6	6
MeOH/THF, 4/1	1	3	4	1	–	5	5	1
MeOH/EtOAc, 1/2	0	0	3	1	3	–	4	5
MeOH/CH_2Cl_2, 1/2	2	3	3	2	2	3	–	3
MeOH/THF, 1/2	0	1	2	0	1	2	1	–
Chiralpak IC								
MeOH	–	12	6	6	2	5	5	1
2PrOH	12	–	9	9	9	12	12	8
MeOH/EtOAc, 4/1	5	8	–	4	3	7	5	2
MeOH/CH_2Cl_2, 4/1	4	10	3	–	2	6	4	2
MeOH/THF, 4/1	6	13	8	8	–	8	9	1
MeOH/EtOAc, 1/2	3	11	6	6	2	–	5	1
MeOH/CH_2Cl_2, 1/2	2	9	3	2	2	4	–	1
MeOH/THF, 1/2	14	21	16	18	10	16	17	–

On IA, 8 separations are only achieved using MeOH and 13 using 2PrOH as modifier (Table 4). For IB, 7 and 2 such separations are generated by MeOH and 2PrOH, respectively; while for IC both modifiers generate 12 unique separations. The impact of the modifiers was rather unpredictable, as is generally the case in chiral separations. In some cases the number of enantioseparations was rather similar with both modifiers, in other cases it differed significantly, as demonstrated in Fig. 2.

3.2. Comparison of immobilized and coated phases

As stated earlier, a comparison of immobilized and coated polysaccharide-based stationary phases, claimed to have the same selector, should be done with caution, since possibly the higher-order structure of the selector is altered during the immobilization process. To verify differences, obtained separations on the immobilized columns are compared with those on the coated columns with the same selector (Fig. 3). Two mobile phases are evaluated, *i.e.* one with 20% methanol and one with 20% 2-propanol.

Chiralpak IA and IB tend to separate less racemates with a given mobile phase than their coated equivalents. Chiralpak IC generates a similar separation rate as Sepapak-5. A number of compounds are separated on the immobilized phases and not on the coated equivalents and *vice versa.* Hence, a certain degree of complementarity is seen; mainly for Chiralpak IA and IC. Racemates separated on both the coated and immobilized phases, tend to have a higher resolution on the coated. The different coated CSPs with the same selector also behave different in terms of obtained resolutions. AD-H generates higher R_s than Amycoat using both modifiers (MeOH and 2PrOH). LC-1 gives better separations than OD-H and Cellucoat with both modifiers.

Next, we considered retention on the coated and immobilized phases. Important to notice is that the analytes potentially interact as neutral salts. Basic molecules form a salt-pair with TFA and acidic ones with IPA. Hence, the main enantioselective or aspecific interactions will be neutral. This may result in shorter retention times compared to the single use of additives in the MP [4,27].

The retention factors of the last eluting enantiomers of a given racemate are generally slightly higher on the coated CSPs than on the immobilized equivalent (Fig. 4). The results using the mobile phase with 20% 2PrOH are similar (data not shown). These results can be correlated to those observed earlier, where lower resolutions on the immobilized CSPs were observed. The retention difference is an indication that the (enantioselective) interactions are more frequent on the coated CSPs. This observation is in line with our expectations: the structure of the chiral selector is most likely altered when it is immobilized, and possibly is the reason for a changed enantioselectivity. For Chiralpak IC and Sepapak-5 with MeOH as modifier the above trends seem less pronounced.

3.3. Use of alternative organic modifiers

One of the main benefits of the immobilized chiral stationary phases is their versatility towards organic solvents. Atypical modifiers as ethyl acetate, tetrahydrofurane and dichloromethane, that dissolve a polysaccharide coating, can be used on these phases, potentially offering a unique selectivity. In this next stage of the study, the enantioselectivity of the immobilized columns was evaluated using these three modifiers. Preliminary tests showed that up to a ratio of 70/30 (v/v), CO_2/atypical modifier, the mobile-phase strength was insufficient to elute most racemates. This is consistent with information described in the literature [6]. For this reason, we chose to use mixtures of methanol and atypical modifier, *i.e.* 4/1 and 1/2 mixtures of MeOH with EtOAc, THF or CH_2Cl_2 (Table 2).

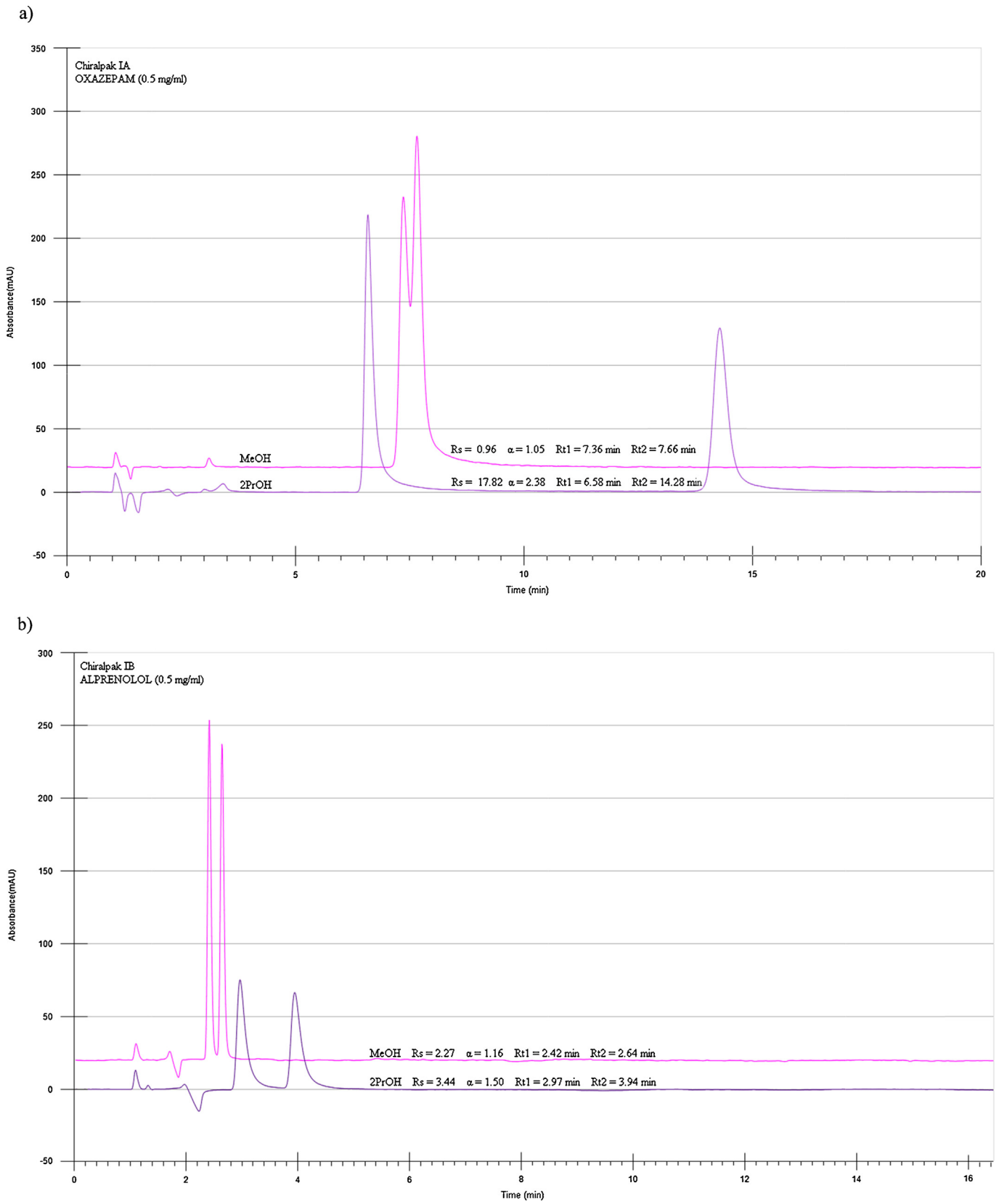


Fig. 2. Chromatograms obtained with 20% (MeOH + 0.1% IPA + 0.1% TFA) and (2PrOH + 0.1% IPA + 0.1% TFA) in the mobile phase for (a) oxazepam using Chiralpak IA and (b) alprenolol using Chiralpak IB.

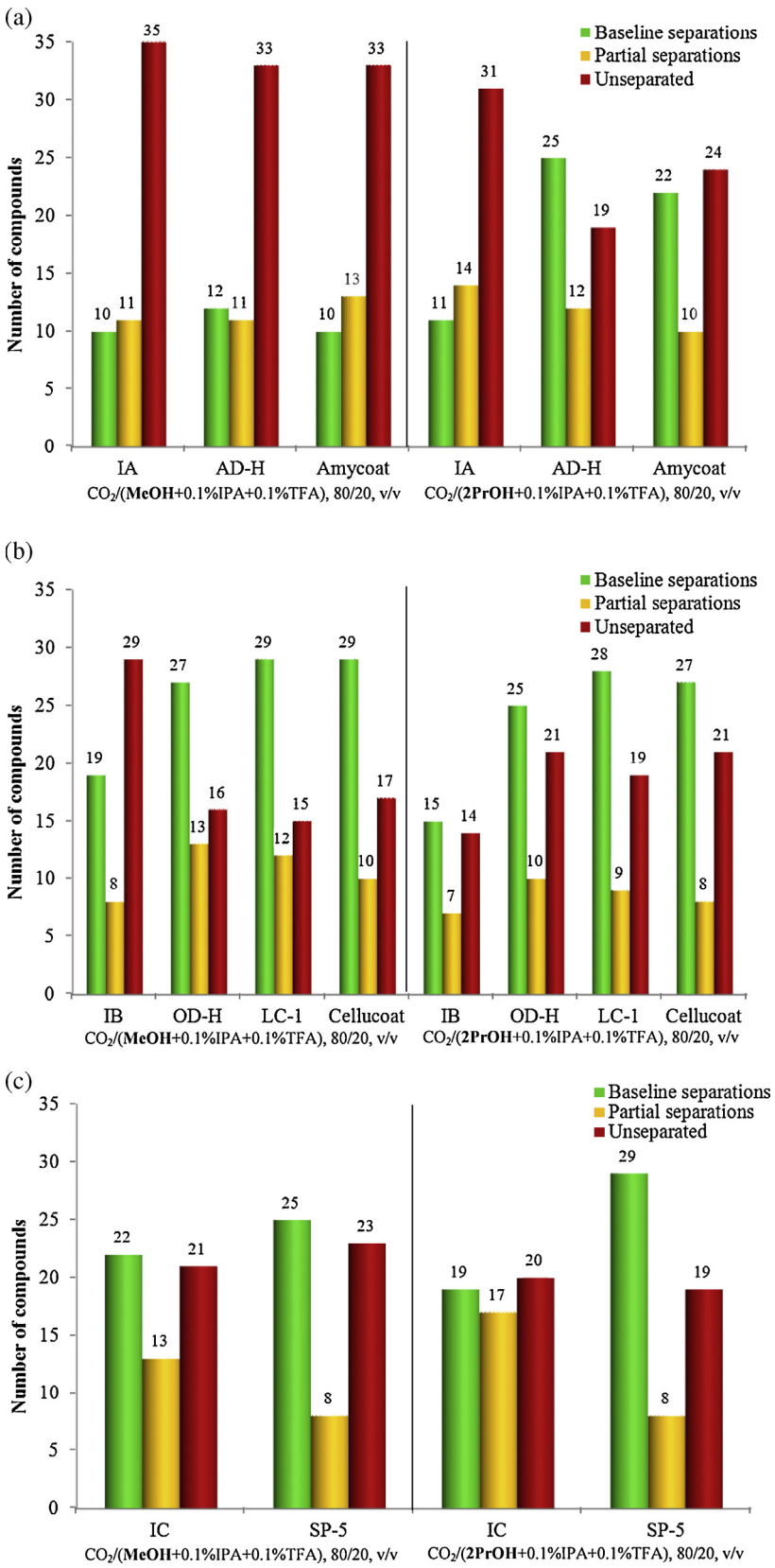


Fig. 3. Performances of the immobilized phases (a) Chiralpak IA, (b) IB, and (c) IC *vs.* coated phases in terms of the absolute number of baseline and partial separations.

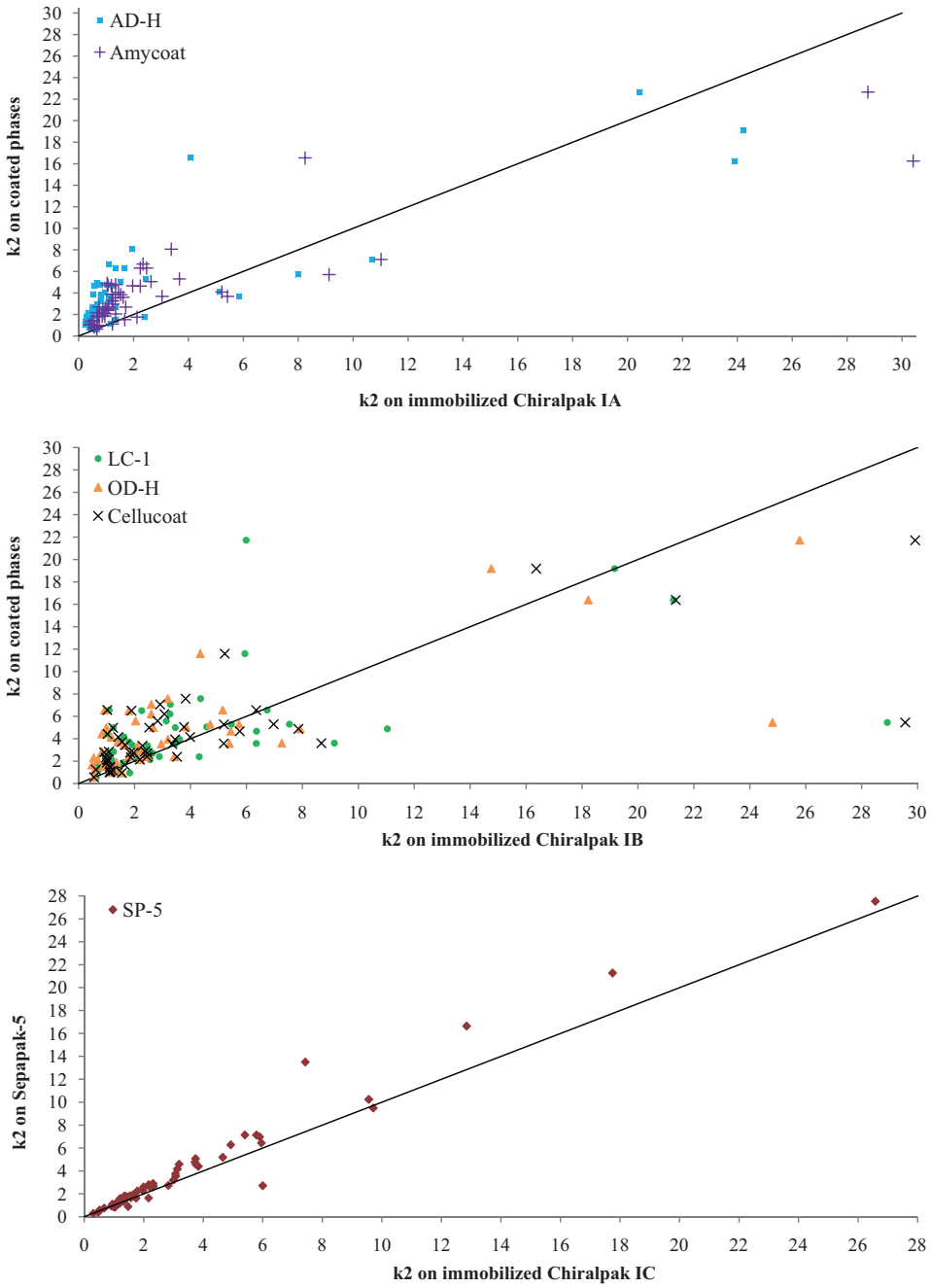


Fig. 4. Comparison of the retention factors of the last eluting enantiomer peak of the 56 compounds on the coated and the equivalent immobilized stationary phase using a mobile phase containing 20% (MeOH + 0.1% IPA + 0.1% TFA).

MeOH was used because its higher solvent strength than 2PrOH, its lower viscosity and because, in our experience, it provides slightly higher success rates on polysaccharide-based CSPs. The choice for the ratios MeOH/atypical modifier was based on literature data [6,7]. Two mixtures were evaluated: one with a higher fraction of MeOH and one with a higher fraction of atypical modifier. We also tried mixing the atypical solvents with lower fractions of MeOH (<33%), but the solvent strengths of the resulting modifier mixtures were insufficient to elute the compounds. The alternative solvents also generate a higher background UV-absorption signal, which may complicate their use at higher fractions.

The final MPs evaluated were thus composed of 75/25 (v/v), CO_2/modifiers (Table 2). This composition was achieved by mixing the solvents with the appropriate flow rates (0.75 ml/min for the modifier mixture and 2.25 ml/min for the carbon dioxide). We used a slightly higher fraction of modifier mixture than when using MeOH or 2PrOH, *i.e.* 25% instead of 20%, because the solvent strengths of the atypical modifiers are lower. Therefore, to allow elution within a similar time frame the modifier fraction was increased.

The success rates on the immobilized CSPs under these conditions were expressed in terms of baseline-, partially-, and not separated compounds (Fig. 5). The separation rates on these CSPs do not differ much when changing to atypical modifier compositions. Relatively small differences in the numbers of baseline and partial separations are seen, thus the impact on the success rate of the organic modifier type is rather modest.

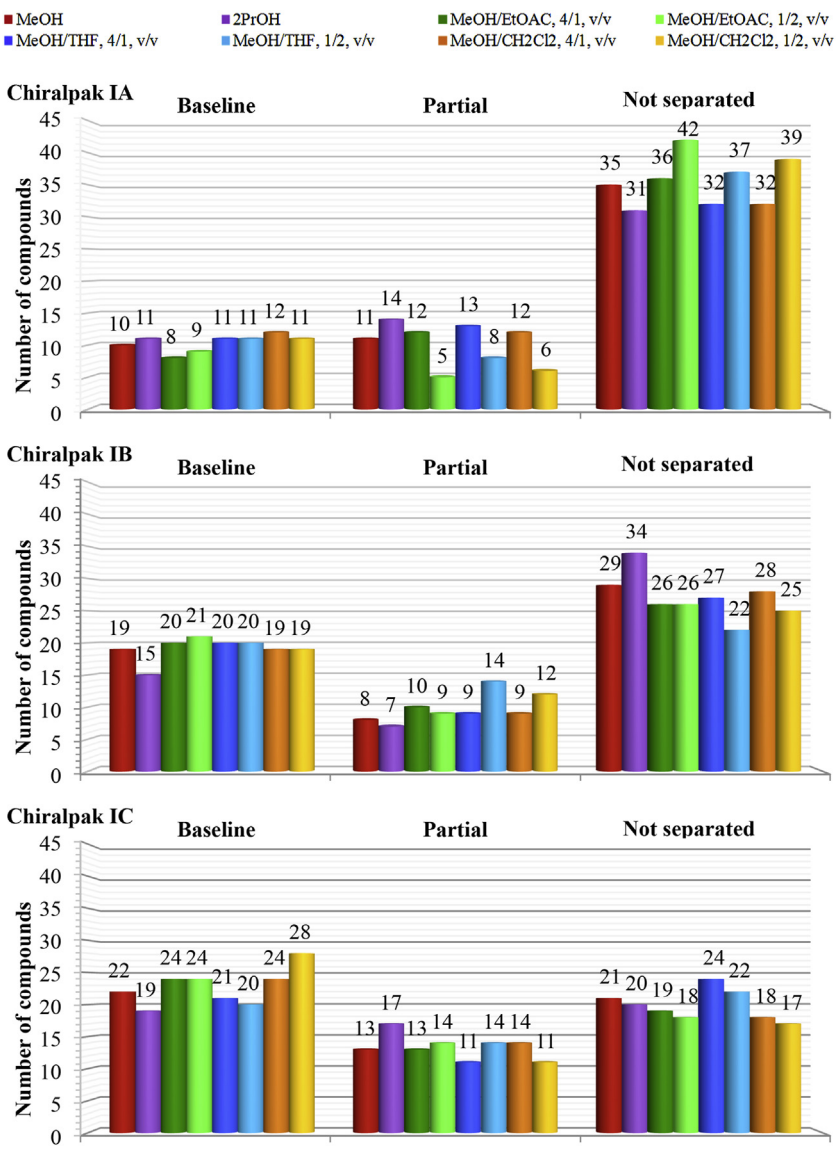


Fig. 5. Success rates on the immobilized chiral columns using different modifiers and modifier mixtures.

The most successful mobile-phase composition is different for each stationary phase. For Chiralpak IB, the mobile phases with atypical modifiers perform slightly better than those with only MeOH or 2-PrOH. For IA and IC some MPs with atypical modifiers provide more separations, but some also less. Overall, as above, Chiralpak IC provides most baseline and partial enantioseparations and thus has the highest success rate under these conditions. Conclusively, it can be stated that the separation rate of the immobilized phases remains rather similar for the different MPs. However, it is important to underline the fact that a significant amount of MeOH is present in each mixture with an atypical solvent. For this reason, the effect of methanol potentially influences the general effect of the mixture with atypical modifiers on the enantioselective behaviour. However, in the 1/2, MeOH/atypical solvent mixtures, the fractions of methanol are much lower and the modifier should be less influential. As explained earlier, it is not desirable to further decrease the methanol fraction, since the mobile phase strength would decrease too much.

Most systems on Chiralpak IA or IB, provide no resolution for more than half of the racemates. These results were linked to the retention times of the unresolved compounds on these systems (Fig. 6). On all immobilized systems (IA, IB, IC), the retention time for the vast majority of unresolved compounds situates below 5 min. This implies that the failure to separate them potentially results from a lack of proper interaction with the stationary phases. The mobile phase with 2PrOH is an exception to this trend, and generally yields longer retention times for the unresolved compounds. Hence, in this case, the lack of resolution can be appointed to low enantiorecognition ability.

To investigate the effect of the modifier type on the enantiorecognition pattern of the immobilized CSPs, a Principal Component Analysis (PCA) was performed on the separation data (R_S). An autoscaling of the data (resolution) matrix is performed to give a similar importance to each of the compounds.

The CSP is the main factor for the enantioseparation pattern (Fig. 7). Systems with the same stationary phase are grouped and separated from systems with another chiral selector. The mobile-phase composition has a smaller influence on the separation patterns. Systems 10 (2PrOH and IB) and 18 (2PrOH and IC) are exceptions to this trend. They are outlying the systems using the same chiral selector. The plot loadings reveal that the outlying position of system 10 is determined by its failure to separate meptazinol and pindolol, while all other systems using IB yield separations for these compounds. Alprenolol is only separated on IC using 2PrOH

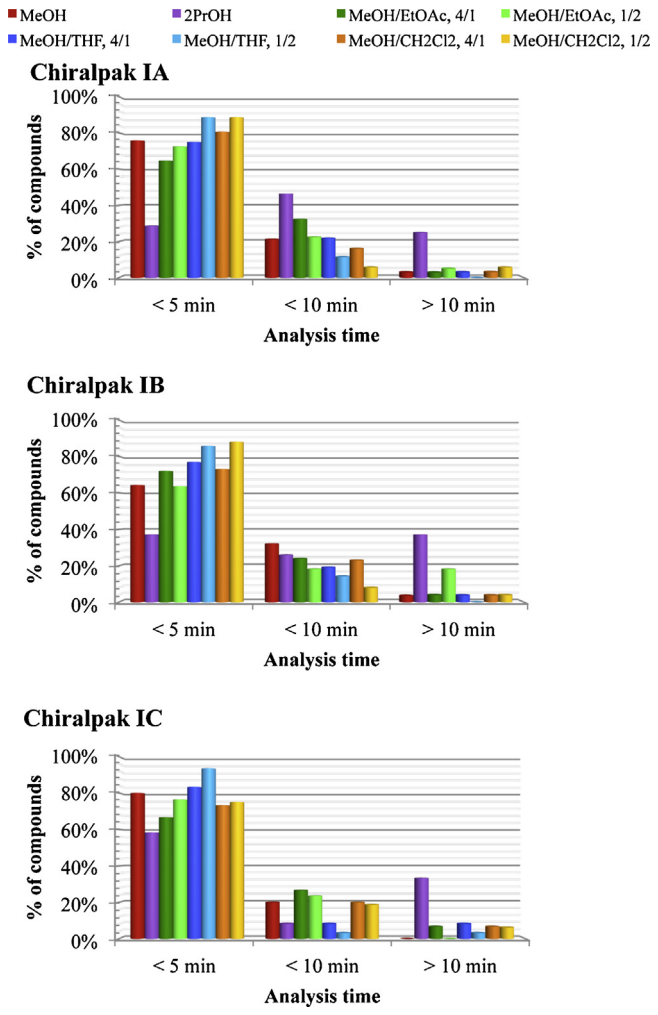


Fig. 6. Retention time of the unresolved compounds ($R_s = 0$) on the different chromatographic systems. The results are expressed as % of the unresolved compounds since their absolute number is different on each system.

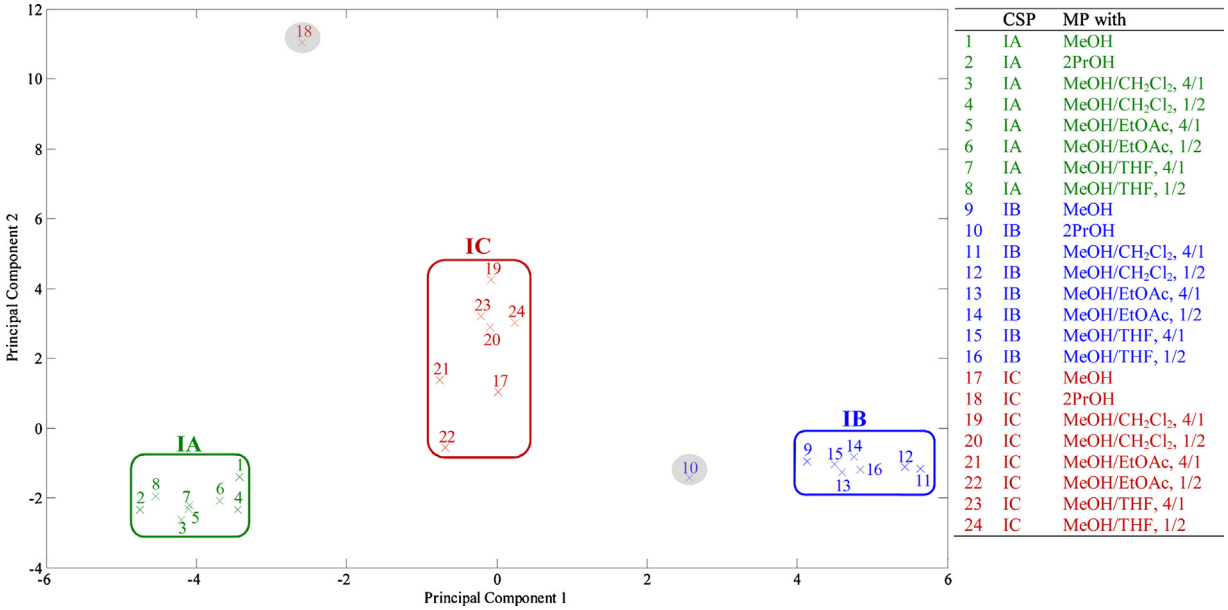
and not with the other MPs, this explains the outlying position of system 18.

The complementarity between the different mobile phases was determined (Table 4). This complementarity is reflected in the number of compounds separated with one MP but not with another. A separation is considered unique, when only one of two MPs yields a separation on a given CSP. On all immobilized CSPs, there is a large complementarity between the MP with 2PrOH and all others, *i.e.* high numbers of unique separations are seen either with the other modifiers (all CSPs). On Chiralpak IB, the complementarity between the MPs is more limited compared to the other immobilized CSPs. From these results it is clear that although similar success rates are achieved using different MPs on a given CSP, the separated compounds are not always the same.

3.4. Applicability of atypical modifiers for screening purposes

The success rate on each immobilized stationary phase is somewhat different for each MP. Chiralpak IA fails to separate more than 25/56 compounds (45%) with any MP (Fig. 5). The MP with 2PrOH is most successful and separates 25 compounds. Chiralpak IB performs better than IA and enables the separation of 34/56 racemates (61%) with a mobile phase containing MeOH/THF, 1/2 (v/v). The traditional MPs perform slightly worse than the atypical ones on this CSP. 2PrOH yields the lowest separation rate (22 compounds), followed by MeOH (27 separations). Chiralpak IC performs best and yields a separation for 39/56 (70%) compounds with the most successful modifier, *i.e.* MeOH/CH_2Cl_2, v/v, 1/2. On this CSP, the traditional and atypical modifiers yield similar success rates.

However, as mentioned earlier, the complementarity of chromatographic systems besides the enantioselectivity is also important when defining a screening approach. In such approach, one aims to achieve the broadest enantioselectivity with the smallest number of systems. Two systems that yield only a rather limited number of enantioseparations will not be considered successful. However, if these separations are unique, *i.e.* only achieved with one system, cumulatively they can cover a rather broad enantioselective range and thus display a high complementarity. In this context, cumulative success rates are determined. They express the total number of separated racemates (baseline or partially)



	CSP	MP with
1	IA	MeOH
2	IA	2PrOH
3	IA	MeOH/CH_2Cl_2, 4/1
4	IA	MeOH/CH_2Cl_2, 1/2
5	IA	MeOH/EtOAc, 4/1
6	IA	MeOH/EtOAc, 1/2
7	IA	MeOH/THF, 4/1
8	IA	MeOH/THF, 1/2
9	IB	MeOH
10	IB	2PrOH
11	IB	MeOH/CH_2Cl_2, 4/1
12	IB	MeOH/CH_2Cl_2, 1/2
13	IB	MeOH/EtOAc, 4/1
14	IB	MeOH/EtOAc, 1/2
15	IB	MeOH/THF, 4/1
16	IB	MeOH/THF, 1/2
17	IC	MeOH
18	IC	2PrOH
19	IC	MeOH/CH_2Cl_2, 4/1
20	IC	MeOH/CH_2Cl_2, 1/2
21	IC	MeOH/EtOAc, 4/1
22	IC	MeOH/EtOAc, 1/2
23	IC	MeOH/THF, 4/1
24	IC	MeOH/THF, 1/2

Fig. 7. PCA score plot of the enantioresolution patterns on the different chromatographic systems with immobilized CSPs. The chromatographic systems using the same chiral stationary phase are marked in separate boxes.

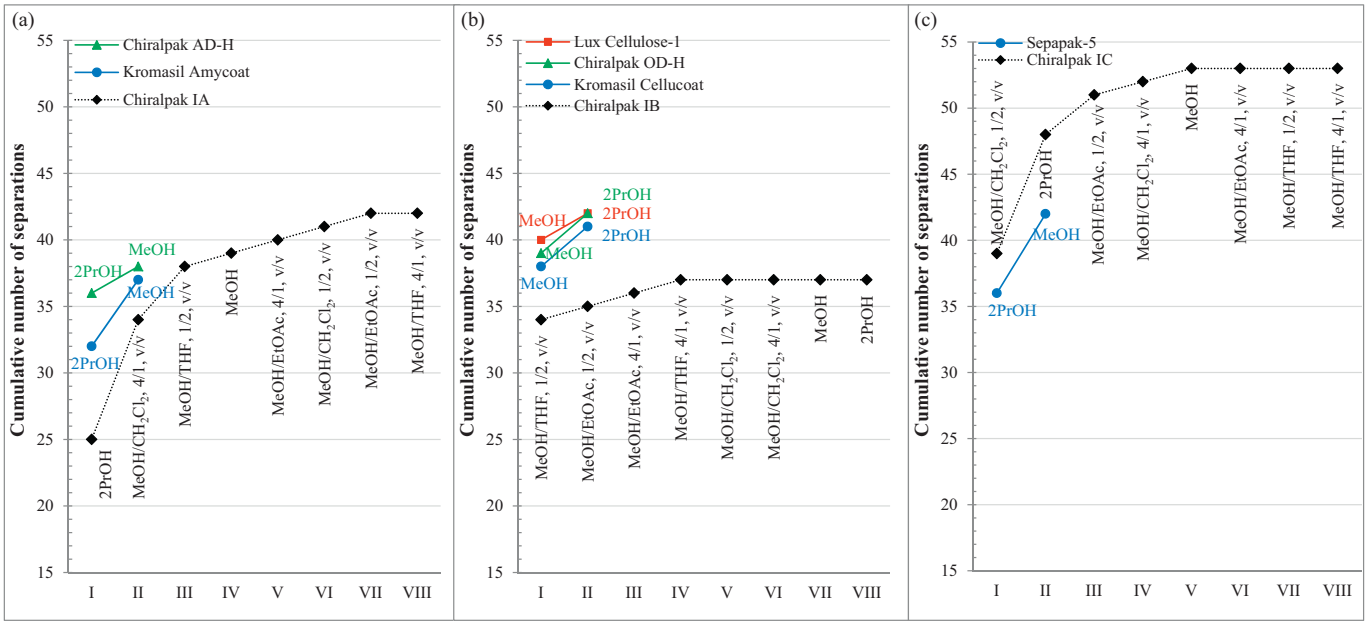


Fig. 8. Cumulative success rates achieved on the immobilized (a) Chiralpak IA, (b) Chiralpak IB and (c) Chiralpak IC, and on the equivalent coated CSPs. The modifiers used in each step are indicated.

after sequentially screening on some selected chromatographic systems. Typically, the screening starts with a system generating most enantioseparations followed by (a) system(s) generating most additional separations. For each immobilized CSP the cumulative success rate is determined and compared to that on their coated equivalents (Fig. 8).

For Chiralpak IA, the highest number of separations (25/56) is achieved using 2PrOH. Most complementary is the MP with MeOH/CH_2Cl_2, 4/1 (v/v). Nine extra separations are achieved by screening the latter MP secondly. Next, some complementarity is also found in the MP with MeOH/THF, 1/2 (v/v); which allows the separation of four extra racemates. The other mobile phase compositions only deliver a limited degree of complementarity. By screening five extra MPs, only four additional separations were gained. In total, 42/56 compounds are separable on Chiralpak IA, using the eight mobile phases of this study. To achieve this result seven mobile phase compositions have to be screened. The cumulative success rate after evaluating two MPs, is somewhat lower for Chiralpak IA (34 compounds) than for its coated equivalents, *i.e.* Chiralpak AD-H (38 compounds) and Kromasil Amycoat (37 compounds). Screening Chiralpak IA only with the conventional modifiers, 2PrOH and MeOH would yield a cumulative separation rate of 33. Hence, eight separations can be gained by screening with non-traditional modifiers.

On Chiralpak IB the mobile phase containing a mixture of MeOH/THF, 1/2 (v/v); is much more successful than the best on IA (34 *vs.* 25 separations). Nevertheless, the total number of separable compounds on Chiralpak IB is lower than on IA (37 *vs.* 42). In other words, although the success rates of some individual mobile phases on IB are higher; their complementarity is limited. After screening the first MP, three extra MPs deliver only three additional separations. The success rates of the coated equivalents of Chiralpak IB are higher even when only two MPs are used, *i.e.* 42 separations for Lux Cellulose-1 and Chiralcel OD-H and 41 for Kromasil Cellucoat. Screening Chiralpak IB only with MeOH and 2PrOH would give a cumulative separation rate of 29. Consequently eight separations can be gained on Chiralpak IB by screening with atypical solvents.

The most successful immobilized CSP is Chiralpak IC. This selector not only displays the highest separation rate for a single MP, but also achieves the highest cumulative separation rate (53/56). The mobile phase with the highest success rate (39 racemates) is MeOH/CH_2Cl_2, 1/2 (v/v). Most complementary is 2PrOH, delivering nine supplementary separations. Subsequently, five additional separations can be achieved by screening three more MPs. This immobilized CSP performs also better than its coated equivalent, Sepapak-5. Screening IC only with MeOH and 2PrOH would result in the separation of 47 racemates. Hence, the gain by screening with non-traditional modifiers (6 separations) is more somewhat limited compared to the other immobilized CSPs, which might be a consequence of the broader enantioselectivity of this phase.

Conclusively, it can be stated that the mixtures with atypical modifiers indeed can deliver a complementary enantioselectivity on the immobilized CSPs. However, for screening purposes, systems with high success rates and large complementarity should be selected, enabling to select an appropriate separation system as fast as possible. In this context, the complementarities of the different MPs on a given CSP are usually too limited. In addition the screening with atypical solvents is somewhat more complicated, since binary mixtures with methanol have to be prepared. From this point of view, it might be more practical to use traditional modifiers in a generic screening approach.

The complementary success rate of Chiralpak IA and IB is lower than those of their coated equivalents. It would thus, be more advisable to try the coated version of these chiral selectors, when using only two different MPs in a screening approach. For Chiralpak IC, the opposite situation is seen. The immobilized version of this selector is more successful and a higher complementary success rate is achieved using only two MPs.

The cumulative success rate of the screening on the immobilized CSPs is similar to that on the coated equivalents. Hence, high success rates are achieved combining the appropriate MPs with the immobilized or coated phases. Only 2PrOH and MeOH are used in the screening of coated phases. For the screening with immobilized phases, THF, EtOAc and CH_2Cl_2 are included besides the traditional modifiers. Thus, from a practical point of view, screening

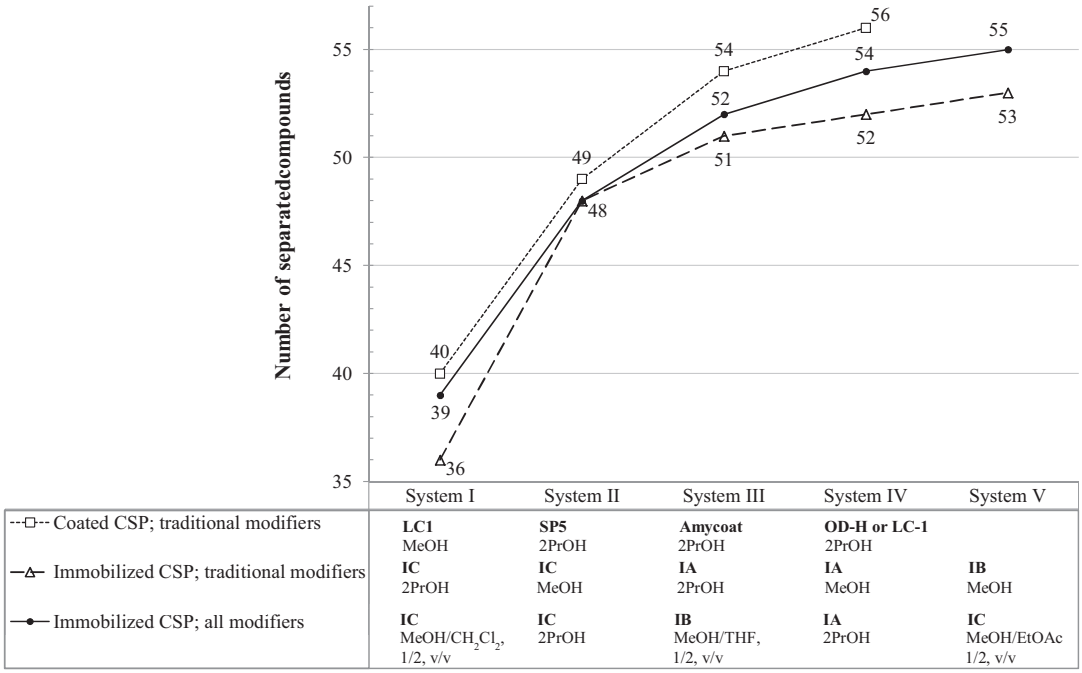


Fig. 9. Cumulative success rates achieved on either immobilized CSP or their coated equivalents.

with the coated stationary phases is simpler. Another possibility is to limit the screening on the immobilized columns to the traditional modifiers, and to reserve the atypical modifiers for cases where no separation is obtained.

However in practise, it seems more interesting (and successful) not to limit screening to one CSP with different MPs, but to screen on different systems including different selectors. For instance, when using three selectors, creating three or four systems, in general high cumulative success rates are obtained. This was already observed in [26] and also here it is seen both for the coated and immobilized CSPs. Such cumulative success rates are shown in Fig. 9, either for the immobilized or the coated CSPs. From Fig. 5 it was already derived that IA, IB, and IC show a different enantioselectivity. Chiralpak IC in combination with MeOH/CH_2Cl_2, 1/2 (v/v) is the system with the highest success rate. The respective systems 18, 16, 2 and 22 (Fig. 7) show the highest degree of complementarity to the initially selected. This is confirmed by the PCA plot (Fig. 7), in which these systems are distant from each other and the initially selected (20). The cumulative success rate reaches 98% (55/56 compounds separated) by screening five chromatographic systems (Fig. 9). The other systems do not deliver any additional separation anymore. However, this screening approach requires the use of four different mobile phases, which is less convenient. For this reason we also determined the cumulative success rate for the immobilized CSPs with only traditional mobile phases (MeOH and 2PrOH-based). This approach yields two separations less, but requires also two mobile phases less. From this point of view it might be more practical to prefer this last approach for screening. The mobile phases with atypical modifiers can then be reserved in case no separation is obtained in the screening.

3.5. Complementarity of the immobilized CSPs to the coated

In earlier research, the enantioselectivity of twelve coated polysaccharide-based stationary phases was investigated [24,25]. The four most successful and complementary systems were selected and included in a screening step that allowed separating the entire test set: (Chiralcel OZ-H/20% (MeOH + 0.1% IPA + 0.1% TFA) → Chiralpak AD-H/20% (2PrOH + 0.1% IPA + 0.1% TFA) → Chiralcel OD-H/20% (MeOH + 0.1% IPA + 0.1% TFA) → Lux Cellulose-4/20% (2PrOH + 0.1% IPA + 0.1% TFA). We verified if it would be of interest to include the immobilized CSPs in this screening step. For this purpose we evaluated all 24 systems with immobilized selectors both using traditional and atypical modifiers.

The coated CSPs generate a somewhat broader enantioselectivity with higher success rates, larger complementarity and possibly fewer systems to screen. Thus, combining coated and immobilized CSPs in a screening attempt seems unnecessary to yield the maximum number of cumulative separations. However, the cumulative success rate of a screening step with only immobilized stationary phases is similar to that of the coated ones. On the immobilized stationary phases, atypical modifiers also need to be used to achieve this highest number of cumulative separations. One compound, nitrendipine, failed to be separated on any immobilized system, while its separation can be achieved on the coated phases.

4. Conclusions

Three immobilized stationary phases, *i.e.* Chiralpak IA, IB and IC, were evaluated in SFC with two traditional modifiers, methanol and 2-propanol. Results showed that the enantioselectivity exhibited by the systems towards the test set is not very large. Chiralpak IC is the only phase able to separate more than half of the test set. Compared to the coated equivalents with the same chiral selector, their enantioresolution capability is lower. This may be because the immobilized chiral selectors have a different geometrical structure. It was also noticed that methanol and 2-propanol generated a strongly complementary separation pattern on the immobilized stationary phases.

In a next step, their performance was investigated in combination with ethyl acetate, tetrahydrofurane, or dichloromethane. For solvent strength/polarity requirements, mixtures of these modifiers with methanol were used in the mobile phase. The success rates of the latter mixtures are somewhat similar to those of MeOH and 2PrOH. However, certain unique separations can be achieved by using these atypical modifiers. In many cases they show a high degree of complementarity towards the traditional modifiers.

The complementarity of the immobilized stationary phases was investigated based on the generated data. A Principal Component Analysis was performed and showed different enantioselectivity between the immobilized phases much more than between modifiers. The immobilized CSPs, allow achieving a similar cumulative separation rate as the equivalent coated phases. In addition, they offer opportunities in case of problems with sample solubility in the mobile phase. In this context, this type of CSPs has great potential for upscaling. However, further research is required to investigate this potential in SFC.

Summarized the complementarity and enantioselectivity of the polysaccharide selectors, regardless being coated or immobilized, seem thus that, applying three of them in three or four chromatographic systems results always in high cumulative success rates. This observation we also saw in another study on polysaccharide-based selectors.

Conflict of interest

The authors declared no conflict of interest.

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