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Enantiomeric separation of newly synthesized amino, thio, and oxy derivatives of monoterpene lactones, amides, and ester applying polysaccharide-based chiral stationary phases in normal-phase mode

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ABSTRACT

New amino, thio, and oxy derivatives of monoterpene lactones, amides, and esters have been synthesized and their enantioselective separations were investigated on seven covalently immobilized polysaccharide-based chiral stationary phases. The effects of basic additives, different short-chain alcohols, and the influence of the temperature on the chromatographic behavior were studied. In addition, relationships between the structure of selector and selectand and the chromatographic parameters were explored to reveal mechanistic details of chiral recognition. Experiments were performed in the temperature range 10–50°C and thermodynamic parameters were calculated from plots of lnα versus 1/T. The separations were generally enthalpy-controlled, but entropy-driven separation was also observed. Special attention has been paid to the enantiomer elution order and several examples are shown how the structural characteristics of the selector, the nature, and the concentration of the polar modifier induce reversal of the enantiomer elution order in the case of the polysaccharide-based selectors.

1. Introduction

The α-methylene-γ-lactone moiety, the key structural feature for numerous natural terpenoids such as arglabin, alantolactone, isoalantolactone, grosheimin, parthenolide, and santonin [1], acts as a Michael acceptor and reacts with nucleophiles (sulfhydryl or amino groups) in enzymes, transcription factors, and other proteins, alkylating them irreversibly [2]. However, these terpenes typically have poor water solubility, and the α-methylene-γ-lactone can exhibit non-selective binding as a Michael acceptor with undesired targets [3]. Therefore, transforming a parent bioactive natural compound to a new and more bioactive one *via* a semi-synthetic approach by the incorporation of heteroatoms (N, O, or S) could enhance aqueous solubility, improve the pharmacokinetic profile, and maintain or even augment the biological activity of the parent molecule [4]. The conjugate addition (1,4-additions) of aza/thia/oxo nucleophiles to Michael acceptors is a powerful technique for new carbon–carbon or carbon–heteroatom bond formation that plays an important role in the synthesis of thio, amino, and hydroxy derivatives of natural lactones [5]. Since biological activities of lactone analogs, in many cases, depend strongly on their stereochemistry, there is a need for analytical methods to determine the enantiomeric purity of lactones and their derivatives. High-performance liquid chromatography (HPLC) employing chiral stationary phases (CSPs) is an effective analytical tool for the resolution of chiral compounds.

Polysaccharide-based selectors represent the most frequently applied CSPs with specific enantio-, diastereo-, and chemoselectivity [6–8]. Phenylcarbamate derivatives of polysaccharides coated onto silica gel were first applied by Okamoto et al. [9]. Subsequently, new perspectives opened up on the application of polysaccharide-based CSPs by incorporation of halomethyl *N*-phenylcarbamate moieties to the cellulose and amylose chains by Chankvetadze and Okamoto [10]. A further development was achieved by immobilization of amylose- or cellulose-based *tris*-(phenylcarbamate) moieties onto silica resulting in very robust CSPs [11]. Very recently, polysaccharide-based *tris*-(phenylcarbamate) moieties were successfully immobilized covalently to superficially porous particles resulting in highly efficient CSPs providing short analysis times [12,13].

Temperature is one of the most important tunable variables in chiral chromatography, also for polysaccharide-based CSPs. To describe how the temperature affects retention and separation in

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chromatographic systems usually the van't Hoff equation is applied,

$$ln\ \alpha = -\frac{\Delta(\Delta H^{\circ})}{RT} + \frac{\Delta(\Delta S^{\circ})}{R} \quad (1)$$

where α is the selectivity factor, $\Delta(\Delta H^{\circ})$ and $\Delta(\Delta S^{\circ})$ are the differences in standard enthalpy and standard entropy, R is the universal gas constant, and T is the temperature in Kelvin. Using the plots of $\ln\alpha$ vs T^{-1} and calculating the values of $\Delta(\Delta H^{\circ})$ and $\Delta(\Delta S^{\circ})$ for pairs of enantiomers, uncertainties related to the determination of the phase ratio can be eliminated [14]. However, as described in an excellent review by Asnin and Stepanova [15], nonselective contributions cannot be eliminated in this way. That is a cautious interpretation is needed when thermodynamics are applied for the characterization of the recognition process.

The focus of the present manuscript is on the study of the enantioseparation of newly synthesized amino, thio, and oxy derivatives of monoterpene lactones, amides, and esters of pharmaceutical relevance. Their chromatographic behavior was investigated on amylose- and cellulose-based *tris*-(phenylcarbamate)-type CSPs. A detailed study was carried out to investigate the effects of the nature of basic additives, the nature and concentration of alcohol modifiers, the specific structural features of the selectors and analytes, and the temperature on retention, selectivity, and resolution of the enantiomeric analytes. Since the configurations of all samples are known, the enantiomer elution order (EEO) was determined in all cases.

2. Materials and methods

2.1. Chemicals and reagents

The synthesis route, the physical and chemical properties of the newly synthesized compounds as well as related analytical data are presented in Supplementary Information. The names of the studied analytes are listed in Table S1.

Methanol (MeOH), ethanol (EtOH), 1-propanol (PrOH), 2-propanol (2-PrOH), 1-butanol (BuOH), and *n*-hexane of HPLC grade, ethylamine (EA), diethylamine (DEA), triethylamine (TEA), triethanolamine (TEOA), and other reagents of analytical reagent grade were purchased from VWR International (Radnor, PA, USA).

2.2. Apparatus and chromatography

HPLC measurements were carried out on a Shimadzu Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A system controller, DGU-20A solvent degasser, an LC-20AB binary pump, an SPD-M20A photodiode array detector with a flow cell (10-mm optical path length), a CTO-20AC column oven, and a SIL-20AC autosampler. LabSolution chromatographic data software (Shimadzu Corporation, Kyoto, Japan) allowed to perform the acquisition and processing of chromatographic data.

The commercially available amylose- or cellulose-based polysaccharide columns applied in this work were amylose *tris*-(3,5-dimethylphenylcarbamate) (Chiralpak **IA)**, cellulose *tris*-

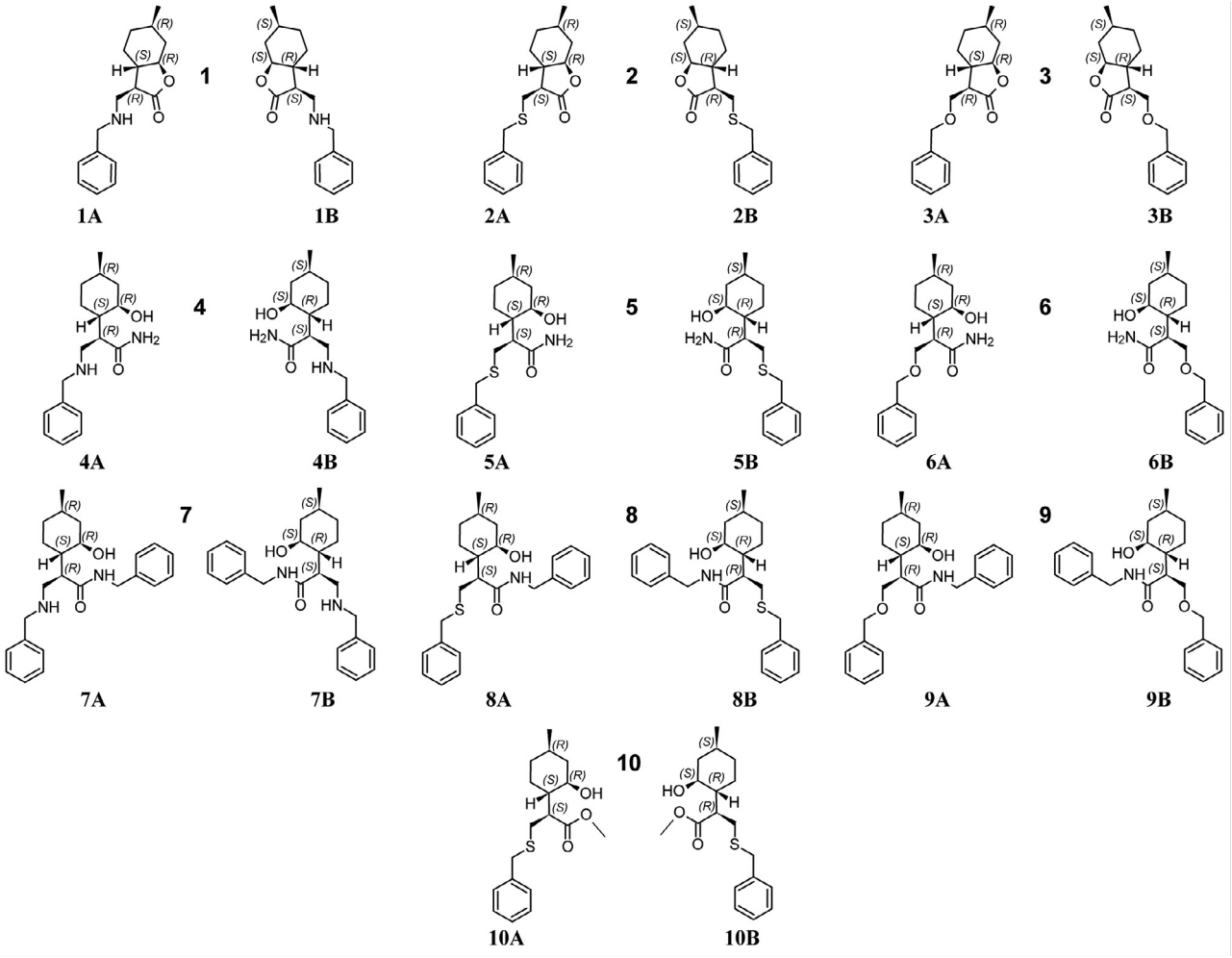

Fig. 1. Structure of the studied analytes.

(3,5-dimethylphenylcarbamate) (Chiralpak **IB**), amylose *tris*-(3,5-dichlorophenylcarbamate) (Chiralpak **IE**), and cellulose *tris*-(3,5-dichlorophenylcarbamate) (Chiralpak **IC**), as well as amylose *tris*-(3-chlorophenylcarbamate) (Chiralpak **ID**), amylose *tris*-(3-chloro-4-methylphenylcarbamate) (Chiralpak **IF**), and amylose *tris*-(3-chloro-5-methylphenylcarbamate) (Chiralpak **IG**). All columns with the same geometric (250 mm × 4.6 mm I.D.) and particle size (5 µm) were from Chiral Technologies Europe (Illkirch, France). All CSPs employed in this study are immobilized phases and their structures are presented in Figure S1.

Stock solutions of analytes (1.0 mg ml^{-1}) were prepared by dissolution of the solid samples in EtOH, further diluted with the appropriate mobile phase, and injected as 10-µl samples. The hold-up times of the columns were determined by the injection of tri-*t*-butyl benzene.

3. Results and discussions

3.1. Structural features of the investigated analytes

The structure of the studied analytes is presented in Fig. 1. The monoterpene-based analytes employed in this study structurally can be divided into four groups: **a)** analytes **1, 2**, and **3** are benzylamino, benzylsulfanyl, and benzyloxy-derivatives of monoterpene lactones; **b)** ring opening of lactons by NH_3 resulted in **4, 5**, and **6** as benzylamino, benzylsulfanyl and benzyloxy-derivatives of amides; **c)** ring opening of lactones with benzylamines gave **7, 8**, and **9** as *N*-benzyl-benzylamino, *N*-benzyl-benzylsulfanyl, and *N*-benzyl-benzyloxy analogs of amides; **d)** analyte **10** is an ester analog of **5**. The ring-opened analogs of **1, 2**, and **3** contain an extra hydroxy group in the case of **4**–**9**, and extra primary and secondary amino groups in the case of **4**–**6** and **7**–**9**, respectively, capable of H-bonding with the carbamate residue of the selector. Furthermore, the additional benzyl ring in **7**–**9** may be involved in $\pi-\pi$ interactions. The structural differences of these analytes provide an opportunity to study the effects of the structural features on chiral recognition, and to explore relationships between the structure and retention (selectivity). The two- and three-ring systems provide characteristic structural features and lead to molecules with

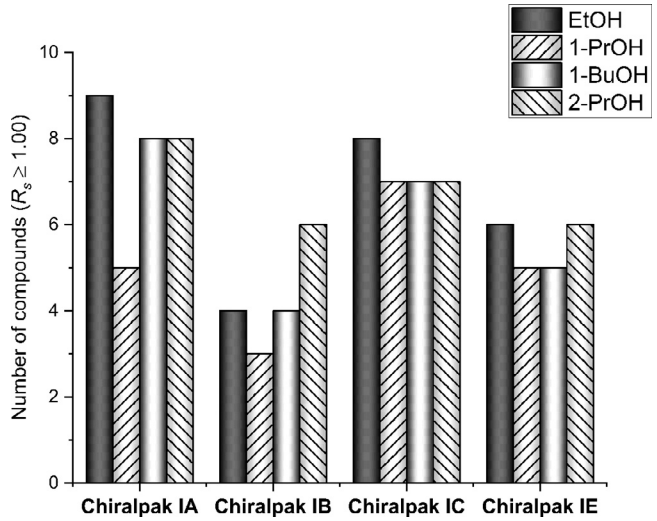

Fig. 2. Effects of the nature of alcohol on the resolution of all studied analytes Chromatographic conditions: columns, Chiralpak **IA, IB, IC**, and **IE**; mobile phase, *n*-hexane/alcohol/DEA, molar concentration of alcohol in *n*-hexane, 3.43 M and concentration of DEA in mobile phase, 0.1 %(*v/v*); flow rate, 1.0 ml min^{-1}; detection, 220 nm; temperature, 25°C; symbols, EtOH, ; 1-PrOH, ; 1-BuOH, ; 2-PrOH, .

Table 1
Chromatographic data, k_1, α, R_S and enantiomer elution order (EEO) for analytes **1–10** on polysaccharide-based chiral stationary phases in normal-phase mode.

Sample	Column	k_1	α	R_s	EEO
1	IA	2.39	1.26	4.78	*A* < *B*
	IB	0.81	1.08	0.95	*B* < *A*
	IE	3.34	1.06	1.30	*B* < *A*
	IC	2.28	1.19	3.44	*B* < *A*
	IF	5.74	1.28	5.30	*A* < *B*
	IG	1.90	1.07	0.99	*A* < *B*
	ID	2.16	1.11	1.95	*A* < *B*
2	IA	2.08	2.25	10.63	*B* < *A*
	IB	0.72	1.00	0.00	-
	IE	1.86	1.00	0.00	-
	IC	2.04	1.00	0.00	-
	IF	3.36	1.17	3.67	*B* < *A*
	IG	3.46	1.53	9.30	*B* < *A*
	ID	1.32	1.09	1.40	*B* < *A*
3	IA	1.95	1.45	6.84	*A* < *B*
	IB	0.76	1.15	1.67	*B* < *A*
	IE	1.80	1.05	0.71	*A* < *B*
	IC	2.15	1.38	6.64	*B* < *A*
	IF	2.20	1.23	4.31	*A* < *B*
	IG	2.91	2.26	17.30	*A* < *B*
	ID	1.12	1.13	1.85	*A* < *B*
4	IA	1.41	1.46	5.34	*A* < *B*
	IB	0.68	1.00	0.00	-
	IE	1.97	1.09	1.42	*B* < *A*
	IC	1.43	1.48	5.59	*B* < *A*
	IF	1.73	3.06	20.76	*B* < *A*
	IG	2.89	2.40	16.20	*B* < *A*
	ID	1.06	2.05	11.36	*B* < *A*
5	IA	2.03	1.14	2.00	*B* < *A*
	IB	0.72	1.08	0.55	*A* < *B*
	IE	2.00	1.85	10.92	*B* < *A*
	IC	1.71	1.56	6.33	*B* < *A*
	IF	2.29	1.68	8.91	*B* < *A*
	IG	2.97	2.35	13.67	*B* < *A*
	ID	1.43	1.18	2.35	*B* < *A*
6	IA	1.85	1.08	1.29	*A* < *B*
	IB	0.70	1.00	0.00	-
	IE	2.00	1.41	6.18	*B* < *A*
	IC	1.95	1.55	6.91	*B* < *A*
	IF	2.17	1.23	3.59	*B* < *A*
	IG	3.21	1.53	7.19	*B* < *A*
	ID	1.33	1.13	1.65	*B* < *A*
7	IA	1.29	1.05	0.44	*B* < *A*
	IB	0.35	1.31	1.26	*A* < *B*
	IE	1.44	1.00	0.00	-
	IC	0.57	1.15	0.84	*A* < *B*
	IF	1.41	1.00	0.00	-
	IG	2.27	1.31	3.87	*B* < *A*
	ID	1.02	1.00	0.00	-
8	IA	1.90	1.10	1.41	*A* < *B*
	IB	0.47	1.18	1.15	*A* < *B*
	IE	1.57	1.36	4.43	*B* < *A*
	IC	0.79	1.19	1.46	*A* < *B*
	IF	1.51	1.43	5.03	*B* < *A*
	IG	3.07	1.57	6.78	*B* < *A*
	ID	1.25	1.27	2.96	*B* < *A*
9	IA	1.86	1.35	4.69	*A* < *B*
	IB	0.46	1.29	1.50	*A* < *B*
	IE	1.48	1.24	3.27	*B* < *A*
	IC	0.85	1.00	0.00	-
	IF	1.64	1.23	3.22	*B* < *A*
	IG	3.75	1.16	2.34	*B* < *A*
	ID	1.41	1.15	1.90	*B* < *A*
10	IA	1.10	1.09	1.11	*A* < *B*
	IB	0.42	1.13	0.71	*A* < *B*
	IE	1.08	1.00	0.00	-
	IC	0.62	1.99	7.07	*A* < *B*
	IF	1.64	1.18	2.75	*A* < *B*
	IG	2.21	1.04	0.23	*B* < *A*
	ID	0.85	1.00	0.00	-

Chromatographic conditions: columns, Chiralpak **IA, IB, IC, ID, IE, IF**, and **IG**; mobile phase, *n*-hexane/EtOH/DEA (80/20/0.1 *v/v/v*), molar concentration of EtOH in *n*-hexane, 3.43 M; flow rate, 1.0 ml min^{-1}; detection, 220 nm; temperature, 25 °C.

different polarities and van der Waals volumes. For analytes **1**–**10** the polarity values (log P) are 2.91, 4.00, 3.13, 1.45, 2.54, 1.67, 3.70, 4.56, 3.85, and 3.44, respectively, while the van der Waals volumes are 276 Å, 270.5 Å, 266.7 Å, 292.5 Å, 298.8 Å, 289.2 Å, 380.8 Å, 387.3 Å, 377.5 Å, and 313.9 Å, respectively. All data were calculated with the Marvin Sketch v. 17.29 software (ChemAxon, Budapest).

3.2. Selection of the basic additive and the polar modifier

Polysaccharide-based CSPs are employed most frequently in normal-phase (NP) mode. Depending on structural characteristics, attractive forces such as H-bonding, dipole-dipole, $\pi-\pi$ interactions, and repulsive forces such as steric hindrance might be taken into account. The most frequently applied mobile phases are mixtures of a nonpolar hydrocarbon (typically *n*-hexane or *n*-heptane) and an alcohol of low molecular weight (*e.g.*, EtOH, 1-PrOH, 1-BuOH, or 2-PrOH) [6–8,16]. Polar basic additives can mask underivatized silanols and reduce peak tailing [17].

Additives can have an impact on the selector-selectand association within the chiral cavities, thus the effects of mobile phase additives might be CSP-dependent. However, our earlier results showed that the basic additives usually exerted a slight effect on the chromatographic parameters independently from the CSP applied [18]. For the investigation of the effect of the nature of basic additive, separations were carried out on the Chiralpak **IA** column as the most widely used stationary phase among the

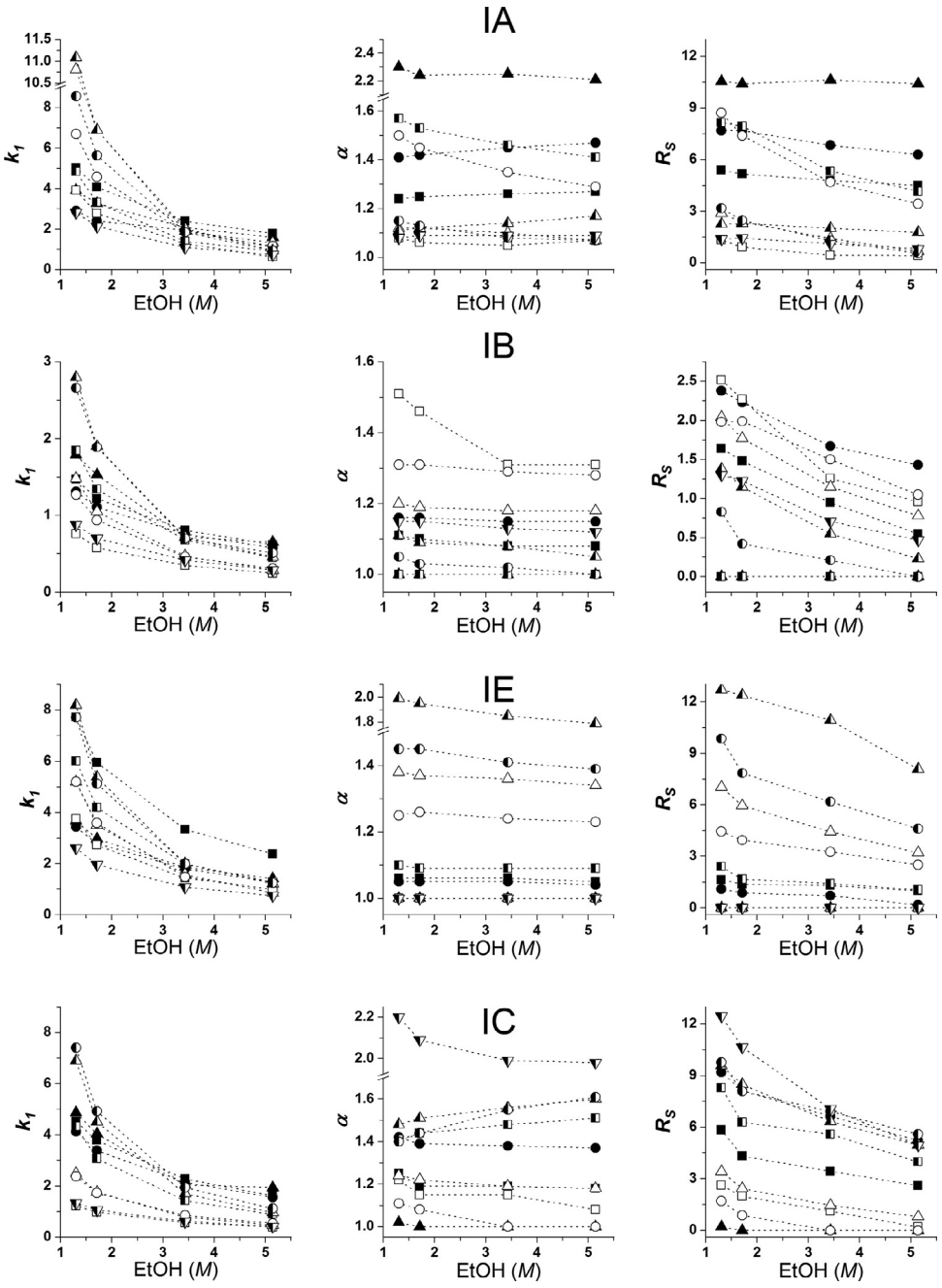

Fig. 3. Effects of EtOH concentration on the retention factor (k_1), separation factor (α), and resolution (R_S) for the separation of the studied analytes Chromatographic conditions: columns, Chiralpak **IA, IB, IE**, and **IC**; mobile phase, *n*-hexane/EtOH/DEA, all containing 0.1 %(*v*/*v*) DEA; the concentration of EtOH: 1.30, 1.71, 3.43 and 5.14 M; flow rate 1.0 ml min^{-1}; detection at 220 nm; temperature, 25°C; symbols, analyte **1**, ■; **2**, ▲; **3**, ●; **4**, ◧; **5**, ◭; **6**, ◐; **7**, □; **8**, △; **9**, ○; **10**, ▽.

polysaccharide-based CSPs in the presence of EA, DEA, TEA, or TEOA in a mobile phase of *n*-hexane/EtOH/base 80/20/0.1 (*v/v/v*) composition, where the selected bases differ in the degree and nature of their alkyl substitution on the nitrogen atom. In the case of analytes **1, 2, 3, 4**, and **7**, the experimental results depicted in Figure S2 reveal that the nature of base exerted only a slight effect on k_1, α, and R_S values, indicating that the bases participate in the separation process in a similar manner. Their competition for the binding sites of the stationary phase and contribution to the reduction of band broadening are practically independent of their nature. Since the nature of the base was found to exert a negligible effect on the chromatographic parameters, in all further experiments DEA was used as a basic additive.

The nature and concentration of the alcohol can have a deep impact on the retention and stereorecognition processes with cellulose- and amylose-based CSPs. The higher-order structure of the polysaccharide-derivative chains, having an essential role in the process of enantiorecognition, has been shown to be modified by alcohols as additives in different ways [19–21]. That is, the nature of alcohol is expected to affect the three-dimensional structure of the selector and the interactions formed between the selector and analytes, depending on the size and the structure of the analytes. To study the effect of the nature of alcohol on the chromatographic parameters, EtOH, 1-PrOH, 1-BuOH, and 2-PrOH as polar modifiers were applied. Based on their structural similarities, four polysaccharide-based CSPs, *i.e.,* amylose- and cellulose-based *tris*-(3,5-dimethylphenylcarbamate) (Chiralpak **IA** and **IB)** and *tris*-(3,5-dichlorophenylcarbamate) (Chiralpak **IE** and **IC)** were selected, and experiments were carried out in *n*-hexane/alcohol/0.1 %(*v/v*) DEA mobile phase systems. The concentration of different alcohols was kept at a constant level of 3.43 M, which corresponds to mobile phase compositions of *n*-hexane/EtOH/DEA 80/20/0.1 (*v/v/v*), *n*-hexane/1-PrOH/DEA 74.6/25.4/0.1 (*v/v/v*), *n*-hexane/1-BuOH/DEA 68.7/31.3/0.1 (*v/v/v*), and *n*-hexane/2-PrOH/DEA 73.8/26.2/0.1 (*v/v/v*). The data obtained with the change of the nature of alcohol are presented in Table S2. Under NP conditions, increasing the apolar character of the alcohol usually results in enhanced analyte retention [22,23]. Note, however, that opposite observations have also been described [24]. Under the conditions applied in this study, retentions were most frequently the highest with EtOH or 2-PrOH, and often the lowest with 1-BuOH, but no general trends could be observed in the variation of the retention factors with the nature (polarity) of the alcohol. Specifically, k_1 values of analytes **1**–**10** ranged for EtOH, 1-PrOH, 1-BuOH, and 2-PrOH between 0.35–3.34, 0.25–3.48, 0.15–3.56, and 0.28–4.79, respectively. The nature of the alcohol affects the enantioselectivity, but no clear relationship between enantioselectivity and polarity of the alcohol can be explored. Fig. 2 summarizes the number of effective separations when $R_S \geq 1.00$ was reached on four different CSPs applying EtOH, 1-PrOH, 1-BuOH, and 2-PrOH. In most cases, EtOH led to higher resolutions, but in several cases, 2-PrOH proved to be a better choice. Taking these results into account further experiments were carried out in the presence of EtOH or 2-PrOH.

3.3. Effects of mobile phase composition

The variation of the alcohol concentration serves most often for the modulation of the chromatographic behavior in NP mode [19,22–24]. The effect of alcohol concentration on the chromatographic parameters for monoterpene-based analogs was studied first in *n*-hexane/EtOH/DEA mobile phase systems. Analyses were carried out on two pairs of structurally similar CSPs, namely Chiralpak **IA, IB**, and **IE, IC**, with mobile phase compositions of *n*-hexane/EtOH/DEA containing EtOH of 1.30, 1.71, 3.43, and 5.14 M [which correspond to *n*-hexane/EtOH ratios of 92/8, 90/10, 80/20, and 70/30 (*v/v*), respectively]. Furthermore, all mobile phases contained the same amount of 0.1 %(*v/v*) DEA as basic additive. As depicted in Fig. 3, in these mobile phase systems a typical NP behavior was observed for all studied CSPs: the retention decreased with

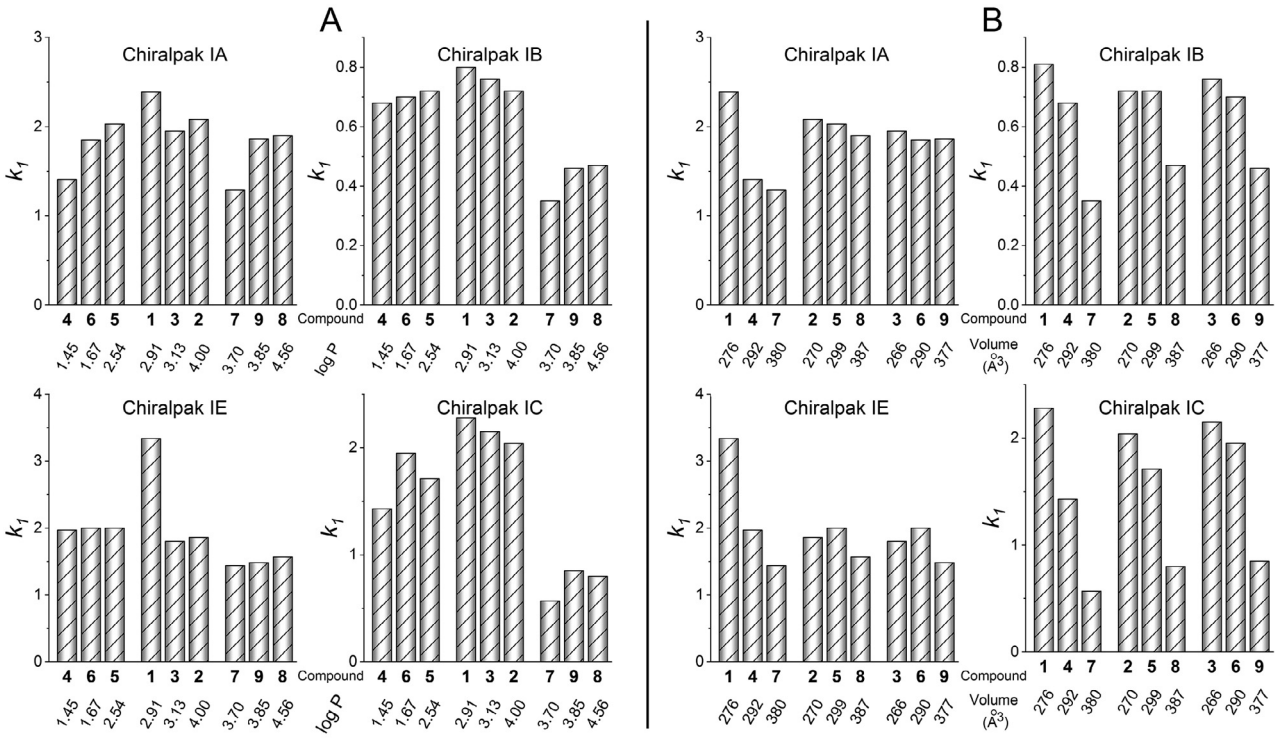

Fig. 4. Effects of **A**, polarity (log P), and **B**, the volume of the molecule (Å^3) on retention factor (k_1) for the separation of the studied analytes Chromatographic conditions: columns, Chiralpak **IA, IB, IE**, and **IC**; mobile phase, *n*-hexane/EtOH/DEA 80/20 (*v/v/v*); flow rate 1.0 ml min^{-1}; detection at 220 nm; temperature, 25°C.

increasing EtOH content. With the increase of mobile phase polarity, the strength of H-bonding between the selector and selectand decreases, while the solubility of analytes in the mobile phase increases [19] resulting in a marked decrease in k_1 for all analytes. Consistent with this observation, retention of analytes **4, 5**, and **6**, being the most polar compounds (as supported by the lowest log P values, 1.45, 2.54, 1.67, respectively,) decreased the most. Compared to the changes observed in retentions, enantioselectivity usually exhibited only a small variation, generally slightly decreased with increasing EtOH content. Regarding resolution, R_S decreased more significantly, in most cases in parallel with the change of k_1.

In the second series of experiments, 2-PrOH was applied at the same molar concentration as EtOH in the *n*-hexane/2-PrOH/DEA mobile phase system. The applied columns were the same as mentioned above and on the basis of structural analogies, analytes **1**–**4** and **7** were selected as model compounds. A comparison of the results obtained with the application of EtOH (depicted in Fig. 3) and 2-PrOH (depicted in Figure S3) reveals that the analytes behave essentially in a similar manner in both mobile phase systems. Specifically, k_1 decreased with increasing alcohol content on all the four studied CSPs independently from the nature of alcohol. Retention factors obtained with EtOH and 2-PrOH were similar on Chiralpak **IB, IE**, and **IC**. However, on Chiralpak **IA**, especially at low alcohol content, much higher k_1 values were registered when applying EtOH as a polar modifier. This clearly indicates that the retentive properties of the Chiralpak **IA** column are more sensitive to the nature of alcohol when the mobile phase contains a low concentration of the polar modifier. As concerns the α values, they changed similarly in both mobile phase systems in most cases. However, some differences could be observed in the behavior of compounds **4** and **7**. On Chiralpak **IE** α values for **4** decreased, while for **7** they increased with increasing 2-PrOH content. On Chiralpak **IC** the trend was the opposite. Concerning resolutions, rather divergent correlations can be seen. With increasing alcohol content R_S decreases or increases for **4** and **7** on Chiralpak **IE**, respectively, while a decrease was registered for both analytes on Chiralpak **IC**.

3.4. Structure–retention (selectivity) relationships

Table 1 depicts the chromatographic parameters of the separation of all analytes obtained on the studied columns applying the same mobile phase [*n*-hexane/EtOH/DEA 80/20/0.1 (*v/v/v*)].

The effect of polysaccharide backbone can be evaluated by the comparison of the chromatographic data obtained with the same *tris*-(3,5-dimethylphenylcarbamate) derivatives (Chiralpak **IA** *vs.* **IB**), and *tris*-(3,5-dichlorophenylcarbamate) (Chiralpak **IE** *vs.* **IC**) of amylose or cellulose, respectively. Data summarized in Table 1 show that higher retention factors could be observed on amylose- than on cellulose-based CSP. The only exceptions are **2** and **3**, where k_1 was higher on Chiralpak **IC** than on Chiralpak **IE**.

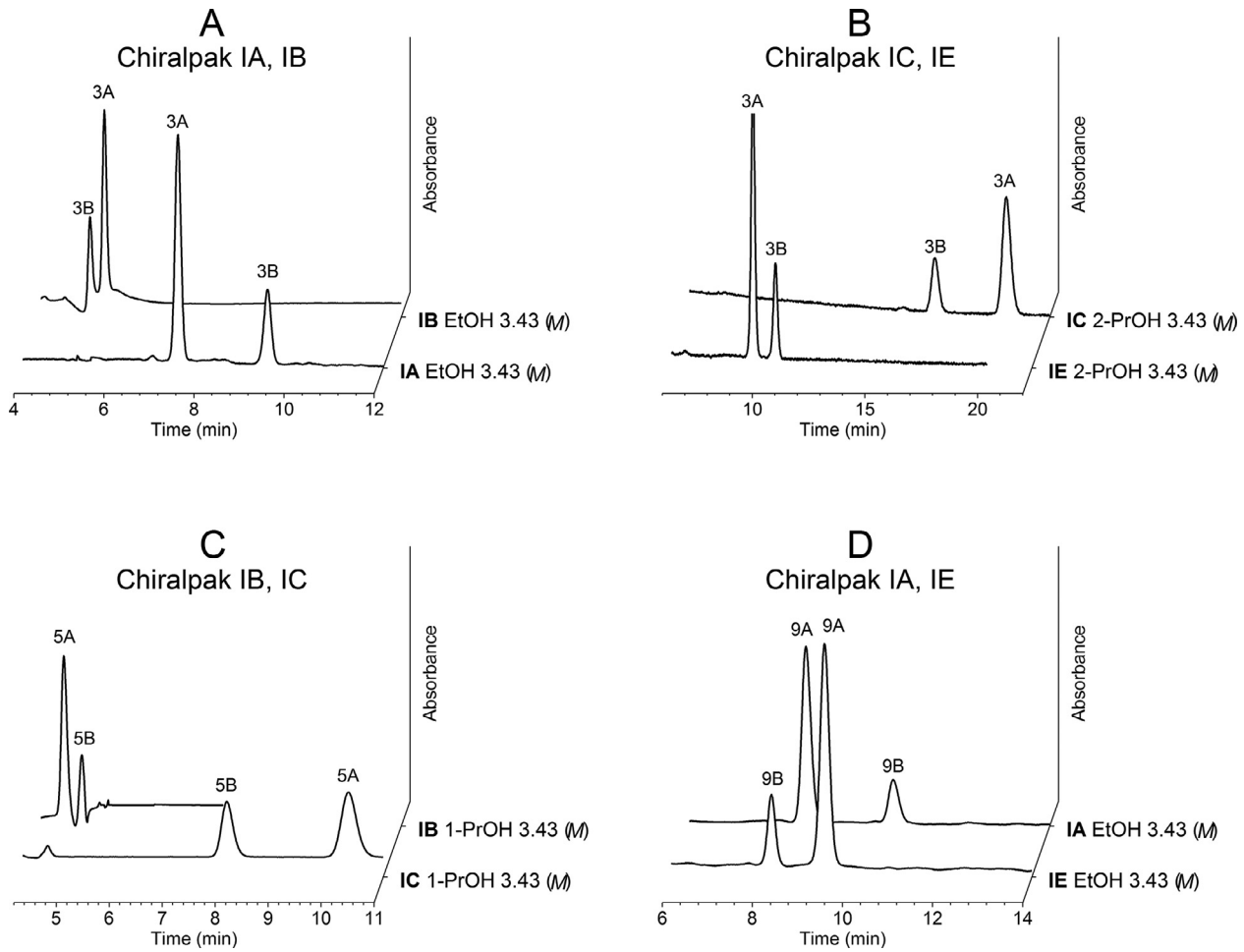

Fig. 5. Representative chromatograms for the change of the enantiomer elution order with the change of polysaccharide backbone or *tris*-(phenylcarbamate) residues of the polysaccharide chain Chromatographic conditions: analytes, **A** and **B, 3; C, 5; D, 9**; columns, **A**, Chiralpak **IA** and **IB; B**, Chiralpak **IE** and **IC; C**, Chiralpak **IB** and **IC; D**, Chiralpak **IA** and **IE**; mobile phase, **A** and **D**, *n*-hexane containing 3.43 M EtOH; **B**, *n*-hexane containing 3.43 M 2-PrOH; **C**, *n*-hexane containing 3.43 M 1-PrOH; all mobile phase contained 0.1 %(*v/v*) DEA; flow rate 1.0 ml min^{-1}; detection at 220 nm; temperature, 25°C.

These results indicate that analytes fit usually better into the chiral grooves of the helical chain of amylose than in that of cellulose. Besides the influence of the backbone of the polysaccharide chain, α and R_S are markedly affected by the nature of the carbamate residues attached to the polysaccharide chain. In this eluent system in the case of *tris*-(3,5-dimethylphenylcarbamate) derivative (Chiralpak **IA** *vs.* Chiralpak **IB**), except for analyte **7**, the amylose-based CSP provided higher α and R_S, while in the case of *tris*-(3,5-dichlorophenylcarbamate) derivative (Chiralpak **IC** *vs.* Chiralpak **IE**), the cellulose-based CSP exhibited better separation for analytes **1, 3, 4, 6, 7** and **10**.

A comparison of the effect of 3,5-dimethylphenylcarbamate *vs.* 3,5-dichlorophenylcarbamate substitution on the same polysaccharide backbone (Chiralpak **IA** *vs.* **IE**, Chiralpak **IB** *vs.* **IC**) reveals that with a few exceptions, retention is higher on CSPs substituted with 3,5-dichlorophenylcarbamate. This is probably due to the enhanced hydrogen bonding interaction between the halogen-substituted selector and the selectands. In the case of amylose, higher selectivity factors and resolution values were obtained with *tris*-(3,5-dimethylphenylcarbamate)-based CSP than with *tris*-(3,5-dichlorophenylcarbamate)-based CSP with the exceptions of **5, 6**, and **8**. In contrast, an opposite trend was observed on cellulose-based CSPs with higher α and R_S registered on *tris*-(3,5-dichlorophenylcarbamate)-based CSP (exceptions were analytes **7** and **9**).

The effects of the position of methyl and chloro substituents on the phenyl moiety (Chiralpak **IF** *vs.* Chiralpak **IG**) can also be analyzed on the basis of data listed in Table 1. In most cases, higher k_1, α, and R_S were registered on Chiralpak **IG** than on Chiralpak **IF** providing evidence for the significant role played by steric effects of the substituents of the phenylcarbamate moiety on chiral recognition. It is interesting to note that Chiralpak **ID** possessing *tris*-(3-chlorophenylcarbamate) moieties exhibited the lowest chiral recognition ability (analyte **4** is an exception).

Analytes **1, 2**, and **3**, as well as **4, 5**, and **6** are benzylamino, benzylsulfanyl, and benzyloxy derivatives of monoterpene lactones and amides, respectively, while **7, 8**, and **9** are *N*-benzyl-benzylamino, *N*-benzyl-benzylsulfanyl, and *N*-benzyl-benzyloxy derivatives of amides. Analyte **10** is an ester analog of **5** (Fig. 1). These structural differences result in different polarities and sizes of molecules which should be related to chromatographic behavior and chiral recognition.

Exchanging the amino group for oxy or thio group in monoterpene lactones, amides, and *N*-benzyl amides, respectively, resulted in a slight decrease in polarity (log P increases). Fig. 4A depicts results obtained with Chiralpak **IA, IB, IE**, and **IC** columns for the dependence of retention factor of the first eluting enantiomer (k_1) on polarity (log P) applying the *n*-hexane/EtOH/DEA 80/20/0.1 (*v/v/v*) mobile phase system. According to the results of amino, oxy, and thio derivatives of monoterpene amides (**4, 6, 5**) and monoterpene *N*-benzyl amides (**7, 9, 8**), k_1 decreases slightly with increasing polarity of the molecules due to the enhanced interactions between the analyte and the polar component of the mobile phase. However, monoterpene lactones, **1, 3**, and **2** behave in a different way, with the most polar analyte **1** retaining the most. In this case, the lactone ring probably plays a more prominent role in the interac-

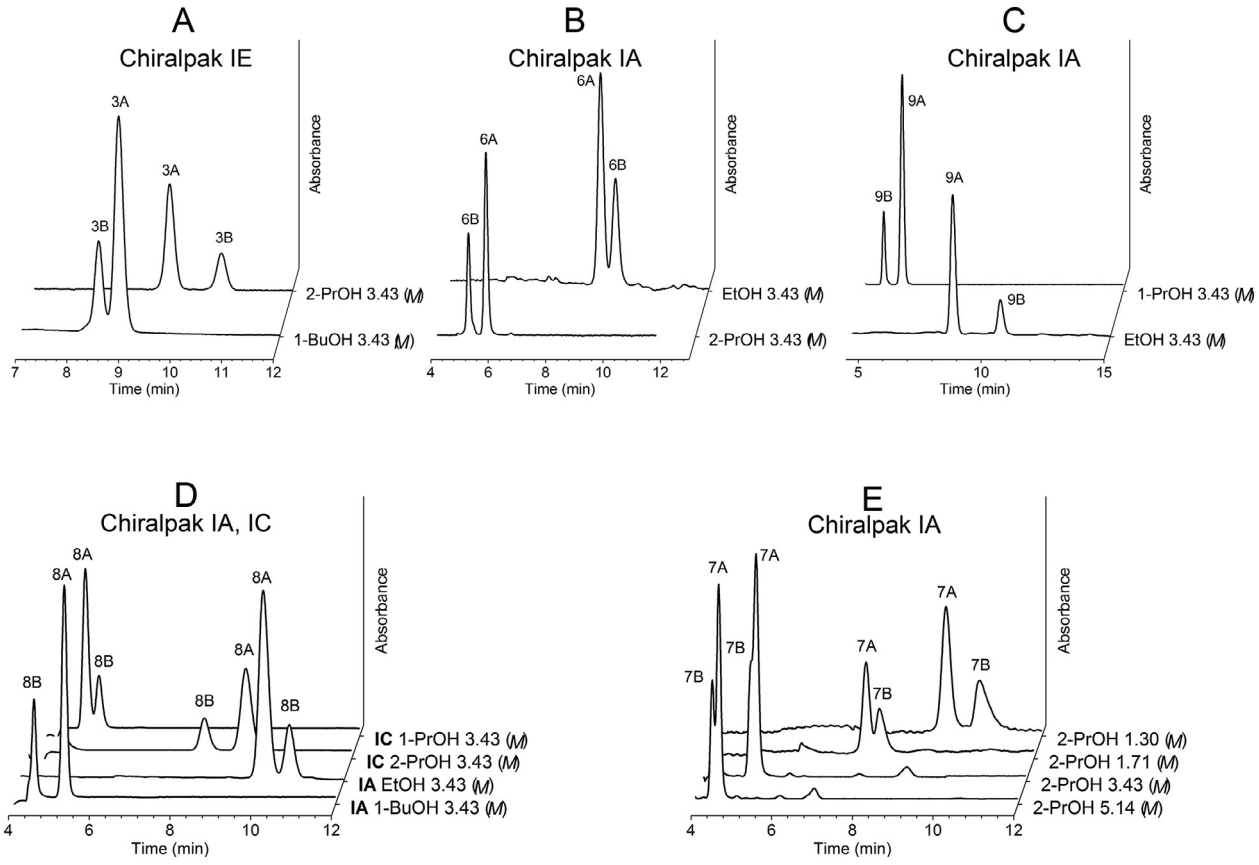

Fig. 6. Representative chromatograms for the change of the enantiomer elution order with the change of the nature and concentration of alcohol modifier Chromatographic conditions: analytes, **A, 3; B, 6; C, 9, D, 8; E, 7**; columns, **A**, Chiralpak **IE; B, C** and **E**, Chiralpak **IA; D**, Chiralpak **IA** and **IC**; mobile phase, **A**, *n*-hexane containing 3.43 M 1-PrOH or 1-BuOH, **B**, *n*-hexane containing 3.43 M EtOH or 2-PrOH; **C**, *n*-hexane containing 3.43 M EtOH or 1-PrOH; **D**, *n*-hexane containing 3.43 M EtOH or 1-PrOH (Chiralpak **IA**) or 3.43 M 1-PrOH and 2-PrOH (Chiralpak **IC**); **E**, *n*-hexane containing 1.30, 1.71, 3.43 and 5.14 M 2-PrOH; all mobile phase contained 0.1 %(*v/v*) DEA; flow rate 1.0 ml min^{-1}; detection at 220 nm; temperature, 25°C.

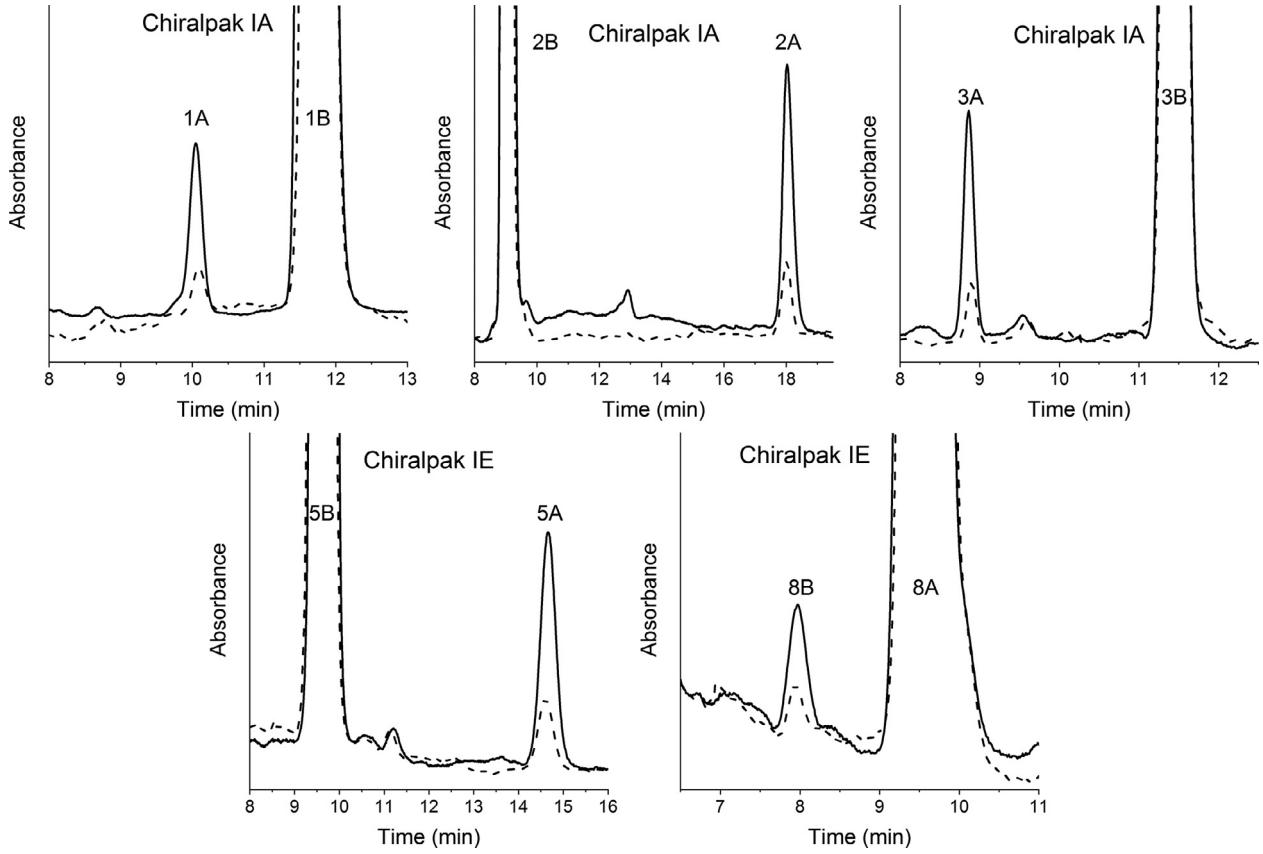

Fig. 7. Selected chromatograms to illustrate the determination of enantiomeric impurities. The dotted line represents the chromatogram of the sample containing one (major) enantiomer, the continuous line represents when the sample is spiked with 0.1% enantiomeric impurity Chromatographic conditions: analytes, **1, 2, 3, 5**, and **8**; columns, Chiralpak **IA** and **IE**; mobile phase, *n*-hexane/EtOH/DEA 80/20/0.1 (*v/v/v*); flow rate 1.0 ml min^{-1}; detection at 220 nm; temperature, 25°C.

tions with the selector reducing the possible effects of the polarity of the analytes. A comparison of analyte **5** with its ester analog (**10**) reveals, that the carbamate moiety in **5** is capable of enhanced H-bonding interactions with the carbamate group of the selector. Generally, higher k_1, α, and R_S were registered for **5** indicating improved enantiorecognition.

Fig. 4B shows the elution behavior of monoterpene lactones and their ring-opened analogs, *i.e.,* **1, 4, 7**, and **2, 5, 8**, as well as **3, 6, 9**, as a function of the volume of the molecule. Results revealed a marked correlation between retention and the size of the molecule. In most cases, on Chiralpak **IA, IB, IE**, and **IC** columns, k_1 increased in the sequence **7** < **4** < **1, 8** < **5** < **2**, and **9** < **6** < **3**, showing that molecules with smaller size possess higher retention. This finding suggests that a molecule with a smaller size fits better into the chiral cavities of the polysaccharide chains and, therefore, selector–selectand interactions are enhanced. These results are in line with the general understanding that the polarity, size, and structure of the selectands, together with the mobile phase composition, contribute to the observed chromatographic behavior in the case of polysaccharide-based CSPs.

3.5. Enantiomer elution order and determination of minor enantiomeric impurity

The biological activity of chiral pharmaceuticals depends strongly on their stereochemistry; therefore, the chromatographic identification and quantification of the enantiomers have primary importance. In a homologous series of compounds, when the configuration of one enantiomer is known, EEO on some selectors like macrocyclic glycopeptides [25], or *Cinchona* alkaloids [26] can often be predicted. On polysaccharide phases, several factors determine EEO, and numerous examples can be found in the literature for its dependence on CSP, temperature, and eluent composition [11,17,27–32]. In Table 1, EEO observed on the seven studied columns applied under the same chromatographic conditions are summarized.

Fig. 5 depicts chromatograms to illustrate the influence of the polysaccharide-based selectors on the EEO under constant mobile phase composition. EEO reversal was observed when the backbone of the polysaccharide chain or the nature of the carbamate moiety changed. EEO reversed in the case of analyte **3** between the columns having amylose or cellulose *tris*-(3,5-dimethylphenylcarbamate) selector (Fig. 5A) and between the columns having amylose- or cellulose *tris*-(3,5-dichlorophenylcarbamate) selector (Fig. 5B). Reversals in EEO took place when *tris*-(3,5-dimethylphenylcarbamate) was replaced with *tris*-(3,5-dichlorophenylcarbamate) on cellulose-based columns in the case of analyte **5** (Fig. 5C) and on amylose-based columns using analyte **9** (Fig. 5D). Interestingly, as shown in Fig. 6, not only the selector but also the nature and concentration of the polar component of the applied mobile phase exerted a great effect on EEO. Figs. 6A–6D represent typical examples, when reversal of EEO took place due to the change of the nature of alcohol, while Fig. 6E displays chromatograms to show how the variation of 2-PrOH concentration in *n*-hexane on Chiralpak **IA** alters the elution behavior of the enantiomers of analyte **7**.

It has now become evident that in some cases EEO can be manipulated with polysaccharide-based CSPs which can be beneficial in the determination of EEO. Since no theories exist today for the interpretation of the EEO, the identification of the chromatographic

peaks remains vital. From a practical point of view, the determination of the enantiomeric purity of a pharmaceutical product is of utmost importance. According to the FDA recommendation, it must be possible to quantify 0.1% minor component in the presence of the major one. Fig. 7 depicts examples for the determination of minor components in the presence of the major one in a thousandfold excess for some analytes, providing evidence that the developed method is applicable for the quantitative determination of enantiomeric impurities.

3.6. Effect of temperature and assessment of thermodynamic parameters

Despite the limitations of the van't Hoff equation (the non-chiral and chiral components of separation cannot be distinguished [15]), its application to study the temperature dependence of retention and selectivity may provide valuable information on the chiral recognition process [33–36]. To investigate the effects of temperature on chromatographic parameters, a variable-temperature study was carried out with all analytes on four polysaccharide-based CSPs, namely Chiralpak **IA, IB, IE**, and **IC** over the temperature range 10–50 °C. Note, that some analytes were not separable in the whole temperature range (see footnote to Table 2.) To avoid very long retention at lower temperatures, the *n*-hexane/EtOH/DEA 80/20/0.1 (*v/v/v*) mobile phase composition was selected. In general, both the retention factor (k_1) and the selectivity (α) decreased with increasing temperature (Table S3). However, with increasing temperature k_1 decreased in the case of analyte **1** on Chiralpak **IE, 2** on Chiralpak **IC, 5** on Chiralpak **IA** and **IB**, and **7** on Chiralpak **IC**, while α slightly increased. Employing polysaccharide phases for enantioseparations, this phenomenon has been reported in several papers [22,28,29,37,38]. In most cases, R_S also decreased with increasing temperature, but in a few cases, an increase or a maximum curve was reached for the change in resolution (Table S3). To characterize the separations from a thermodynamic point of view, van't Hoff plots were constructed and evaluated as described before.

According to the data in Table 2, $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ exhibit both negative and positive values. Negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values indicate a stronger complex formation between CSP and the second eluting enantiomer, whereas negative entropy is less favorable for enantioseparation. In such case, the enantioseparation was enthalpically-driven and α decreased with increasing temperature. When $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values were both positive, α in-

Table 2
Thermodynamic parameters, $\Delta(\Delta H^o)$, $\Delta(\Delta S^o)$, $T\times\Delta(\Delta S^o)$, $\Delta(\Delta G^o)$, correlation coefficients (R^2) and Q values of the studied analytes on **Chiralpak IA, IB, IC**, and **IE** CSPs.

Analyte	$-\Delta(\Delta H^o)$ (kJ/mol)	$-\Delta(\Delta S^o)$ (J/(mol*K)	Correlation coefficients (R^2)	$-T\times\Delta(\Delta S^o)_{298K}$ (kJ/mol)	$-\Delta(\Delta G^o)_{298K}$ (kJ/mol)	Q
Chiralpak IA						
1	3.7	9.4	0.983	2.8	0.9	1.3
2	8.2	20.7	0.998	6.2	2.0	1.3
3	4.9	13.7	0.999	4.1	0.8	1.2
4	5.0	13.8	0.997	4.1	0.9	1.2
5	-1.3	-5.4	0.999	-1.6	0.3	0.8
6*	3.6	11.5	0.999	3.4	0.2	1.1
7*	1.9	5.7	0.994	1.7	0.2	1.1
8**	0.6	1.3	0.959	0.4	0.2	1.5
9	1.6	2.9	0.961	0.9	0.7	1.8
10	0.5	1.3	0.958	0.4	0.1	1.4
Chiralpak IB						
1	0.9	2.3	0.997	0.7	0.2	1.3
3	0.8	1.7	0.987	0.5	0.3	1.6
5	-2.5	-9.0	0.993	-2.7	0.1	0.9
6	0.6	1.1	0.929	0.3	0.3	1.9
7	1.7	4.5	0.965	1.3	0.4	1.3
8	1.0	1.9	0.977	0.6	0.4	1.7
9	1.2	2.9	0.977	0.9	0.4	1.4
10*	1.7	5.0	0.959	1.5	0.2	1.1
Chiralpak IE						
1	-0.8	-2.6	0.976	-0.8	0.1	0.9
3*	1.4	4.3	0.992	1.3	0.1	1.1
4	0.9	2.4	0.999	0.7	0.2	1.3
5	6.3	16.0	0.999	4.8	1.5	1.3
6	3.6	9.3	0.999	2.8	0.8	1.3
7*	1.7	5.5	0.984	1.6	0.1	1.1
8	2.6	6.4	0.999	1.9	0.7	1.4
9	2.2	5.8	0.999	1.7	0.5	1.3
Chiralpak IC						
1	0.8	1.1	0.984	0.3	0.5	2.5
2***	-1.3	-4.5	0.994	-1.3	0.1	0.9
3	2.2	4.7	0.992	1.4	0.8	1.6
4	4.3	11.2	0.991	3.3	1.0	1.3
5	5.6	14.9	0.998	4.5	1.1	1.3
6	4.8	12.5	0.998	3.7	1.1	1.3
7	-1.3	-5.3	0.982	-1.6	0.3	0.8
8	1.4	3.3	0.994	1.0	0.4	1.4
10	8.0	21.1	0.998	6.3	1.8	1.3

Chromatographic conditions: column, **Chiralpak IA, IB, IC, IE**; mobile phase, *n*-hexane/EtOH/DEA (80/20/0.1 *v/v/v*); flow rate, 1.0 ml min^{-1}; detection 220 nm; $Q = \Delta(\Delta H°)/298\ x\ \Delta(\Delta S°)$. Temperature range: * 10 – 30 °C, **10 – 40 C, *** 30 – 50 °C.

creased with increasing temperature. In this case, the change in the adsorption enthalpy exerted a positive effect on the enantioselectivity, and the positive $\Delta(\Delta S°)$ compensated for the positive $\Delta(\Delta H°)$ resulting in negative $\Delta(\Delta G°)$. Under these circumstances, the enantioseparation is entropically-driven.

A comparison of the studied polysaccharide-based CSPs from a thermodynamic point of view allows to formulate some general trends. Of the four CSPs, the most negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values were obtained most frequently on the amylose-based Chiralpak **IA** and **IE**. Effects of phenylcarbamate derivatives can also be derived. In the case of amylose, slight differences can be found between *tris*-(3,5-dimethylphenylcarbamate) and *tris*-(3,5-dichlorophenylcarbamate). In contrast, in the case of cellulose, generally more negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values were obtained for the *tris*-(3,5-dichlorophenylcarbamate) derivative (Chiralpak **IC** *vs.* **IB**).

To estimate the enthalpy/entropy contribution to the free energy, Q values were calculated at 298 K [$Q = \Delta(\Delta H°)/298\ x\ \Delta(\Delta S°)$]. According to data in Table 2, the discrimination process, in most cases, was enthalpically-driven with $Q > 1.0$ indicating the relatively higher contribution of enthalpy to the free energy. However, in a few cases, $Q < 1.0$ suggesting a predominant entropic contribution to the free energy, leading to entropically-controlled enantioseparations.

4. Conclusions

The aims of this work were to develop liquid chromatographic methods for the enantiomeric separation of amino, thio, and oxy derivatives of monoterpene lactones, amides, and ester and explore the chromatographic properties of these newly synthesized compounds. Applying covalently immobilized polysaccharide-based chiral stationary phases in normal-phase mode efficient separations were achieved. Effects of mobile phase compositions were investigated to gain insights into the chiral recognition processes. Independently from its nature basic additive was found to improve peak shapes without markedly affecting the enantioselectivity. Under the applied conditions, typical normal-phase behavior was observed for all studied analytes, thus changing the nature or concentration of alcohol additive was found to offer a simple option for method optimization. Some general trends could be recognized by the comparison of the effects of the nature of the polysaccharide backbone or the phenylcarbamate moiety. Usually, higher retention could be observed on amylose-based than on cellulose-based CSPs, indicating a better fit in the chiral grooves of amylose for the analytes studied. A comparison of the separation characteristics of the selectors suggests that the H-bonding interactions between the halogen-substituted selector and the studied selectands play an important role.

Relationships between the chromatographic parameters and the chemical properties, such as polarity and volume of the molecule, were explored, however, the relationship between selectivity, resolution, and chemical structure could not always be established. Examples shown for the reversal of the enantiomer elution order call attention to the importance of the identification of individual peaks by the enantiomers with known configurations in case of polysaccharide-based CSPs.

Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Attila Bajtai: Investigation, Writing – original draft, Visualization, Writing – review & editing. **Gábor Németi:** Investigation, Writing – original draft, Writing – review & editing. **Tam Minh Le:** Resources, Writing – original draft. **Zsolt Szakonyi:** Resources, Writing – original draft. **István Ilisz:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463050.

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