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Chiral separations in normal-phase liquid chromatography: Enantioselectivity of recently commercialized polysaccharide-based selectors. Part II. Optimization of enantioselectivity

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ABSTRACT

Earlier, a set of pharmaceuticals with different chemical structures has been used to evaluate the enantioselectivity of four recently commercialized polysaccharide-based chiral stationary phases, Lux® Cellulose-1/Sepapak® 1, Lux® Cellulose-2/Sepapak® 2, Lux® Amylose-2/Sepapak® 3 and Lux Cellulose-4/Sepapak® 4 and of three Daicel columns, Chiralpak® AD-H, Chiralcel® OD-H and Chiralcel® OJ-H, using the screening conditions of an existing generic separation strategy in normal-phase liquid chromatography (NPLC). In this study, the applicability of the optimization steps of the existing separation strategy was examined using 44 drugs (70 optimization cases) representing the three possible resolution situations that occur after screening. Optimizations are demonstrated by modifying parameters such as polar modifier percentages, temperatures, flow rates and additives concentration. Changing the percentage of polar modifier was found to have the largest influence on the resolution. The resolution, peak shape and the analysis time were nicely improved for 49/70 cases (70%) after the application of the original optimization steps. The introduction of some modifications to the original optimization increased this number from 49 to 62 cases, i.e. from 70% to 88.6%. Finally, an updated generic separation strategy in NPLC was proposed.

1. Introduction

Chiral separations play an important role in drug discovery and development since enantiomers of drug compounds may possess quite different pharmacological and toxicological properties [1,2]. Enantiomers are difficult to analyze since they have similar physical and chemical properties. They differ only in the way they rotate plane-polarized light and in their behavior in a chiral environment. Enantiomeric separation may be performed by either indirect or direct methods. Indirect methods are based on the formation of diastereomers through chemical derivatization with a "chiral" reagent. Interactions of enantiomers with a chiral selector, which can be added to the mobile phase or coated/bonded onto a chromatographic support creating a chiral stationary phase (CSP), are the basis of the direct methods. Direct methods, based on CSPs, are most frequently used in chromatographic separation techniques since they are rapid and suitable to resolve racemates on both analytical and preparative scale. Different separation techniques have been reported for chiral separations, including gas chromatography (GC) [3–5], high-performance liquid chromatography (HPLC) [6–14], supercritical fluid chromatography (SFC) [15–22], capillary electrophoresis (CE) [23–27] and capillary electrochromatography (CEC) [28–30]. The most widely used analytical method is the direct separation of enantiomers using high-performance liquid chromatography (HPLC) with chiral stationary phases (CSPs) because of its simplicity, accuracy, high reproducibility, broad UV detection range and the variety of mobile phases that can be used.

Chiral discrimination is a very complex phenomenon. Therefore, it is almost impossible to predict which CSP and mobile phase combination will provide the best separation [31]. Screening of a set of CSPs that offers a broad spectrum of enantioselectivity has been an alternative approach to the desired trial-and-error methods, in order to find the best CSP to develop enantiomeric separations in the pharmaceutical industry. The aim of such screening step is not to achieve optimal separations, but to serve as a "filter" to rapidly determine which combination of CSP and mobile phase conditions has the potential of providing acceptable resolution, and which can constitute a good starting point for further method optimization [31]. Separation strategies (which involve both screening and optimization steps) for separation of chiral molecules in normal-phase (NP), reversed-phase (RP) and polar organic solvent (POSC)

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Table 1
Classification of the test compounds according to their resolution during screening [34].

Compound	Resolution	
	Rs > 1.5	1 < Rs < 1.5
Acebutolol	**AD-EtOH**, OD-IPA, **LuxC1**, **LuxA2-EtOH**	OD-EtOH, LuxC2-EtOH
Acenocoumarol	OD, LuxC1, **LuxC2-EtOH**, **LuxC4-EtOH**	–
Alprenolol	AD, OD-EtOH, LuxC1	LuxC2-EtOH, LuxA2-EtOH, LuxC4
Ambucetamide	AD, **OD-EtOH**, LuxC1, LuxA2	–
Atenolol	**OD-EtOH**, **LuxC1-EtOH**, **LuxA2-EtOH**	–
Atropine	**AD-EtOH**, LuxC1-IPA, **LuxC2-EtOH**, LuxA2-EtOH, LuxC4-EtOH	AD-IPA
Bupranolol	AD, LuxC1	**OD-EtOH**, LuxC2-IPA, **LuxA2**
Carazolol	AD-EtOH, OD-EtOH, LuxC1, LuxC2-IPA, LuxA2, LuxC4-EtOH	AD-IPA
Carbinoxamine	AD-IPA, OD-IPA, LuxC1, LuxC2-PA, LuxA2	AD-EtOH, OD-EtOH, LuxC2-EtOH, LuxC4-IPA
Carvedilol	**LuxA2-EtOH**	–
Chlorpheniramine	AD-IPA, LuxA2-IPA	**AD-EtOH**
Chlorthalidone	–	**OD-EtOH**
Dimethindene	LuxC1	**AD-EtOH, OD, LuxC2-EtOH, LuxA2**
Ephedrine	**AD-EtOH**, OD-IPA, LuxC1, LuxA2-EtOH, LuxC4	AD-IPA, **OD-EtOH**
Fenoprofen	AD, **LuxA2**, LuxC4-IPA	OD-IPA, **LuxC1**, LuxC2,
Flurbiprofen	AD	OD-IPA, **LuxC1**, **LuxA2**
Hexobarbital	AD-IPA, LuxC1, **LuxC2-EtOH**, **LuxA2**, **LuxC4-EtOH**	**OD**
Ibuprofen	LuxC2-IPA	**LuxC1**
Isothipendyl	AD-IPA, LuxC1-IPA	**AD-EtOH**, OD-IPA, **LuxC1-EtOH**, LuxA2-IPA
Mandelic acid	AD-IPA, OD, LuxC1, LuxA2, LuxC4	**AD-EtOH**, **LuxC2**
Mebeverine	OD, LuxC1, LuxC2-IPA,	**AD**, LuxA2-EtOH
Mepindolol	LuxC2-EtOH, LuxA2-EtOH, LuxC4-EtOH	**AD**, LuxA2-IPA
Meptazinol	LuxC2-EtOH	AD-IPA, **OD**, Lux-C1
Methadone	–	AD-IPA, **LuxC2-EtOH**, LuxA2-IPA, **LuxC4-EtOH**
Nadolol	**AD**, OD-EtOH,LuxC2-EtOH	LuxA2-EtOH
Nicardipine	–	AD-IPA, OD-IPA, **LuxC1**, LuxC2-IPA0, LuxA2-IPA0, LuxC4-IPA
Nimodipine	LuxC2-EtOH, LuxA2	OD-IPA, LuxC1, LuxC4-IPA
Nisoldipine	AD, OD-EtOH, LuxC1-IPA, LuxC2-IPA	OD-IPA,LuxC1-EtOH
Nitrendipine	–	**AD**, LuxC4-IPA
Oxazepam	OD, **LuxC1-EtOH**, LuxA2-EtOH	–
Praziquantel	AD-IPA, **OD-EtOH**, LuxC1-EtOH	–
Promethazine	–	AD-IPA, OD, **LuxA2**
Propiomazine	LuxA2-EtOH	**AD**, LuxC1-IPA, LuxA2-IPA
Propranolol	AD-EtOH, **OD**, LuxC1, LuxC2, LuxC4-IPA	**LuxC4-EtOH**
Salmeterol	**LuxC2-EtOH**	AD-EtOH
Sotalol	**AD-EtOH**, **LuxC2-EtOH**, **LuxA2-EtOH**	LuxC1-EtOH
Sulpiride	–	**OD-EtOH**, **LuxC1-EtOH**
Suprofen	AD-EtOH, LuxC2-IPA, **LuxA2-EtOH**, LuxC4	**LuxC2-EtOH**
Terbutaline	**AD-EtOH**	LuxC2-EtOH, LuxC4-EtOH
Tetramisol	AD, OD, LuxC1, **LuxC2**, **LuxA2**, **LuxC4-EtOH**	–
Verapamil	AD-IPA, **LuxA2-EtOH**	AD-EtOH, LuxC2-EtOH
Warfarine	**AD**, LuxC2, LuxC4-EtOH	–
Naproxen and naringenin were not resolved by any of the tested CSPs		

Mobile phases: EtOH = heptane–ethanol–diethylamine (DEA) or triflouroacetic acid (TFA) (90:10:0.1, v/v/v) and IPA = heptane–isopropanol–DEA or TFA (90:10:0.1, v/v/v). – = not observed within the analyzed period. Columns: AD = Chiralpak AD, OD = Chiralcel OD, LuxC1 = Lux Cellulose-1, LuxC2 = Lux Cellulose-2, LuxA2 = Lux Amylose-2 and LuxC4 = Lux Cellulose-4. Conditions in bold are those tested in this study for the applicability of the optimization steps.

liquid chromatography modes [10,31,32], capillary electrophoresis (CE) [33] and capillary electrochromatography (CEC) [28,29] have earlier been developed in our laboratory.

In the first part of this study [34], four recently commercialized polysaccharide-based CSPs, Lux Cellulose-1 (cellulose tris(3,5-dimethylphenylcarbamate)), Lux Cellulose-2 (cellulose tris(3-chloro-4-methylphenylcarbamate)), Lux Amylose-2 (amylose tris(5-chloro-2-methylphenylcarbamate)) and Lux Cellulose-4 (cellulose tris(4-chloro-3-methylphenylcarbamate)), formerly known as Sepapak-1, Sepapak-2, Sepapak-3 and Sepapak-4, respectively, were investigated for their generic enantioselectivity on a set of 61 racemic compounds by applying the screening conditions of an existing chiral separation strategy in normal-phase liquid chromatography (NPLC) [10]. Three of these columns have a chlorinated polysaccharide chiral selector. A total of 54 compounds out of 61 (88.5%) were resolved by at least one of these four CSPs. The degree of separation was either partial or baseline, and often required further optimization.

In the present study, the optimization schemes proposed by Matthijs et al. [10] were examined for their applicability on the four columns, as well as on the three Daicel columns, Chiralpak AD, Chiralcel OD, and Chiralcel OJ, which were used in the existing strategy. Forty four pharmaceuticals (70 optimization situations) were selected as a test set representing the three possible resolution profiles (baseline, partial and no resolution, see further) that are obtained after screening. These compounds were subjected to analysis time optimization (27 optimization cases), peak shape optimization (5 optimization cases), resolution optimization (38 optimization cases), depending on the results achieved in the screening step.

2. Experimental

2.1. Chemicals and reagents

A test set consisting of 44 racemic compounds, either not-partially or baseline resolved at screening conditions [34] were selected for this study: acebutolol, alprenolol, atenolol, atropine, chlorthalidone, ephedrine, fenoprofen, ibuprofen, mandelic acid, naproxen, naringenin, nadolol, oxazepam, praziquantel, promethazine, sulpiride, suprofen, tetramisole, warfarin (all from Sigma–Aldrich, Steinheim, Germany), acenocoumarol, dimethin-

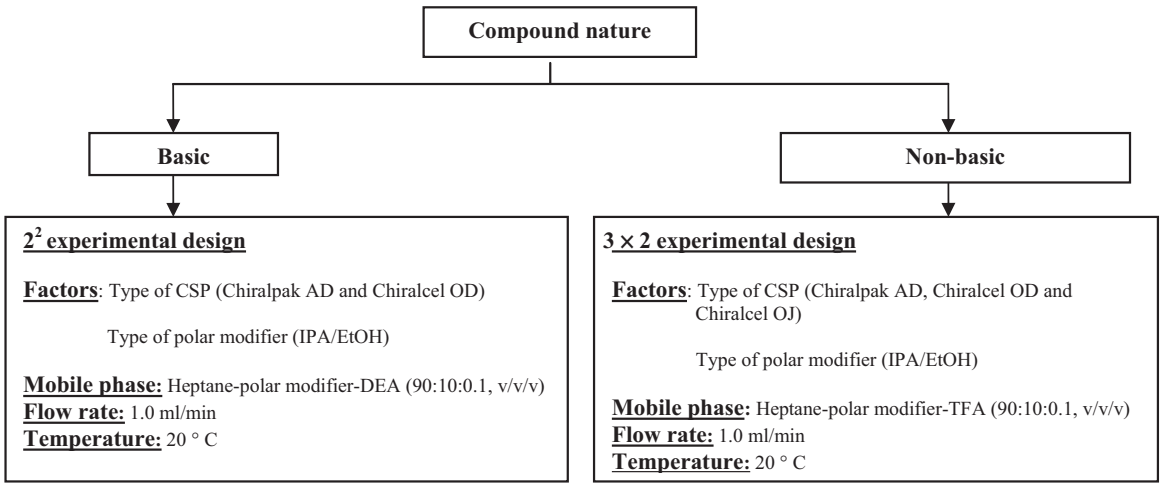

Fig. 1. General screening in NPLC, extracted from [10].

dene (from Novartis, Basel, Switzerland), metoprolol (Astra Hassle AB, Lund, Sweden), nimodipine, nisoldipine, nitrendipine (Bayer, Leverkusen, Germany), propranolol and verapamil (Fluka, Neu-Ulm, Switzerland), carvedilol (Boehringer, Mannheim, Germany), flurbiprofen (ICN Biomedicals, Ohio, USA), mebeverine (Duphar, Amsterdam, The Netherlands), nicardipine (UCB, Brussels, Belgium), sotalol (Merck, Darmstadt, Germany), terbutaline (Astra-Draco AB, Lund, Sweden), bupranolol, carazolol, salmeterol, methadone, carbinoxamine, chlorpheniramine, hexobarbital, isothipendyl, mepindolol, meptazinol and propiomazine (gifts from different origins).

HPLC grade n-heptane was purchased from BDH (Poole, UK), absolute ethanol (EtOH), methanol (MeOH) and isopropanol (IPA), HPLC grade, from Merck (Darmstadt, Germany). Diethylamine (DEA) was obtained from UCB (Brussels, Belgium) and trifluoroacetic acid (TFA) from Sigma (Steinheim, Germany).

All sample solutions have a concentration of about 0.1 mg/ml either in ethanol or isopropanol depending on the modifier used in the mobile phase.

2.2. Chromatographic conditions

The chromatographic system consisted of a HP 1050 automatic injector and a UV detector (Agilent Technologies, Palo Alto, CA), a HP 1100 quaternary pump (Agilent Technologies) and a membrane degasser. The columns were thermostatted using an electric oven (Jones chromatography model 7956, Glamorgan, U.K.).

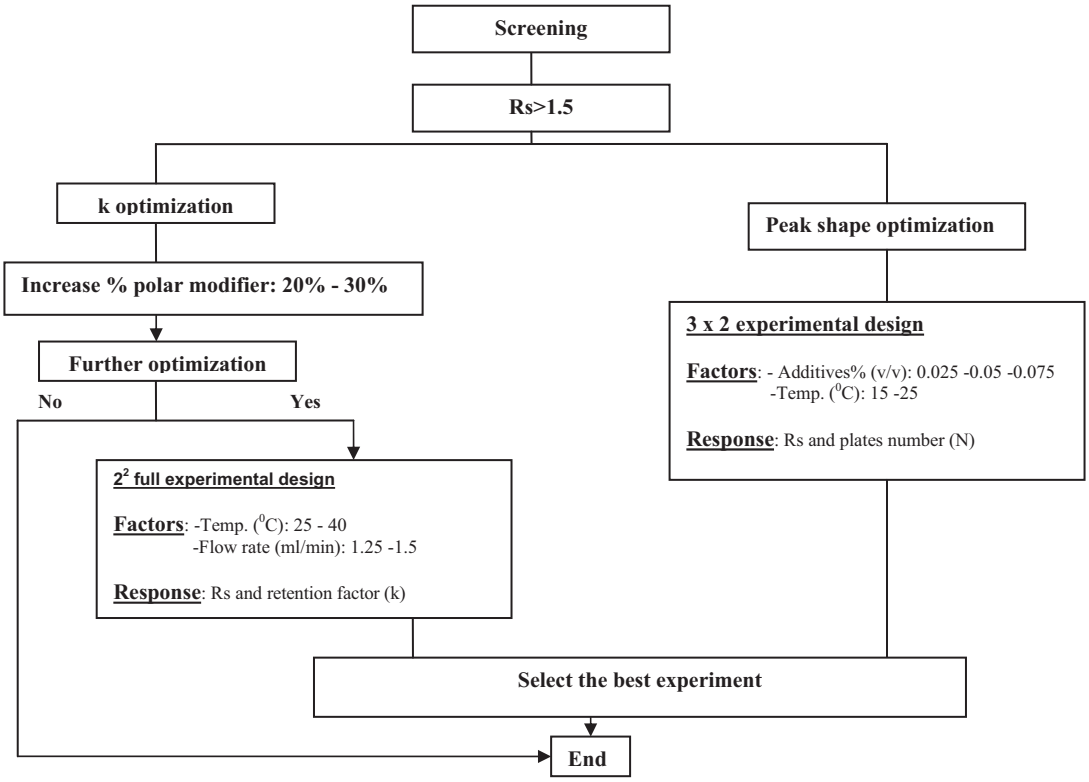

Fig. 2. Optimization 1 in NPLC, extracted from [10].

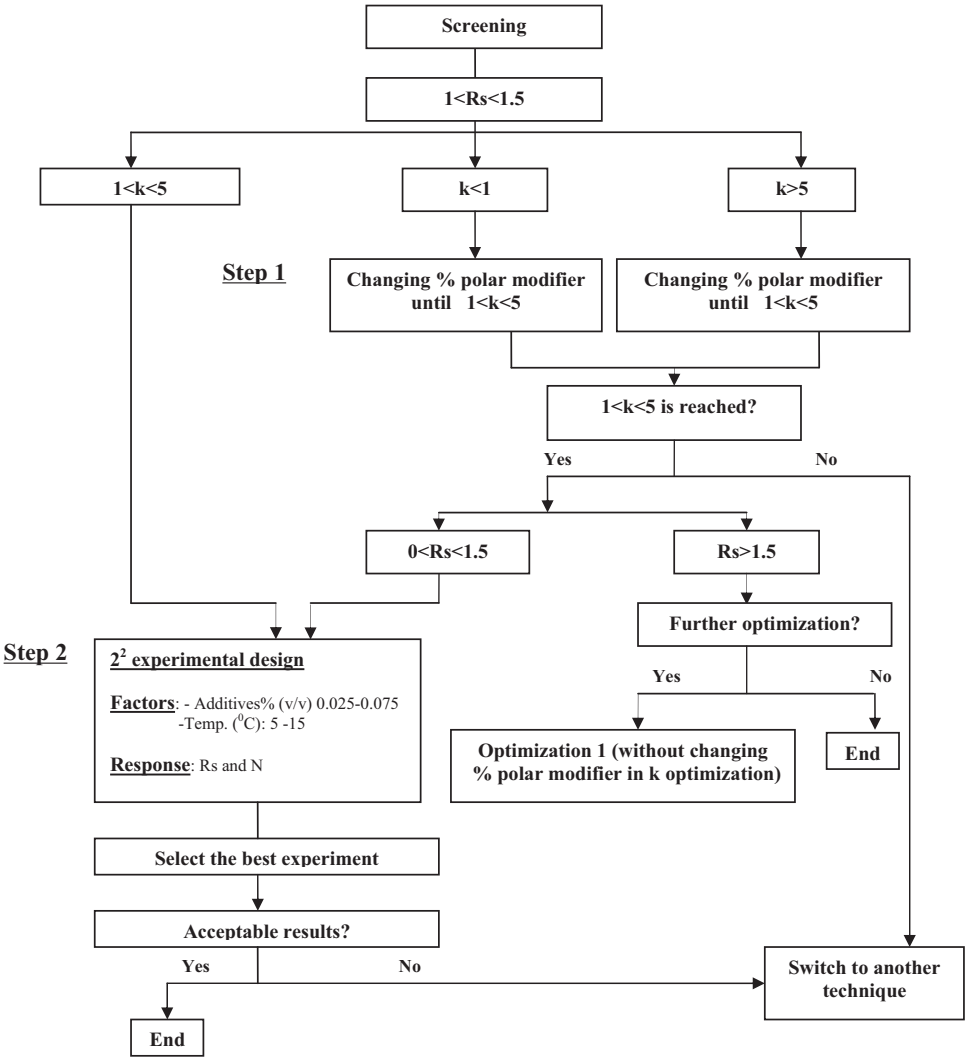

Fig. 3. Optimization 2 in NPLC, extracted from [10].

The injection volume of each sample was 5 μl. The analyses were executed at a temperature of 20 °C with a mobile phase flow rate of 1 ml/min. The detection wavelength was set at 220 nm to enable the detection of every compound. The four recently commercialized columns were from Phenomenex (Torrance, CA, USA), while Chiralpak® AD-H, Chiralcel® OD-H and Chiralcel® OJ-H were from Chiral Technologies Europe (Illkirch, France). The particle size in all columns was 5 μm and the dimensions were 250 mm × 4.6 mm.

2.3. Data processing

Analytical data were acquired and treated with the Hewlett-Packard Chemstation for LC software package (Rev.A.10.02, Agilent Technologies 1990–2003).

Resolution values (Rs) were calculated according to the United States Pharmacopeia [35]:

$$Rs = \frac{2(tr_2 - tr_1)}{(w_1 + w_2)} \quad (1)$$

where tr_1 and tr_2 are the retention times in minutes of the first and the last eluting peak of a pair, respectively, while w_1 and w_2 are the baseline widths in minutes of these peaks (determined by the tangents method).

3. Results and discussion

After evaluating the enantioselectivities of the four recently commercialized polysaccharide-based CSPs and of the three Daicel columns using the screening conditions proposed by Matthijs et al. [10] in NPLC (Fig. 1), the former four columns succeeded in the enantioseparation of 54/61 (88.5%) compounds of which 44 showed baseline resolution, whereas the Daicel columns achieved 53/61 (86.9%) separations of which 38 were baseline resolved. Once the screening step is performed in a separation strategy further optimizations can be performed starting with the most promising combination of CSP and mobile phase conditions (e.g. best resolution achieved to this point). Three resolution profiles might arise from the screening step: (a) baseline resolution (Rs > 1.5); (b) partial resolution (0 < Rs < 1.5); and (c) no resolution (Rs = 0). When baseline resolution is achieved and the results are satisfactory, method development can be ended, unless further optimization is required to meet special needs. For example: (i) when the analysis time (AT) is too long, (ii) the peak shape is not appropriate, (iii) when impurity determinations are required or (iv) better resolution than 1.5 is needed. In these cases, Optimization Scheme 1 (Fig. 2, Section 3.1.1) is proposed to achieve these requirements. In the second situation, when enantioselectivity with limited separation is observed during screening, it is recommended to perform Optimization Scheme 2 (Fig. 3, Section 3.1.2) to enhance reten-

Table 2
Retention factor optimization: changing polar modifier concentration.

Column	Substance	Screening results		20% EtOH		30% EtOH	
		Rs	*k*	Rs	*k*	Rs	*k*
Lux Cellulose-1							
	Atenolol	**4.46**	9.43	**4.34**	*3.13*	**3.63**	*1.50*
	Carazolol	**3.49**	11.27	**2.84**	*3.70*	**2.42**	*1.86*
	Oxazepam	**8.59**	11.82	**7.62**	*3.81*	**6.70**	*2.14*
Lux Cellulose-2							
	Acenocoumarol	**1.74**	18.70	1.22	*4.40*	0.99	*2.57*
	Hexobarbital	**10.42**	16.50	**9.28**	6.60	**8.53**	*5.00*
	Salmeterol	**3.80**	15.95	**2.34**	*3.00*	**1.80**	*1.50*
	Sotalol	**4.02**	22.33	**2.93**	*5.00*	**2.22**	*2.50*
	Tetramisol	**6.76**	11.50	**5.28**	*3.80*	**4.53**	*2.40*
Lux Amylose-2							
	Acebutolol	**6.25**	13.53	**4.10**	*2.65*	**3.04**	*1.31*
	Atenolol	**3.04**	24.08	1.20	*4.38*	0.92	*2.07*
	Carvedilol	**1.56**	24.27	**2.04**	*4.40*	**1.67**	*2.21*
	Fenoprofen	**9.56**	12.22	**3.78**	*1.15*	**2.76**	0.58
	Hexobarbital	**23.14**	15.25	**19.90**	*4.00*	**18.57**	*3.10*
	Sotalol	**6.62**	18.48	**3.85**	*3.47*	**2.86**	*1.70*
	Tetramisol	**7.72**	11.40	**6.24**	*3.66*	**5.27**	*2.29*
	Verapamil	**4.69**	13.23	**4.03**	*4.59*	**3.27**	*3.03*
	Suprofen	**4.09**	15.04	**3.16**	*4.61*	**2.72**	*2.78*
Lux Cellulose-4							
	Acenocoumarol	**1.68**	23.67	1.25	5.50	0.82	*2.70*
	Hexobarbital	**5.69**	16.92	**4.25**	6.60	**3.75**	*4.40*
	Tetramisol	**5.73**	17.47	**3.16**	*4.50*	**2.79**	*2.80*
Chiralpak AD							
	Acebutolol	**2.58**	11.75	**2.00**	*2.43*	**1.60**	*1.13*
	Nadolol	**4.33**	15.00	**2.83/5.4/10.6**	11.45	**2.4/4.7/6.8**	*5.00*
	Sotalol	**7.33**	18.86	**4.02**	*4.28*	**3.40**	*2.11*
	Terbutaline	**1.93**	15.98	n.p.	0.00	n.p.	*0.00*
	Warfarine	**8.20**	13.05	**7.50**	*3.47*	**6.12**	*1.89*
Chiralcel OD							
	Atenolol	**2.23**	13.36	**1.85**	*2.96*	**1.55**	*1.50*
	Praziquantel	**3.44**	10.79	**2.06**	*4.43*	**1.80**	*3.12*

Mobile phases: heptane–EtOH–DEA or TFA (variable:variable:0.1, v/v/v), flow rate 1.00 ml/min and $T = 20\,^{\circ}C$. n.p. = no peak observed after 60 min of analysis. Values in bold indicate baseline resolution. Values in italics indicate $1 < k < 5$.

tion and separation quality. Whenever no separation is observed for basic compounds in the screening step, screening Chiralcel® OJ-H or a different modifier (such as methanol) are proposed in the screening step before exploring some other separation mode. To evaluate the utility of the proposed optimization schemes to the set of new CSPs, 44 compounds representing the three resolution cases (see Table 1) were selected from the previous study. Here, it is important to note that the choice of these compounds did not take into account the final column selection that should be made for an updated version of the method development strategy. In this way, an extended set of compounds was selected, which simplifies the general conclusions about the utility of the optimiza-

Table 3
Retention factor optimization: changing temperature and flow rate.

Column	Substance	Factors		Response		Screening results	
		T (°C)	Flow rate (ml/min)	*k*	Rs	*k*	Rs
Chiralpak AD							
	Terbutaline[a]	25	1.25	n.p.	n.p.	15.98	1.93
		25	1.5	n.p.	n.p.		
		40	1.25	n.p.	n.p.		
		40	1.5	n.p.	n.p.		
Lux Amylose-2							
	Atenolol[a]	25	1.25	18.9	1.19	24.08	3.04
		25	1.5	18.8	1.07		
		40	1.25	17	1.39		
		40	**1.5**	**16.9**	**1.51**		
Lux Cellulose-4							
	Acenocoumarol[b]	25	**1.25**	**14**	**2.15**	23.67	1.68
		25	**1.5**	**13.9**	**2**		
		40	**1.25**	**12.6**	**2.6**		
		40	**1.5**	**13.7**	**2.31**		
Lux Cellulose-2							
	Acenocoumarol[b]	25	1.25	10.8	1.39	18.7	1.74
		25	1.5	10.9	1.27		
		40	**1.25**	**10**	**1.53**		
		40	1.5	10	1.4		

[a] Heptane–EtOH–DEA (90:10:0.1, v/v/v).
[b] Heptane–EtOH–TFA (90:10:0.1, v/v/v). Values in bold indicate baseline resolution.

Table 4
Peak shape optimization.

Column	Substance	Parameters		Response			Screening results		
		T (°C)	% DEA	Rs	*N*	As	Rs	*N*	As
Chiralpak AD									
	Atropine[a]	15	0.025	1.98	2114/2128	0.65/0.80	2.53	3102/1885	0.63/0.66
		15	0.05	2.38	2735/2934	0.66/0.88			
		15	0.075	2.32	2527/2841	0.7/0.7			
		25	0.025	2.45	3020/3007	0.61/0.63			
		25	0.05	2.53	3102/2977	0.61/0.65			
		25	**0.075**	**2.65**	**3508/3142**	**0.75/0.71**			
	Ephedrine[a]								
		15	0.025	1.79	1616/1761	0.42/0.50	1.86	2324/3216	0.38/0.54
		15	0.05	1.78	1723/1608	0.42/0.5			
		15	0.075	1.75	1741/1470	0.39/0.50			
		25	0.025	1.79	2382/2580	0.45/0.54			
		25	0.05	1.86	2324/3216	0.40/0.53			
		25	**0.075**	**2.08**	**2707/4351**	**0.42/0.63**			
Chiralcel OD									
	Ambucetamide	15	0.025	9.03	1953/1431	0.60/0.72	9.11	1958/1566	0.60/0.58
		15	0.05	8.97	1880/1398	0.61/0.68			
		15	0.075	8.68	1706/1317	0.55/0.68			
		25	0.025	8.87	2100/1661	0.53/0.61			
		25	**0.05**	**8.92**	**2167/1624**	**0.56/0.64**			
		25	0.075	8.97	2176/1604	0.54/0.67			
	Propranolol[a]								
		15	0.025	2.88	1642/1772	0.56/0.65	3.74	2035/2085	0.45/0.42
		15	0.05	2.9	1640/1781	0.55/0.65			
		15	0.075	2.95	1630/1811	0.57/0.66			
		25	**0.025**	**3.10**	**2990/3023**	**0.54/0.56**			
		25	0.05	3.10	2001/2071	0.51/0.56			
		25	0.075	3.14	1988/2066	0.51/0.56			
Lux Cellulose-2									
	Atropine[a]	15	0.025	6.12	4653/4584	0.80/0.72	6.2	4834/4590	0.62/0.93
		15	0.05	6.20	5957/5920	0.77/0.81			
		15	0.075	6.15	6057/6358	0.97/0.79			
		25	**0.025**	**6.55**	**6157/6258**	**0.92/0.94**			
		25	0.05	6.45	6587/5457	0.58/0.94			
		25	0.075	6.38	5815/6307	0.79/0.81			

[a] Mobile phase = heptane–EtOH–DEA (90:10:varied, v/v/v), flow rate 1.00 ml/min. The best results are indicated in bold.

Table 5
Retention factor optimization (optimization 2 step 1) for compounds that have $k < 1$ or $k > 5$.

Column	Substance	2.5% polar modifier		5% polar modifier		20% polar modifier		Screening results	
		Rs	*k*	Rs	*k*	Rs	*k*	Rs	*k*
Chiralpak AD									
	Dimethindene[a]	**1.86**	*1.20*	**1.83**	*1.07*			1.21	0.74
Chiralcel OD									
	Bupranolol[a]	**3.02**	*1.14*	**1.71**	0.62			1.17	0.55
	Carbinoxamine[a]	1.39	*1.21*	0.90	*0.79*			0.83	0.65
	Ephedrine[a]	0.96	*2.00*	**1.79**	*1.21*			1.00	0.98
	Meptazinol[a]	1.15	*2.18*	0.96	*1.09*			0.67	0.75
	Sulpiride[a]					0.00	*3.30*	0.58	11.70
	Chlorthalidone[b]					0.00	*4.80*	0.93	13.44
Lux Cellulose-1									
	Isothipendyl[a]	**1.68**	*1.00*	1.28	0.80			1.09	0.65
	Flurbiprofen[c]	1.38	*2.32*	1.27	*1.20*			1.33	0.90
	Ibuprofen[c]	1.25	*1.02*	0.96	0.82			0.83	0.49
	Sulpiride[a]					0.24	*3.64*	0.90	14.80
Lux Cellulose-2									
	Alprenolol[a]	1.43	*2.05*	1.24	*1.19*			0.87	0.65
	Methadone[a]	1.23	0.66	1.12	0.52			0.73	0.41
Lux Amylose-2									
	Alprenolol[a]	1.04	*1.53*	0.76	*0.88*			0.62	0.76
	Bupranolol[a]	**2.00**	*1.62*	1.35	*0.98*			1.44	0.80
	Promethazine[a]	**1.69**	*1.00*	1.23	0.71			1.38	0.58
	Flurbiprofen[b]	**3.05**	*2.58*	**2.21**	*1.45*			1.10	0.95
Lux Cellulose-4									
	Alprenolol[a]	**1.50**	1.50	1.13	*0.65*			0.99	0.49
	Methadone[a]	1.25	*1.01*	1.29	0.68			1.29	0.51
	Propranolol[a]	**1.55**	*2.70*	1.03	*1.27*			0.61	0.90

Mobile phases. Flow rate 1.00 ml/min and $T = 20$ °C. Values in italics indicate $1 < k < 5$. Values in bold indicate baseline resolution.

[a] Heptane-variable amount of EtOH–0.1% DEA, v/v/v.
[b] Heptane-variable amount of EtOH–0.1% TFA, v/v/v.
[c] Heptane-variable amount of IPA–0.1% TFA, v/v/v.

Table 6
Separation optimization (optimization 2 step 2) for compounds with $1 < k < 5$ after screening.

Column	Substance	Factors		Response		Screening results	
		T (°C)	% additive	Rs	N	Rs	N
Chiralpak AD							
	Chlorphenramine[a]	5	0.025	1.37	3763/5353	0.95	4293/3675
		5	0.075	**1.61**	3671/7711		
		15	0.025	0.92	2161/2377		
		15	0.075	1.22	4264/3830		
	Isothipendyl[a]	5	0.025	1.63	2527/1977	0.79	3406/1767
		5	0.075	**1.66**	2682/2134		
		15	0.025	**1.53**	3086/2334		
		15	0.075	1.49	2932/2100		
	Mebeverine[a]	5	0.025	1.38	2622/2240	1.09	3529/3288
		5	0.075	1.41	2725/2276		
		15	0.025	1.24	3051/2850		
		15	0.075	1.22	2798/2616		
	Mepindolol[c]	5	0.025	0.52	2156/1302	0.33	5653/2587
		5	0.075	0.56	2256/1402		
		15	0.025	0.57	3444/1880		
		15	0.075	0.58	2004/1509		
	Nitrendipine[a]	5	0.025	0.72	2927/1887	1.04	3454/7121
		5	0.075	0.78	3104/2343		
		15	0.025	0.8	1629/5568		
		15	0.075	1.13	3496/8657		
	Propiomazine[a]	5	0.025	0.22	3819/2633	0.75	4062/2901
		5	0.075	0.17	3222/2807		
		15	0.025	0.27	4.379/2800		
		15	0.075	0.21	3526/2625		
	Mandelic acid[b]	5	0.025	0.64	1178/893	0.93	2542/2216
		5	0.075	0.85	1641/2068		
		15	0.025	0.62	1580/765		
		15	0.075	1.07	2729/3565		
Chiralcel OD							
	Hexobarbital[b]	5	0.025	1.22	1649/1634	1.14	3153/2778
		5	0.075	**1.52**	1709/1993		
		15	0.025	1.22	1991/1976		
		15	0.075	1.36	1699/1725		
Lux Cellulose-1							
	Fenoprofen[d]						
		5	0.025	0.5	5863/3703	0.63	5430/4886
		5	0.075	0	1457		
		15	0.025	0.53	5495/4180		
		15	0.075	0	1604		
	Nicardipine[c]	5	0.025	0.92	3396/2767	0.62	6483/5418
		5	0.075	1.16	3136/2480		
		15	0.025	0.91	3811/3126		
		15	0.075	1.12	3809/2862		
	Nimodipine[c]	5	0.025	1.06	4045/3556	0.59	6032/5665
		5	0.075	1.12	3739/3757		
		15	0.025	1.03	4508/3966		
		15	0.075	1.07	3927/4194		
	Nisoldipine[c]	5	0.025	1.34	4211/3811	0.61	5146/4152
		5	0.075	1.22	3111/2946		
		15	0.025	1.16	3493/3899		
		15	0.075	1.24	3733/3694		
Lux Cellulose-2							
	Dimethindene[a]	5	0.025	1.2	6241/6095	0.64	6100/6058
		5	0.075	1.17	6170/6009		
		15	0.025	1.04	6484/6334		
		15	0.075	1.01	6391/6262		
	Mandelic acid[b]	5	0.025	0.73	2002/1640	0.83	2504/1983
		5	0.075	1.14	3916/3574		
		15	0.025	0.95	3300/2735		
		15	0.075	1.15	4241/3877		
	Suprofen[b]	5	0.025	1.43	5698/5128	1.28	5802/5881
		5	0.075	1.47	5719/5693		
		15	0.025	1.33	6321/6202		
		15	0.075	1.35	6362/6320		
Lux Amylose-2							
	Dimethindene[a]	5	0.025	0	957	0.55	9531/7807
		5	0.075	0	712		
		15	0.025	0	964		
		15	0.075	0	534		

Flow rate 1.00 ml/min. Values in bold indicate baseline resolution.
[a] Heptane–EtOH–DEA (90:10:varied, v/v/v).
[b] Heptane–EtOH–TFA (90:10:varied, v/v/v).
[c] Heptane–IPA–DEA (90:10:varied, v/v/v).
[d] Heptane–IPA–TFA (90:10:varied, v/v/v).

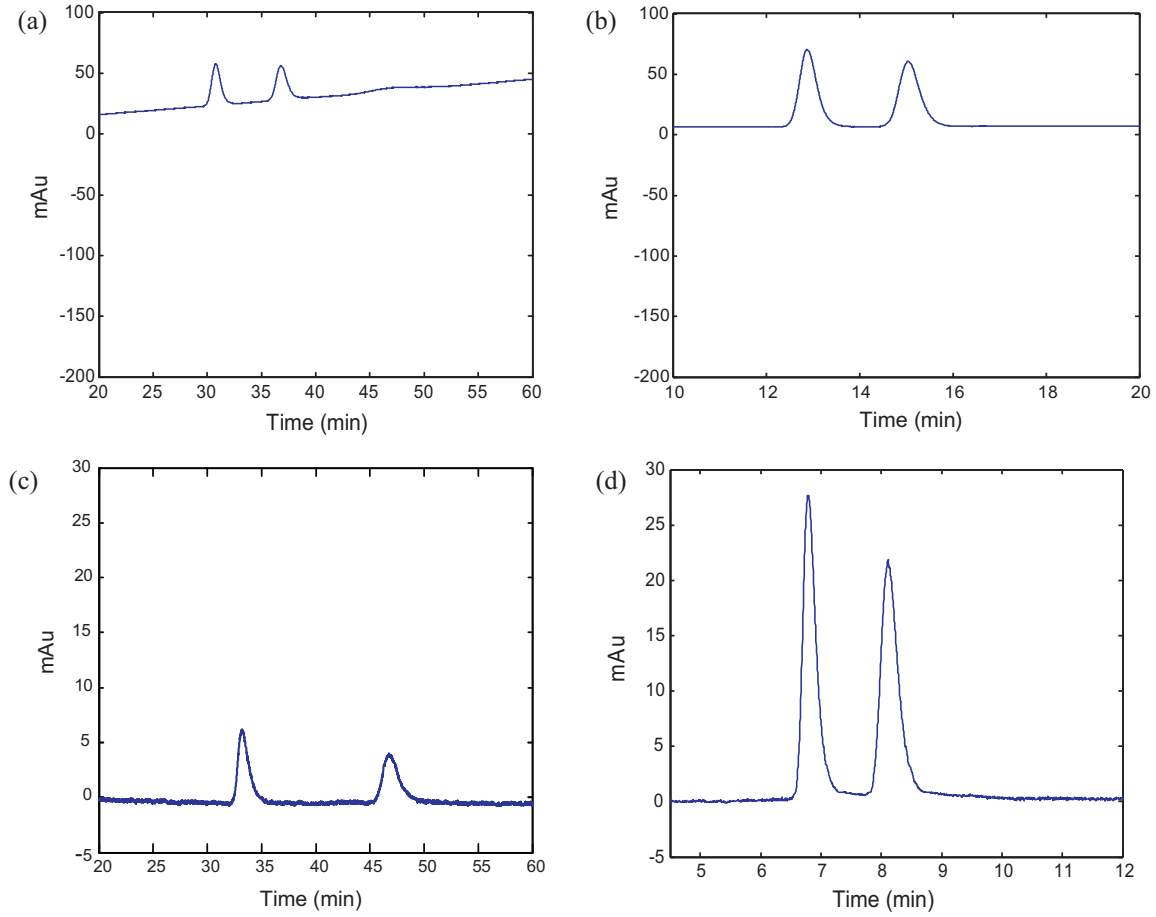

Fig. 4. Chromatograms showing the separation of carazolol (a) and (b) on Lux Cellulose-1 and of sotalol (c) and (d) on Lux Amylose-2 before and after retention factor optimization, respectively. Mobile phase was heptane–EtOH–DEA (90:10:0.1, v/v/v) for both (a) and (c) and heptane–EtOH–DEA (80:20:0.1, v/v/v) for (b) and (d). Flow rate 1.0 ml/min and column temperature 20 °C.

tion schemes. Therefore, the results on both groups of CSPs were considered.

3.1. Optimization strategy

3.1.1. Optimization 1

The aim of optimization scheme shown in Fig. 2 is to adjust analysis time (by reducing retention factor, k) and to improve peak shapes [36–40]. Twenty one chiral drugs (32 optimization cases), that were baseline resolved in one or both generic (screening) mobile phases, were subjected to this optimization.

I. *Optimization* 1, *step* 1: *Analysis time optimization*: The retention factor can be optimizing by adjusting the mobile phase strength (i.e. its modifier content) and column temperature. Each of these factors was varied at two levels, 20 or 30% modifier and 25 or 40 °C, respectively. The flow rate was also varied; it was set to either 1.25 or 1.5 ml/min.

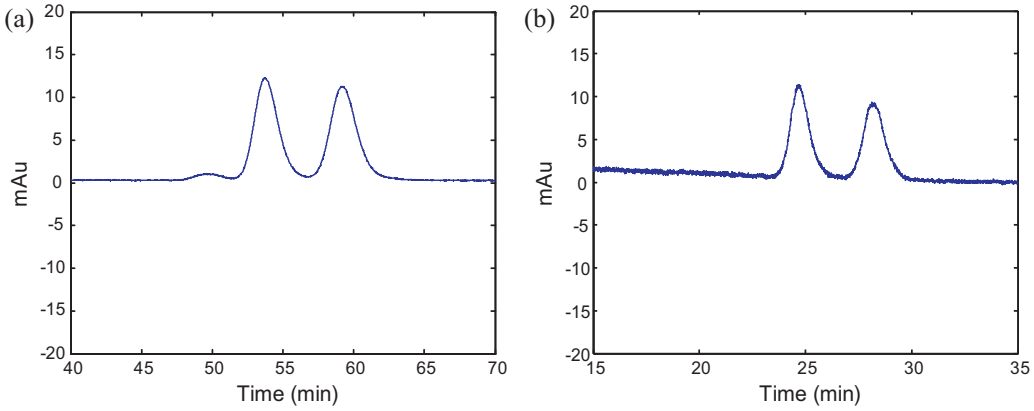

Fig. 5. Chromatograms showing the separation of acenocoumarol on Lux Cellulose-4 (a) before and (b) after optimization, respectively. Chromatographic conditions (a) heptane–EtOH–TFA (90:10:0.1, v/v/v), flow rate 1.0 ml/min and 20 °C (b) heptane–EtOH–TFA (90:10:0.1, v/v/v), flow rate 1.25 ml/min and 40 °C.

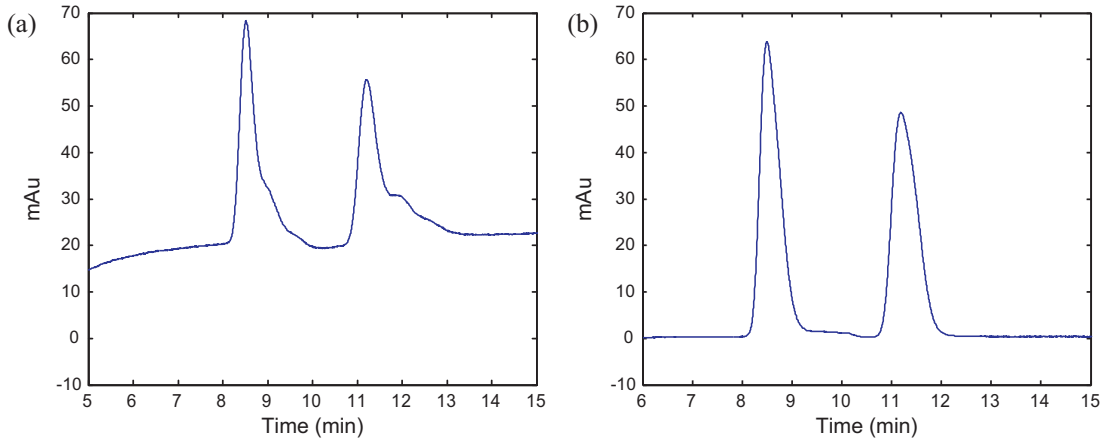

Fig. 6. Chromatograms showing the separation of propranolol on Chiralcel OD (a) before and (b) after peak shape optimization, respectively. Chromatographic conditions (a) heptane–EtOH–DEA (90:10:0.1, v/v/v), flow rate 1.0 ml/min and 20 °C (b) heptane–EtOH–DEA (90:10:0.025, v/v/v), flow rate 1.0 ml/min and 25 °C.

Table 7
Separation optimization (optimization 2 step2) for compounds with 0 < Rs < 1.5 after *k*-optimization.

Columns	Substance	Factors		Response		Screening results	
		T (°C)	% additive	Rs	*N*	Rs	*N*
Chiralcel OD							
	Carbinoxamine[a]	**5**	**0.025**	**2.71**	**3663/3566**	1.39	1309/1361
		5	0.075	2.68	3288/3212		
		15	0.025	2.52	3676/3573		
		15	0.075	2.38	3600/2966		
	Meptazinol[a]	5	0.025	2.27	2686/2303	1.15	1086/1424
		5	0.075	2.23	3251/2568		
		15	0.025	1.91	2557/2360		
		15	**0.075**	**2.29**	**3346/3258**		
Lux Cellulose-1							
	Flurbiprofen[b]	**5**	**0.025**	**1.60**	**4697/4115**		
		5	0.075	1.55	4049/3880		
		15	0.025	1.44	4928/4591		
		15	0.075	1.52	4173/4173		
	Ibuprofen[b]	**5**	**0.025**	**1.38**	**2929/2553**	1.25	3174/2573
		5	0.075	0.96	963/1780		
		15	0.025	1.37	2901/2556		
		15	0.075	0.91	759/1580		
	Sulpiride[c]	5	0.025	0.60	2843/1999		
		5	0.075	0.24	7349/4242		
		15	0.025	0.56	3038/2728		
		15	0.075	0.21	5681/3220	0.90	5414/4734
Lux Cellulose-2							
	Alprenolol[a]	**5**	**0.025**	**2.47**	**4835/5069**	1.43	4701/4923
		5	0.075	1.71	3944/4163		
		15	0.025	2.15	4659/5030		
		15	0.075	1.59	4081/4673		
	Methadone[a]	**5**	**0.025**	**1.81**	**5035/5178**	1.23	4363/4033
		5	0.075	1.59	4806/5199		
		15	0.025	1.65	4877/5174		
		15	0.075	1.41	4375/5057		
Lux Amylose-2							
	Alprenolol[a]	5	0.025	1.19	2631/2673	1.04	6210/5788
		5	0.075	1.05	2991/2865		
		15	0.025	1.08	2854/2846		
		15	0.075	1.02	2653/2675		
Lux Cellulose-4							
	Methadone[a]	5	0.025	1.98	4474/4641	1.29	5772/5832
		5	0.075	2.03	4171/3883		
		15	0.025	2.04	4707/4992		
		15	**0.075**	**2.09**	**3704/5378**		

Flow rate = 1.00 ml/min. Best results are indicated in bold.
[a] Heptane–EtOH–DEA (97.5:2.5:varied, v/v/v).
[b] Heptane–IPA–TFA (97.5:2.5:varied, v/v/v).
[c] Heptane–EtOH–DEA (80:20:varied, v/v/v).

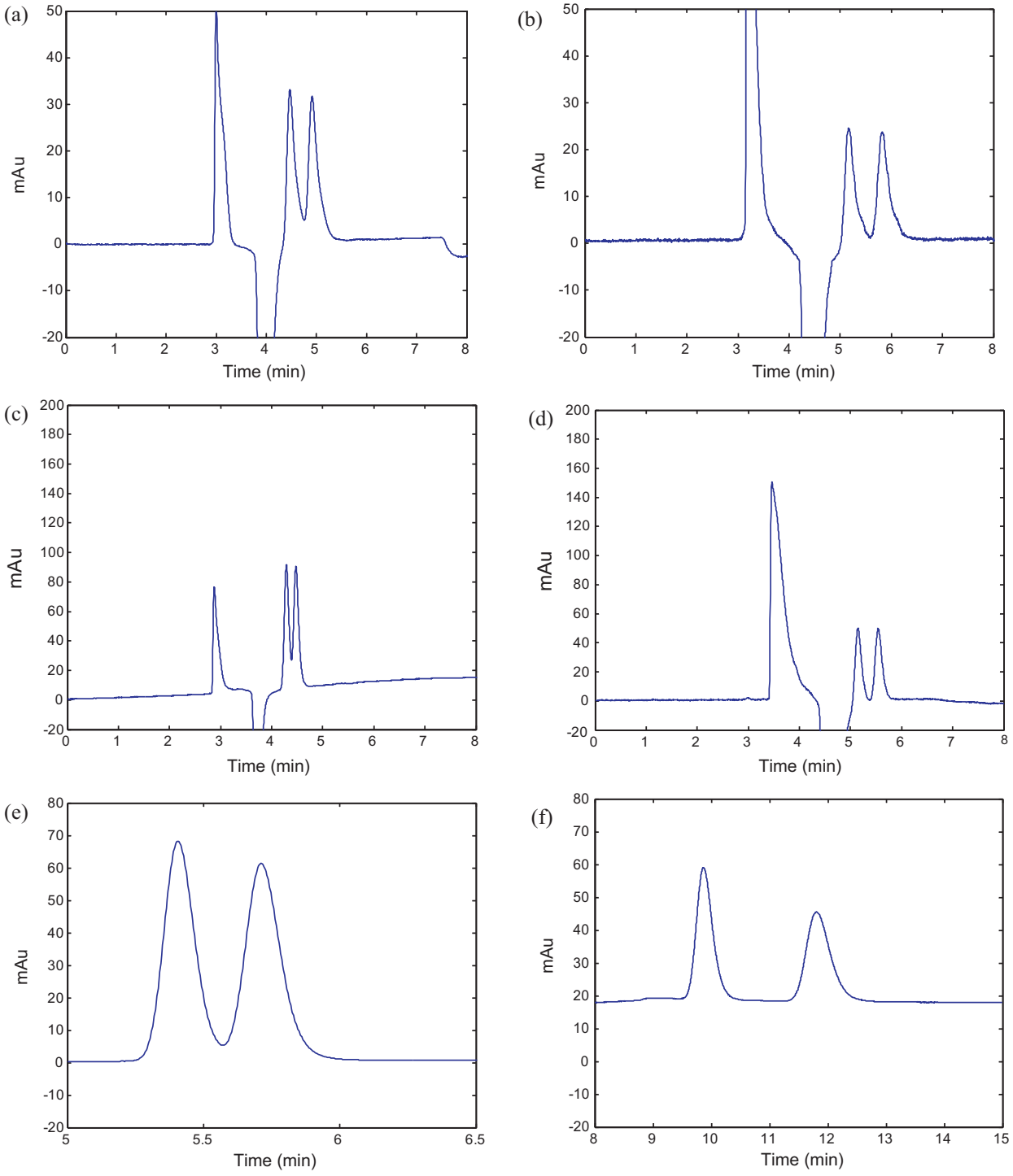

Fig. 7. Chromatograms showing the separation of dimethindene (a) and (b) on Chiralpak AD, isothipendyl (c) and (d) on Lux Cellulose-1 and flurbiprofen (e) and (f) on Lux Amylose-2 before and after *k*-optimization (optimization 2 step 1), respectively. Chromatographic conditions: heptane–EtOH–DEA (90:10:0.1, v/v/v) for (a) and (c), heptane–EtOH–TFA (90:10:0.1, v/v/v) for (e), heptane–EtOH–DEA (97.5:2.5:0.1, v/v/v) for (b) and (d) and heptane–EtOH–TFA (97.5:2.5:0.1, v/v/v) for (f). Flow rate 1.0 ml/min and 20 °C.

Retention factor optimization starts with adjusting modifier content of the mobile phase before any changes in the column temperature are explored. In this step, the eluting strength of the mobile phase is increased compared to the generic conditions applied in the screening step. Next, by increasing column temperature and mobile phase flow rate, the analysis time can be further reduced [41,42]. The considered responses are Rs and retention factor (k).

Twenty seven out of the 32 selected optimization cases could use analysis time optimization and were subjected to Optimization 1, step 1. As mentioned above, in step 1 the modifier content was raised to either 20 or 30%. Retention factors were successfully reduced to $1 < k < 5$ in 23/27 cases (85%); see Table 2 and Fig. 4. Moreover, improved resolution and additional peaks were observed for nadolol. Compounds that could not be optimized by adjusting k (4 cases: acenocoumarol on both Lux

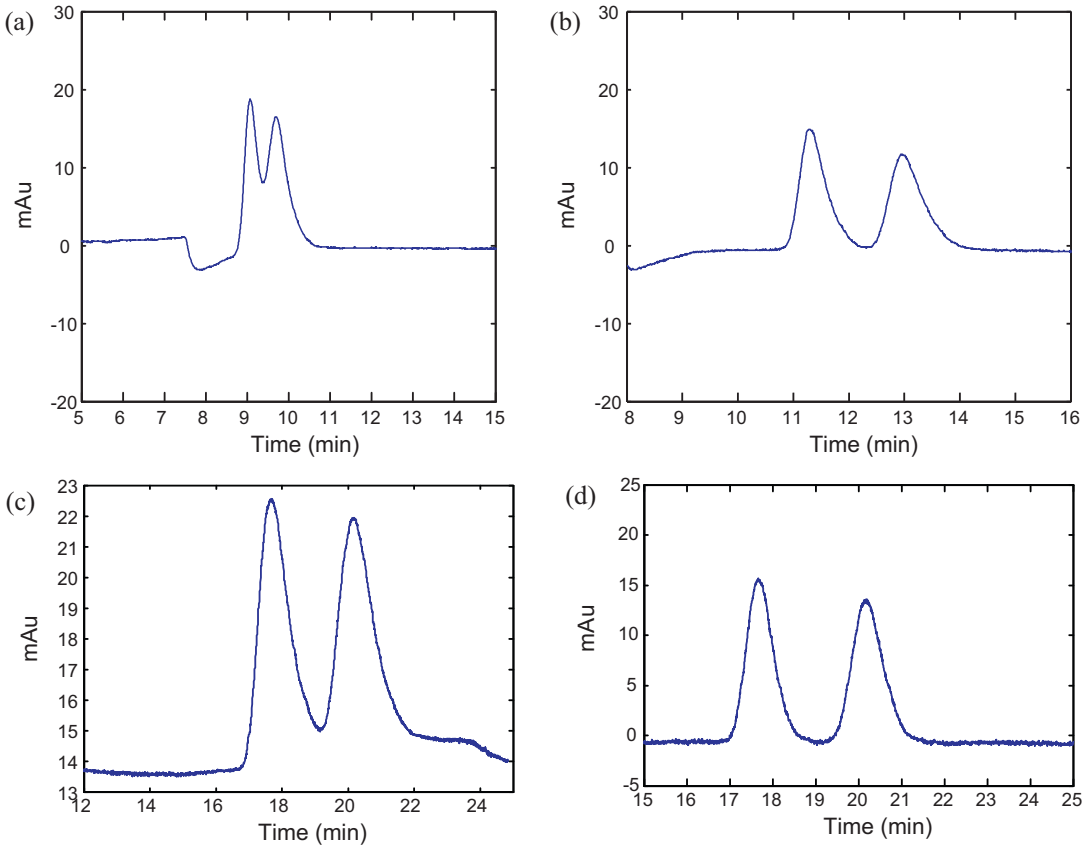

Fig. 8. Chromatograms showing the separation of isothipendyl (a) and (b) on Chiralpak AD and hexobarbital (c) and (d) on Chiralcel OD before and after optimization 2 step 2, respectively. Chromatographic conditions: heptane–EtOH–DEA (90:10:0.1, v/v/v) and 20 °C for (a), heptane–EtOH–TFA (90:10:0.1, v/v/v) and 20 °C for (c), heptane–EtOH–DEA (90:10:0.075, v/v/v) and 5 °C for (b) and heptane–EtOH–TFA (90:10:0.075, v/v/v) and 5 °C for (d). Flow rate 1.0 ml/min.

Cellulose-2 and Lux Cellulose-4, atenolol on Lux Amylose-2, and terbutaline on Chiralpak AD) were further subjected to column temperature and flow rate optimization, using a 2^2 full factorial design. These four experiments were performed using mobile phase conditions giving the best resolution, namely 10% ethanol in heptane. The results obtained showed an improvement in the retention factor of acenocoumarol on Lux Cellulose-2 and Lux Cellulose-4 and of atenolol on Lux Amylose-2 (Table 3 and Fig. 5). At the same time, the baseline resolution was preserved. Terbutaline, baseline resolved in the screening stage, is the only compound that lost resolution during Optimization 1, step 1.

Table 8
Results obtained after alternative step 2.

Column	Substance	Factors		Response	
		% polar modifier	T (°C)	Rs	Rs at screening
Chiralpak AD					
	Chlorpheniramine	2.5% EtOH	5	**2.07**	0.95
	Isothipendyl	2.5% EtOH	15	**3.31**	0.79
	Mandelic acid	5% EtOH	15	**1.85**	0.93
	Mebeverine	2.5% EtOH	5	**2.58**	1.09
	Nitrendipine	5% IPA	5	**1.57**	1.04
	Propiomazine	2.5% EtOH	5	**1.89**	0.75
	Mepindolol	5% IPA	20	**1.24**	0.33
Chiralcel OD					
	Hexobarbital	2.5%EtOH	20	**2.21**	0.63
Lux Cellulose-1					
	Fenoprofen	2.5% IPA	5	**1.64**	0.63
	Nicardipine	5% IPA	15	**2.52**	0.62
	Nimodipine	5% IPA	15	**1.52**	0.59
	Nisoldipine	5% IPA	15	**2.18**	0.61
Lux Cellulose-2					
	Dimethindene	5% EtOH	20	**1.65**	0.64
	Mandelic acid	2.5% EtOH	20	**1.76**	0.83
	Suprofen	2.5% EtOH	20	**1.74**	1.28
Lux Amylose-2					
	Dimethindene	2.5% EtOH	20	1.19	0.55

Heptane percentage and temperature vary, additive% = 0.1% and flow rate 1.00 ml/min. Values in bold indicate baseline resolution.

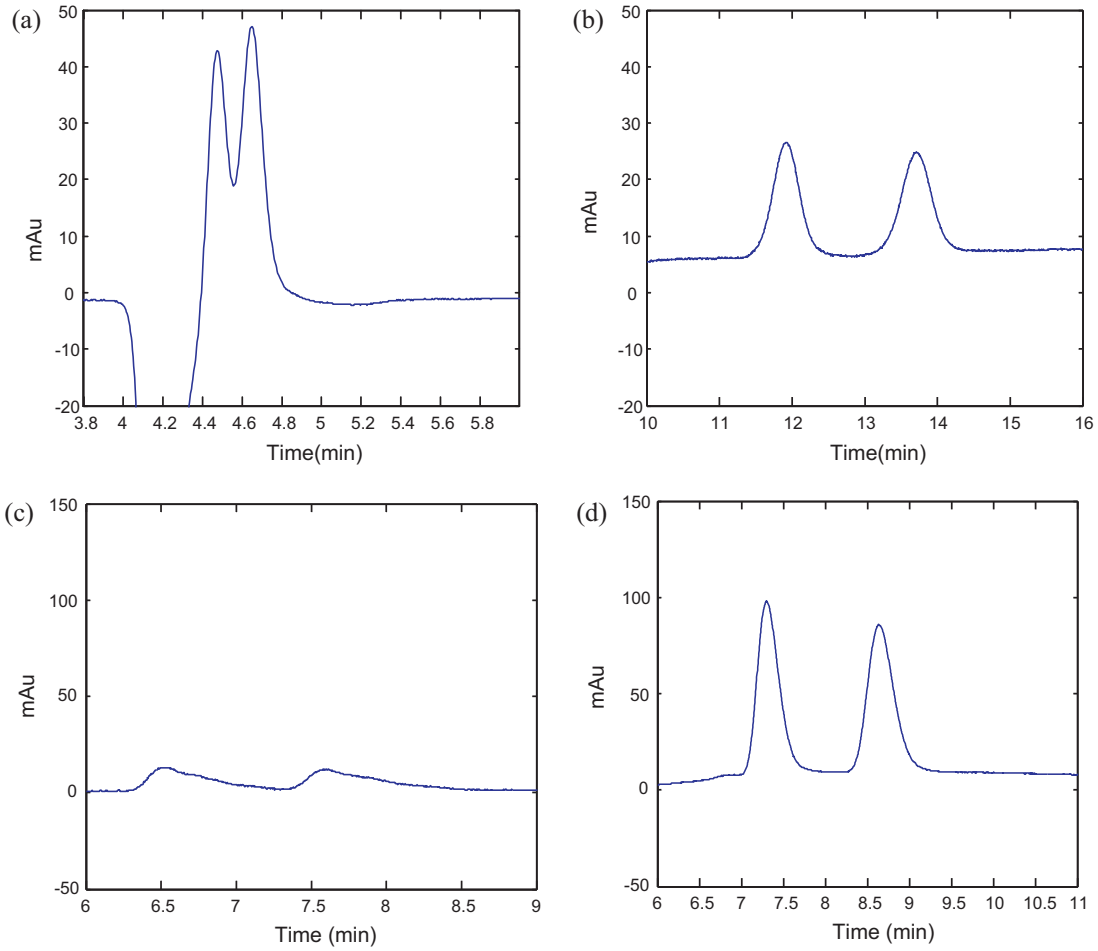

Fig. 9. Chromatograms showing the separation of alprenolol (a) and (b) on Lux Cellulose-2 and carbinoxamine (c) and (d) on Chiralcel OD before and after separation optimization (optimization 2 step 2), respectively. Chromatographic conditions: heptane–EtOH–DEA (97.5:2.5:0.1, v/v/v) and 20 °C for (a) and (c) and heptane–EtOH–DEA (97.5:2.5:0.025, v/v/v) and 5 °C for (b) and (d). Flow rate 1.0 ml/min.

In summary, the proposed retention factor optimization step showed high applicability on both Daicel and Lux columns. Retention factor, and as a consequence the analysis time, was reduced for 26/27 optimization cases (96%), while baseline resolution was preserved.

II. ***Optimization*** 1, ***step*** 2: ***Peak shape optimization***: The addition of mobile phase additives, such as DEA or TFA, to the mobile phase is necessary for the separation of basic or acidic enantiomers. They increase the possibility of analyte interaction with the polysaccharide-based CSP by suppressing the degree of ionization of enantiomers. In addition to increased interaction, additives also improve peak shapes by competing with analytes for adsorption sites [36,40]. Temperature variations can also change the ionization degree of the compound and influence the retention mechanism [42,43]. For peak shape optimization, six experiments are proposed in the original strategy where the additive concentration is examined at three levels (0.025, 0.05 and 0.075%) at two different temperatures (15 and 25 °C) (Fig. 2). Higher concentrations of additives can indeed have a positive effect on the peak shape, but too high concentrations give experimental problems related with stability of the baseline and too high UV absorption [10]

Peak shape optimization was explored for four baseline resolved compounds representing five optimization cases (atropine and ephedrine on Chiralpak AD, metoprolol and propranolol on Chiralcel OD and atropine on Lux Cellulose-2). Plates counts (N) and/or peak shape were improved while preserving baseline resolution (Table 4 and Fig. 6). Atropine and ephedrine on Chiralpak AD had better peak shape and peak symmetry compared to the initial values observed in the screening step without losing their baseline resolution and similar improvements have been observed for compounds examined on both Chiralcel OD and Lux Cellulose-2. These results show the applicability of this optimization step on both new and earlier applied CSPs.

3.1.2. Optimization 2

Optimization 2 (see Fig. 3) is proposed for cases where enantioselectivity was observed, but resolution was insufficient. In the existing strategy, Optimization 2 includes different pathways depending on the value of the retention factor from the best result observed in the screening step (i.e. with highest resolution). When k is below 1 or above 5, optimization starts with adjusting the modifier content (2.5, 5 or 20%) until a k between 1 and 5 is reached. The next step depends on the obtained resolution. (a) If it is higher than 1.5 (baseline resolution) and further optimization (e.g. peak shape) is still required, Optimization 1 is again proposed but without changing the percentage of modifier. (b) If Rs is still below 1.5, an optimization of the additive concentration and the temperature is proposed in four experiments. Both temperature and additive concentration are examined at two levels (Fig. 3). The utility of Optimization 2 on both sets of CSPs was evaluated with 26 drugs (36 optimization cases) displaying a resolution between 0 and 1.5 in the screening step. Compounds were divided into two groups based on the k value obtained during screening. Compounds

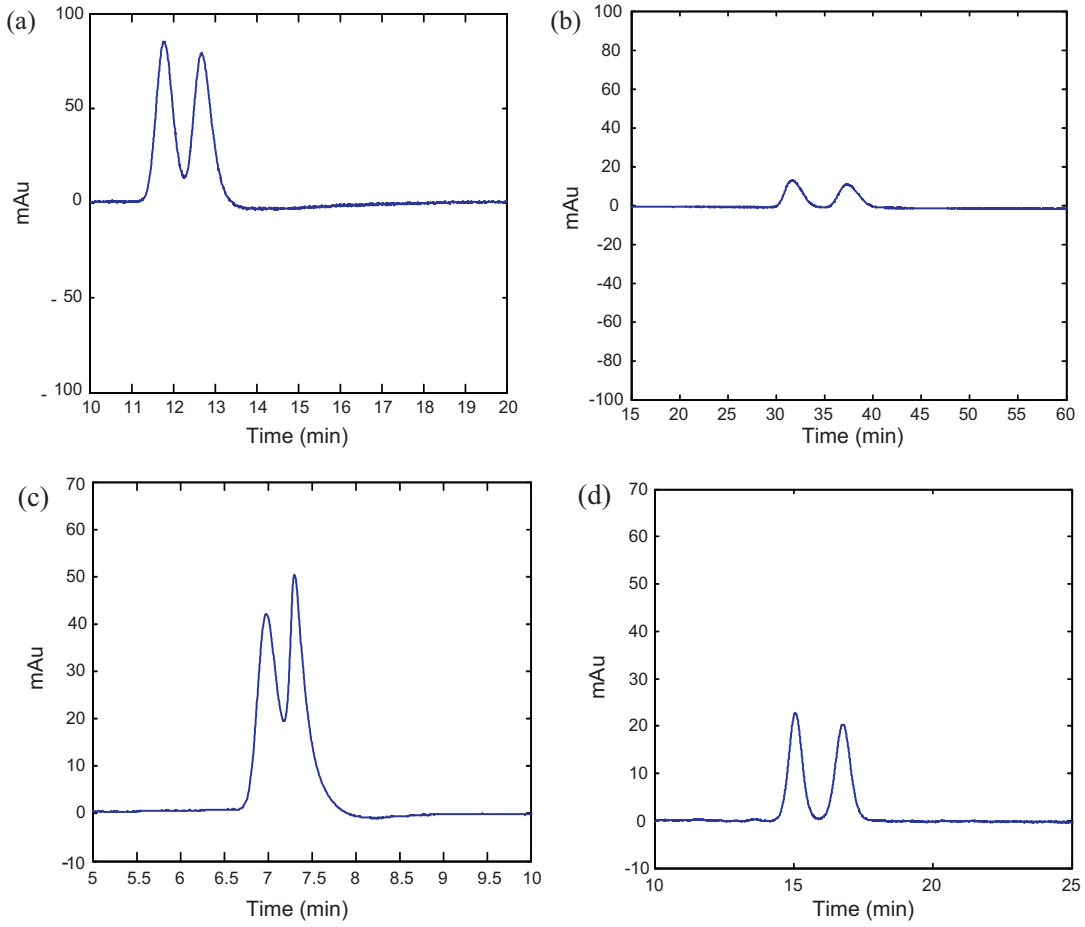

Fig. 10. Chromatograms showing the separation of nimodipine (a) and (b) on Lux Cellulose-1 and nitrendipine (c) and (d) on Chiralpak AD before and after the alternative step 2, respectively. Chromatographic conditions: heptane–IPA–DEA (90:10:0.1, v/v/v) and 20 °C for (a) and (c) and heptane–IPA–DEA (95:5:0.1, v/v/v) and 5 °C for (b) and (d). Flow rate 1.0 ml/min.

with $k < 1$ or $k > 5$ represent one group and those with $1 < k < 5$ the other.

I. ***Optimization*** 2 ***step*** 1: ***Resolution optimization by adjusting retention factor***: This optimization step involves k-optimization for 20 cases that had $k < 1$ or $k > 5$ in the screening step. For these 20 cases, the mobile phase content was adjusted to achieve $1 < k < 5$. Desirable k and baseline resolution were achieved in nine cases (dimethindene on Chiralpak AD, bupranolol and ephedrine on Chiralcel OD, isothipendyl on Lux Cellulose-1, bupranolol, promethazine and flurbiprofen on Lux Amylose-2, and alprenolol and propranolol on Lux Cellulose-4) (Table 5 and Fig. 7). These compounds may be subjected to peak shape or analysis time optimization, if needed, by applying Optimization 1, without changing the modifier content.

In another nine cases (alprenolol and methadone on Lux Cellulose-2, flurbiprofen, ibuprofen and sulpiride on Lux Cellulose-1, alprenolol on Lux Amylose-2, methadone on Lux Cellulose-4, and carbinoxamine and meptazinol on Chiralpak OD), desirable k values were achieved without significant improvement in resolution. Optimization of these compounds will be discussed in the section Optimization 2, step 2.

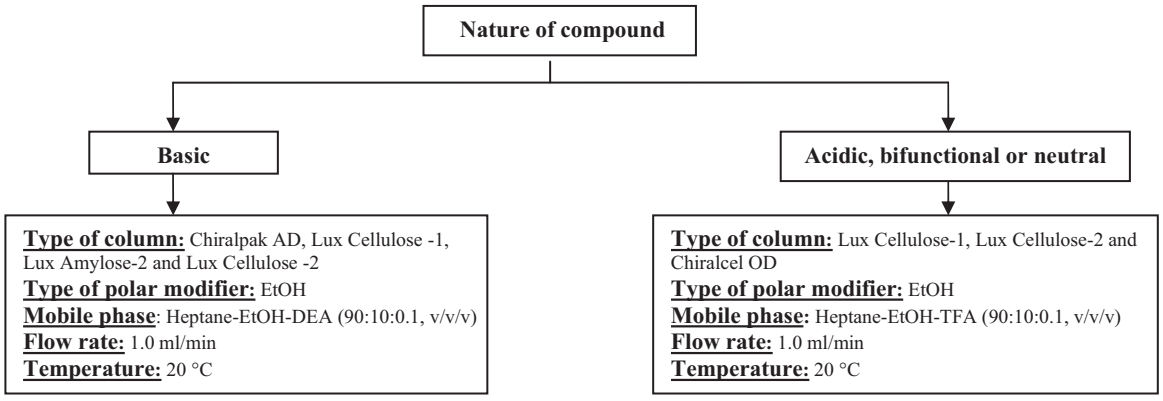

Fig. 11. Updated screening step in NPLC [34].

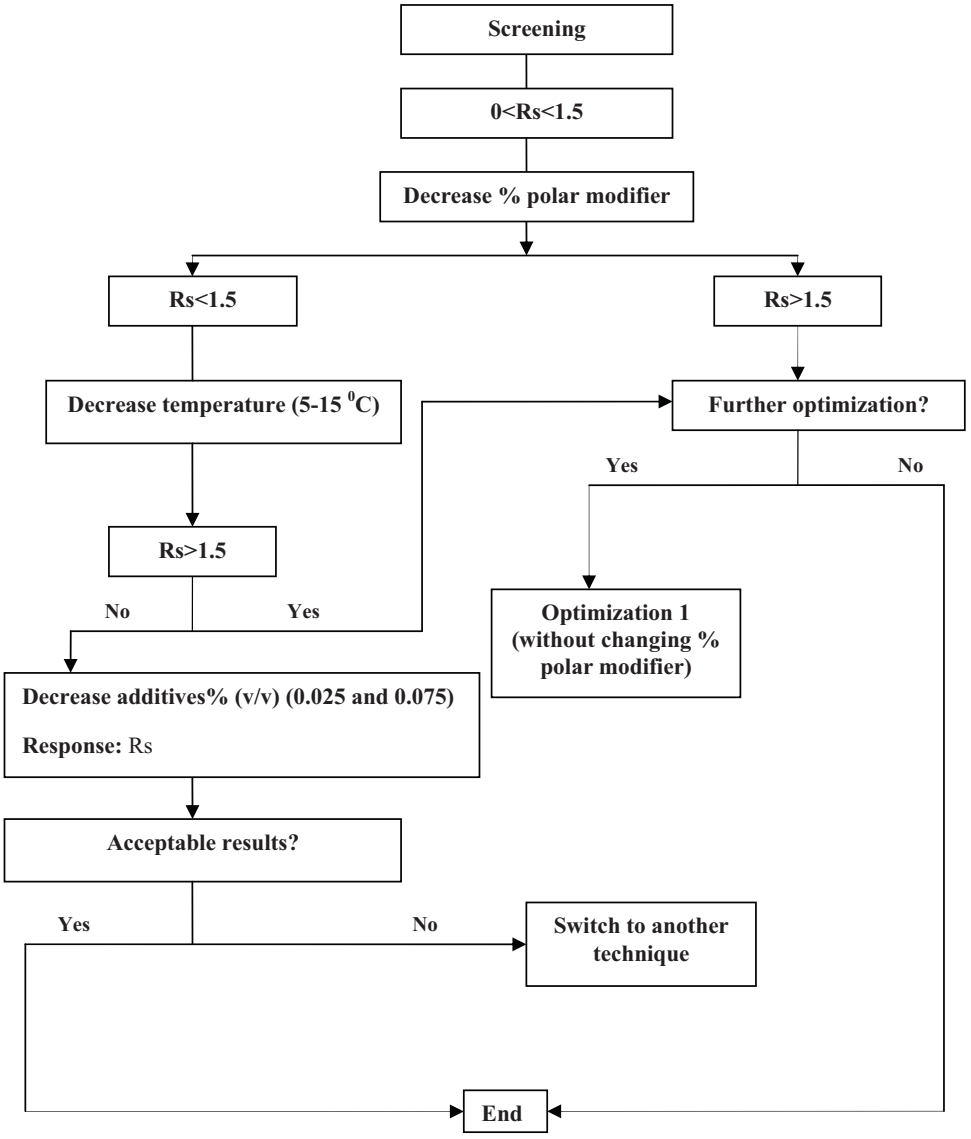

Fig. 12. Updated optimization 2.

Two cases out of the initially studied 20 cases, chlorthalidone and sulpiride on Chiralcel OD (Table 5) lost their resolution after optimization although acceptable k values were observed. In the light of these results Optimization 2 should be revised.

II. ***Optimization*** 2 ***step*** 2: ***Resolution optimization for compounds with*** $1 < k < 5$: The second step in optimization 2 deals with compounds that have desirable k under screening ($1 < k < 5$) or after Optimization 2, step 1. Sixteen optimization cases were subjected directly after screening to a 2^2 experimental design (Fig. 3) where additive concentration and temperature were varied at two levels each, 0.025 and 0.075%, and 5 and 15 °C, respectively. In most cases, the resolution was slightly improved (Table 6), but only three compounds were baseline resolved, i.e. chlorpheniramine and isothipendyl on Chiralpak AD and hexobarbital on Chiralcel OD (Fig. 8). No further optimization is proposed by Matthijs et al. [10] after this step. The results in Table 6 suggest only a limited utility of Optimization 2, step 2. Baseline resolution was achieved for only three cases out of 16.

On the contrary, Optimization 2 worked well when applied to the nine substances partially resolved during Optimization 2 step 1 (alprenolol and methadone on Lux Cellulose-2, flurbiprofen, ibuprofen and sulpiride on Lux Cellulose-1, alprenolol on Lux Amylose-2, methadone on Lux Cellulose-4, and carbinoxamine and meptazinol on Chiralpak OD) with six compounds baseline resolved (Table 7 and Fig. 9). Alprenolol on Lux Amylose-2 and ibuprofen and sulpiride on Lux Cellulose-1 could not be fully resolved.

Overall, the application of Optimization 2 on 36 cases resulted in the baseline resolution of 18/36 cases (50%), leaving 18 cases not baseline resolved. Five of those cases had $k < 1$ or $k > 5$ during screening (alprenolol on Lux Amylose-2, chlorthalidone and sulpiride on Chiralcel OD, and ibuprofen and sulpiride on Lux Cellulose-1) and 13 had $1 < k < 5$.

3.1.3. Suggested revised optimization scheme

In order to increase the total number of baseline resolved compounds during Optimization 2, additional conditions were explored for the 18 cases only partially resolved to this point. For compounds with $k < 1$ or $k > 5$, except chlorthalidone, further optimization was attempted while maintaining 0.1% additive in the mobile phase. The resolution of alprenolol on Lux Amylose-2 with mobile phase heptane–EtOH (97.5:2.5), flow rate 1.0 ml/min, temperature 5 °C and 0.1% DEA, was improved from 1.04 to 1.4 whereas baseline resolution of ibuprofen (Rs = 1.80) was achieved on Lux Cellulose-1 under the same experimental conditions, but now with 0.1% TFA.

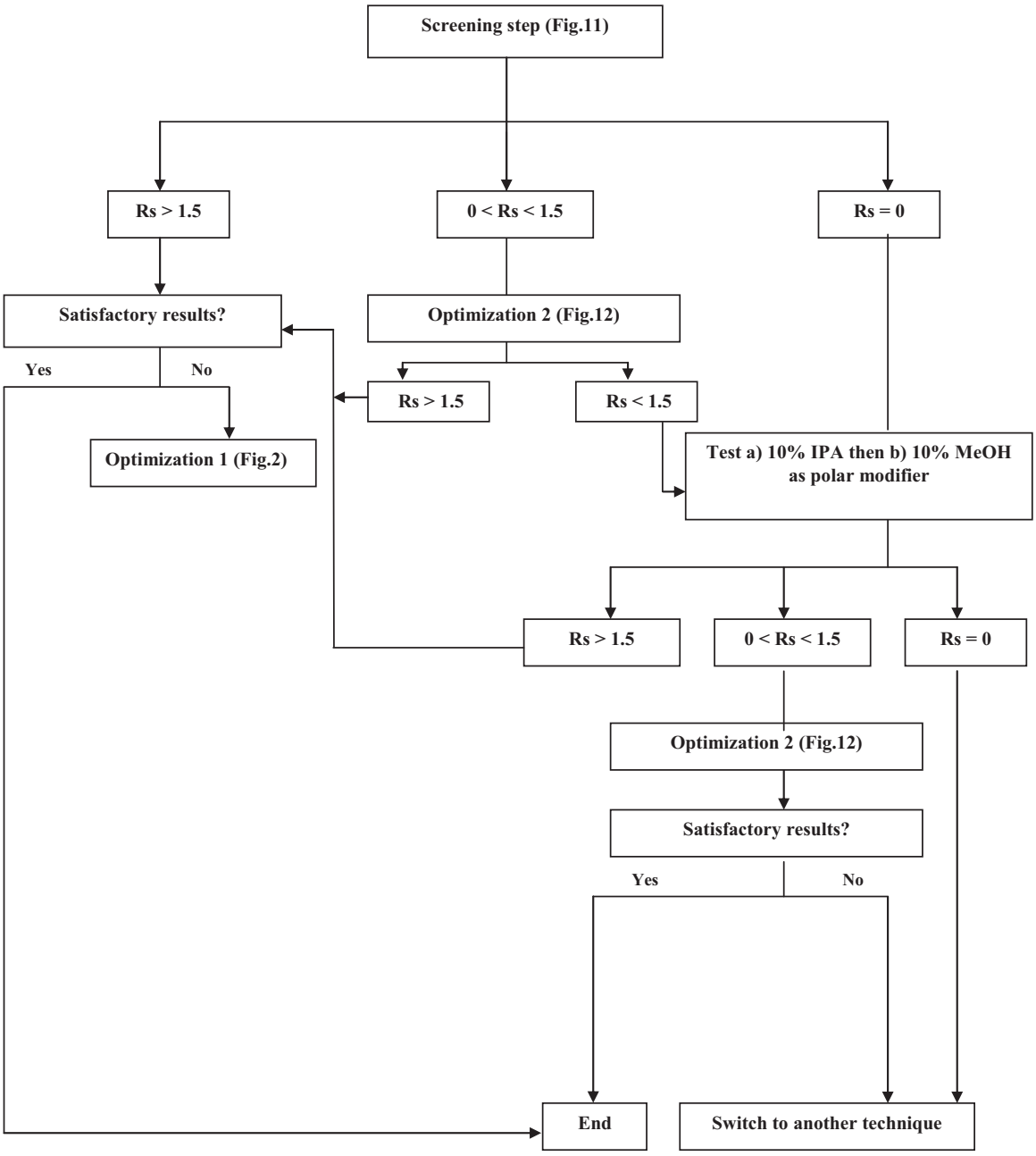

Fig. 13. Updated separation strategy in NPLC.

Chlorthalidone was fully resolved (Rs = 1.95) on Chiralcel OD with heptane–EtOH (90:10), flow rate 1.0 ml/min, temperature 15 °C and 0.075% DEA. Sulpiride (on either Chiralcel OD or Lux Cellulose-1) and alprenolol on Lux Amylose-2 were the compounds which could not be completely separated in spite of all efforts described above. For these compounds, switching to another technique such as POSC, RPLC or CEC might lead to a better resolution.

Alternatively, for compounds having $1 < k < 5$ and only partial resolution, we attempted optimization with a weaker mobile phase, similarly to compounds with $k < 1$. Temperature optimization followed this step if resolution was still less than 1.5, followed by additive optimization if necessary. Results improved with 14/16 cases baseline resolved, 87.5% (Table 8 and Fig. 10). For compounds that could not be baseline separated (dimethindene and mepindolol), the resolution was improved compared to the screening results.

In summary, baseline resolution was observed for 31/36 cases (86%) after applying the suggested optimization conditions compared to only 18/36 (50%) achieved by applying the previously promoted conditions for Optimization 2.

3.1.4. Use of Chiralcel OJ for basic compounds

In the original strategy, the use of Chiralcel OJ is proposed for basic compounds that could not be resolved under generic screening mobile phase conditions. Two basic compounds (2 optimization cases) (naproxen and naringenin) were submitted to this optimization step. The experimental conditions for screening Chiralcel OJ are the same as with the other CSPs. Whenever the separation was not improved, Chiralcel OJ was tested in a mobile phase made of heptane–EtOH, IPA or MeOH-DEA (90:10:.0.1, v/v/v). No improvement in the resolution of these two compounds was achieved. In addition methanol was tested as modifier on all examined columns

but unfortunately no enantioseparation was observed. For these compounds testing another separation technique such as RPLC or SFC might be more effective.

4. Proposed strategy update

The earlier screening strategy proposed by Matthijs et al. [10] was redefined by including a combination of traditional and also chlorine-containing polysaccharide-based CSPs [34]. The updated screening strategy (Fig. 11) demonstrated a high success rate with a higher number of baseline resolved compounds compared to the original one. In the current study, the applicability of the optimization steps defined in the same strategy was examined on both previously mentioned groups of CSPs. It was found that Optimization 1, i.e. optimization of the retention factor and peak shape of initially baseline separated compounds, showed high applicability on both groups of CSPs. For this reason, Optimization 1 (Fig. 2) can be kept in the updated separation strategy without any changes. On the other hand, Optimization 2 (Fig. 3) requires several modifications in order to improve the enantioselectivity of both groups of CSPs and to increase the number of baseline resolved compounds. In the originally proposed Optimization 2 [10], compounds were classified into two categories based on their retention factor obtained during screening (compounds with $k < 1$ or $k > 5$ on the one hand and those with $1 < k < 5$ on the other). In the modified version of Optimization 2 (Fig. 12), no initial classification is done anymore. Compounds showing some degree of separation in the screening step are first optimized by decreasing the modifier concentration (2.5 and 5%), regardless of k value. When baseline resolution is observed, Optimization 1, if required, is applied for optimizing analysis time or peak shape. In situations when resolution is still below 1.5, temperature is varied at two levels (5 and 15 °C), followed by changing the additive concentration, also at two levels (0.025 and 0.075%).

The updated version of Optimization 2 showed a higher improvement of enantioselectivity and led to more baseline resolutions. The number of baseline resolutions obtained upon applying this new optimization step to 36 optimization cases increased to 31 compared to 18 in the earlier defined step. For compounds that showed no enantioselectivity after the screening step, isopropanol and methanol (in this order) should be examined as modifiers on all CSPs. Whenever enantioselectivity is observed, the extended optimization strategy should be followed. If no resolution is seen ($Rs = 0$), another separation technique, such as POSC, RPLC or SFC, may be explored. An updated separation strategy in NPLC taking into account the above results is given in Fig. 13.

5. Conclusions

The applicability of two optimization schemes (Optimization 1 and Optimization 2) of an earlier proposed separation strategy [10] was evaluated on four recently commercialized polysaccharide-based CSPs and on three more classical ones using a test set of 44 chiral drugs (70 optimization cases). Results showed complementarity among both CSPs groups for the baseline separation of the tested compounds. Changing the mobile phase modifier content has the highest influence on resolution compared to parameters, such as temperature and additive concentration.

The two original optimization steps (Optimizations 1 and 2) showed separation improvements and baseline resolution for 49/70 optimization cases (70%) on at least one of the examined CSPs. After revising the optimization scheme, the number of baseline resolutions increased to 62 cases (88.6%). Two substances (naproxen and naringenin) could not be resolved at all on any of the examined CSPs. For these compounds, switching to another separation technique, such as POSC, RPLC or SFC, might lead to a better resolution.

In conclusion, it can be stated that the new screening and optimization strategy for the chiral separation of pharmaceutical in NPLC adds a considerable improvement over the existing one, when a hybrid set of the recently commercialized and traditional CSPs is used.

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