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High-performance liquid chromatographic enantioseparation of azole analogs of monoterpene lactones and amides focusing on the separation characteristics of polysaccharide-based chiral stationary phases

Gábor Németi [a], Róbert Berkecz [a], Tam Minh Le [b], Zsolt Szakonyi [b], Antal Péter [a], István Ilisz [a,*]

[a] Institute of Pharmaceutical Analysis, Interdisciplinary Excellence Centre, University of Szeged, H-6720 Szeged, Somogyi u. 4, Hungary
[b] Institute of Pharmaceutical Chemistry, Interdisciplinary Excellence Centre, University of Szeged, H-6720 Szeged, Eötvös u. 6, Hungary

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ABSTRACT

High-performance liquid chromatography-based enantioseparation of newly prepared azole analogs of monoterpene lactones and amides was studied. Effects of additives and mobile phase composition were evaluated both in normal and polar organic modes. Applying amylose *tris*-(3,5-dimethylphenylcarbamate) selector in normal and polar organic modes acid and base additives were found to affect the peak profiles, without significantly influencing the enantiorecognition ability of the studied selector. In most cases, differences observed in retention times and enantioselectivities were lower than 10 and 20 % under normal phase and polar organic conditions, respectively. Under normal phase conditions decreased retention was observed for all the studied analytes with increased eluent polarity. Interestingly, enantioselectivity was only slightly (<10 %) influenced by the variation in the *n*-hexane/2-propanol ratio between 80/20 and 20/80 *v/v*. In polar organic mode, five different neat solvents (acetonitrile, methanol, ethanol, 1-propanol, and 2-propanol) were tested, and the best results were obtained with acetonitrile and ethanol in the case of Lux Amylose-1 column with enantioresolutions most often above 2. Based on results obtained with amylose and cellulose-based columns the amylose *tris*-(3,5-dimethylphenylcarbamate) selector is found to offer a superior performance both in normal and polar organic modes. When evaluating the possible effects of the selector immobilization, no striking differences were found in the normal phase. Usually, enantioselectivities and resolutions were higher (10–20 %), while retention factors of the first peaks were lower (20–30 %), on the coated-type column. In contrast, in polar organic mode, the retention characteristics and enantiorecognition ability of the coated and immobilized selectors were heavily affected by the nature of the polar solvent.

Special attention has been paid to the history-dependent behavior of polysaccharide-based selectors. A confidence interval-based evaluation is suggested to help comparison of the histereticity observed in different systems. Several examples are shown to confirm that the recently discovered hysteresis is a common characteristic of polysaccharide-based selectors.

1. Introduction

Heterocyclic compounds are of paramount interest in medicinal chemistry. Among them, imidazoles and triazoles, as well-known five-membered heterocyclic ring systems, have important properties in various medicinal agents [1]. The imidazole nucleus forms the main structure of several well-known components of human organisms, and it is frequently found as a constituent of various synthetic drugs, including widely prescribed drugs such as cimetidine, etomidate, ketoconazole, and clotrimazole [2]. Furthermore, drugs that have imidazole functionality also act as anticancer, antiviral, anti-HIV, antiprotozoal, antimycobacterial, anti-inflammatory, analgesic, anxiolytic, and antidiabetic agents, meanwhile, the benzimidazole ring system can be found, for example, in a blockbuster drug structure esomeprazole [3].

1,2,3-Triazoles are key scaffolds in many molecular architectures that display antitubercular, antifungal, anticancer, antibacterial, antioxidant, and antiviral activities [4]. In addition, the 1,2,3-triazole nucleus has also been incorporated into various therapeutically attractive drug candidates, including tazobactam and cefatrizine as an antibiotic, carboxyamidotriazole as an anticancer, mubritinib as a protein kinase

* Corresponding author.
E-mail address: ilisz.istvan@szte.hu (I. Ilisz).

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inhibitor, rufinamide as an anticonvulsant, and clozapine as an antipsychotic drug [5]. In addition, 1,2,4-triazole derivatives possess important pharmacological activities such as antifungal, antiviral, antiasthmatic, anticonvulsant, antidepressant, anti-inflammatory, and insecticidal properties [6]. Since monoterpenes usually possess remarkable biological activities, their coupling with azoles seems promising for preparing enantiomeric *N*-heterocycles and pyrazoles, imidazoles, and triazoles. As a result of the very likely pharmacological differences of the individual enantiomers of the chiral analytes, it is necessary to develop effective methods for their efficient enantioseparations.

High-performance liquid chromatography applying chiral stationary phases (CSPs) is most commonly used in enantioselective separations. After commercialization, polysaccharide-based CSPs soon became the most popular tools in direct HPLC-based enantioseparations. Their wide range of applicability has been proven for the diversity of chiral analytes in the last two decades [7–10]. As a result of the ease of preparation, the so-called coated phases were initially commercialized. Their disadvantageous property is their incompatibility with some solvents (e.g., tetrahydrofuran, ethyl acetate, *tert*-butyl ether, chlorinated solvents, etc.), which may dissolve or swell the polysaccharide coating, drastically reducing or even killing the column's efficiency. By covalently immobilizing the polysaccharide selector onto the surface of the silica support, this limitation factor could be eliminated, and any standard or non-standard solvent became available as a mobile phase component or neat solvent.

Despite the numerous studies on the enantiorecognition mechanism of polysaccharide-based CSPs, an important phenomenon, i.e., their hysteretic behavior, has only been described very recently [11–14]. Zhang and Franco mentioned changes in column selectivity when applying non-standard solvents in the case of Chiralpak IA, the first commercially available immobilized column [15]. They explained the phenomenon with a change in the supramolecular structure of the polymeric chains. Recently, Horváth and Németh provided several examples of the history-dependent retention behavior in the case of the amylose *tris*-(3,5-dimethylphenylcarbamate)-based (ADMPC) selectors [11]. As a realistic explanation, a hindered transition of the higher-order structure of the ADMPC selector was accounted for the observed hysteresis effects. Later, the same authors [12–14] and others [16,17] reported more examples of how the eluents previously used on a particular column affect the column performance. Based on these findings, the hysteretic behavior seems a more general characteristic, at least for the amylose-based CSPs.

The aims of this work were: i) to study the chromatographic behavior of newly synthesized azole analogs of monoterpene lactones and amides by applying amylose- and cellulose-based *tris*-(phenylcarbamate)-type CSPs, ii) to compare the effectiveness of normal-phase (NP) and polar organic (PO) mode separations, iii) to evaluate the possible effects of the mobile phase additives in different chromatographic modes, iv) to compare the separation characteristics of coated and immobilized CSPs, and v) to shed more light on the recently described phenomenon of hysteresis.

2. Materials and methods

2.1. Chemicals and reagents

The structures of the analytes studied are presented in Fig. 1, while their chemical names are listed in Table S1. The physical and chemical properties of the newly synthesized compounds, related analytical data, and the description of the synthetic routes are presented in Supplementary Information.

Acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), and *n*-hexane of HPLC grade, diethylamine (DEA), formic acid (FA) and other reagents of analytical reagent grade were purchased from VWR International (Radnor, PA, USA).

2.2. Apparatus and chromatography

Measurements were carried out on two HPLC systems. The first is a Waters Breeze system consisting of a 1525 binary pump, a 2996 diode array detector, a 717 plus autosampler, and Empower 2 data manager software (Waters Corporation, Milford, MA, USA). The columns were thermostated with a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands). The second HPLC system is a Shimadzu Prominence HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A system controller, a DGU-20A solvent degasser, an LC-20AB binary pump, an SPD-M20A photodiode array detector, a CTO-20AC column oven, and a SIL-20AC autosampler. LabSolution chromatographic data software (Shimadzu Corporation, Kyoto,

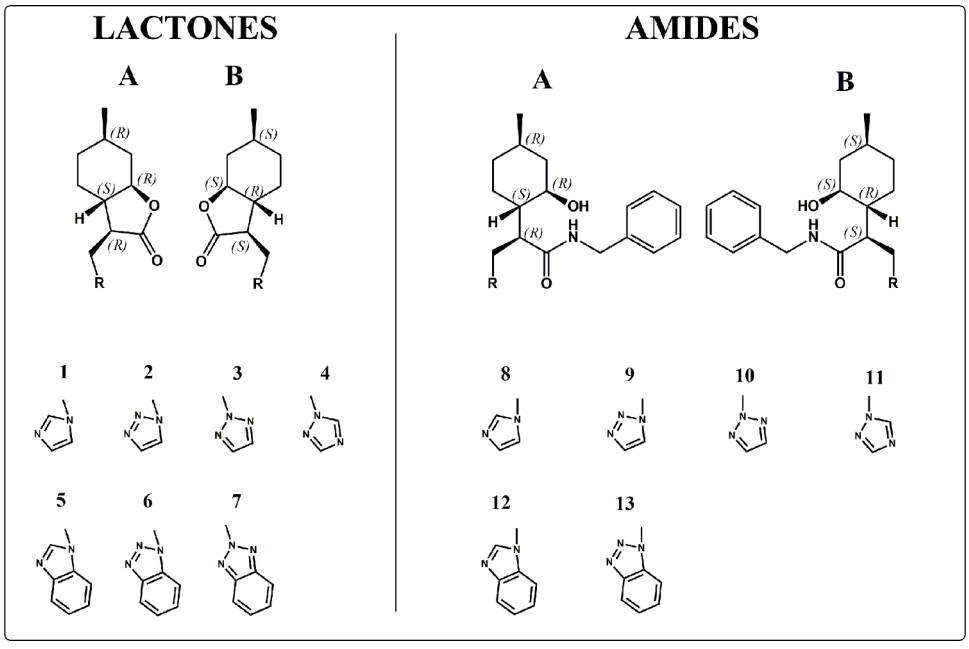

Fig. 1. Structure of the studied analytes.

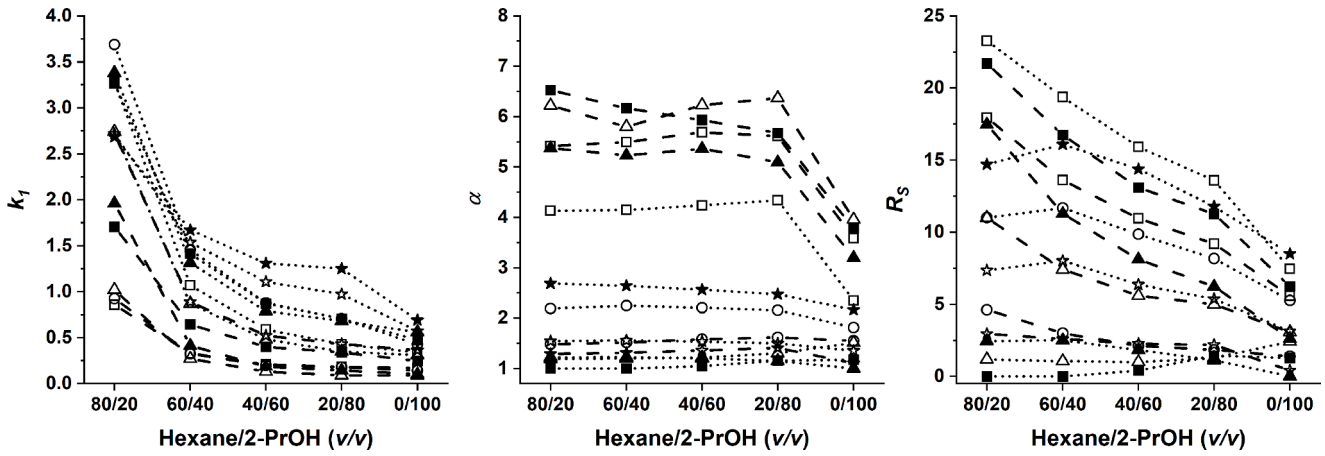

Fig. 2. Effects of eluent composition in normal phase mode on the chromatographic parameters in the separation of azole analogs of monoterpene lactones and amides applying Lux Amylose-1 CSP.
Chromatographic conditions: column, Phenomenex Lux Amylose-1; mobile phase, *n*-hexane/2-PrOH (80/20–0/100 *v/v*); flow rate, 1.0 ml min^{-1} (0.5 ml min^{-1} in 100 % 2-PrOH); detection, 205–215 nm; temperature, 25 °C; symbols for analyte 1,•••△•••; 2,•••□•••; 3,•••☆•••; 4,•••○•••; 5,•••▲•••; 6,•••■•••; 7,•••★•••; 8,—△—; 9,—□—; 10,—☆—; 11,—○—; 12,—▲—; 13,—■—.

Japan) allowed the acquisition and processing of chromatographic data.

The HPLC columns used were Lux Amylose-1 and Lux i-Amylose-1, both with amylose *tris*-(3,5-dimethylphenylcarbamate) selector, Lux Cellulose-1 with cellulose *tris*-(3,5-dimethylphenylcarbamate) selector and Lux Cellulose-4 with cellulose *tris*-(4-chloro-3-methylphenylcarbamate) selector. All columns have the same physical dimensions (250 × 4.6-mm ID, 5-μm particle, Phenomenex, Torrance, CA, USA). The column's hold-up time (t_0) was determined by injecting the solution of tri-*tert*-butyl benzene.

All analytes were dissolved in 2-PrOH in the concentration of 1.0 mg mL^{-1}, further diluted with the eluent when necessary, and injected as a 20-μL sample. The enantiomeric elution order (EEO) was determined for all compounds by adding one of the enantiomers in excess to the racemic mixture.

3. Results and discussions

3.1. Study of mobile phase composition and selector immobilization in the normal-phase mode

The compounds studied (Fig. 1) possess either a lactone (analytes **1**–**7**) or an amide (analytes **8**–**13**) skeleton with an *N*-azole or *N*-benzoazole functional group with different numbers and positions of nitrogen atoms in the ring. The chemical differences between the analytes allow us to explore relationships between enantiorecognition and the chemical structure.

Polysaccharide-type CSPs are often used under normal-phase (NP) conditions with mixtures of a nonpolar hydrocarbon (most commonly *n*-hexane or *n*-heptane) and an alcohol (e.g., EtOH, 1-PrOH, or 2-PrOH). To reduce secondary interactions and achieve favorable peak shapes, applying acid and/or base additives may be advantageous [7–10]. Based on previous results obtained in the case of structurally related compounds [18,19], to investigate the effects of additives, a mobile phase composed of *n*-hexane and 2-PrOH (80/20 *v/v*) was chosen, and DEA and/or FA as an additive was applied at 0.1 *v*% concentration. The results obtained with the Lux Amylose-1 column showed a slight additive effect on chromatographic performance (Table S2). The differences in retention times and enantioselectivities are lower than 10 % in most cases, whereas the resolutions changed a little more significantly. Based on these findings and taking the observed invariance of the EEO into account, it can be stated that for the studied analytes, the additive may affect the peak profile, but it has no significant effect on the enantiorecognition ability of the ADMPC-based CSP applied under NP conditions.

Interestingly, analytes **1** and **8** behaved differently; adding FA resulted in longer retention and erased enantiorecognition. Analytes **1** and **8** have the highest pK_a values among the analytes studied. (The values of pK_a, presented in Table S3, were calculated for the protonated bases with Marvin Sketch v. 22.16, ChemAxon, Budapest.) In the presence of FA, the formation of ion-pairs from the protonated base and the formate ion can be a plausible explanation for the higher retention and the diminished enantiorecognition, however, additional effects of the molecular structure can not be ruled out. When comparing the retention factors of lactones (analytes **1–7**) vs. amides (analytes **8–13**), it is worth mentioning that despite the presence of the extra aromatic ring, amides were less retained in the presence or the absence of a mobile phase additive (Table S2). Further experiments under NP conditions were performed without using any additive.

To explore the influence of mobile phase composition on chromatographic parameters in the case of the Lux Amylose-1 column, mobile phases of *n*-hexane and 2-PrOH were applied in different ratios. The results presented in Fig. 2 show a retention behavior that is often observed under NP conditions [18–21]. Increasing the ratio of the alcoholic modifier resulted in lower retentions for all analytes. The marked decrease observed in retention with increasing polar modifier content can be explained by the increased polarity of the eluent that causes a reduced strength of H-bonding formed between the polysaccharide-based selector and the analytes, and enhanced analyte solubility [18,22]. Interestingly, enantioselectivity is much less affected by the change in the *n*-hexane/2-PrOH ratio, suggesting that in addition to H-bonding, non-selective interactions are markedly reduced. In similar mobile phase systems, the alcohol modifier was found to be incorporated into the ADPMC CSP [23,24]. The displacement of *n*-hexane by the polar modifier led to remarkable alterations in the steric environment of the chiral cavities of the ADMPC selectors. Changes in the higher-order structure of the polysaccharide chains may affect both the selective and non-selective interactions that resulted in the observed chromatographic behavior.

The ADMPC selector showed unexpectedly high enantioselectivities, even when neat 2-PrOH was applied, baseline separations could be achieved for nine analytes out of thirteen (Fig. 2). Regardless of the mobile phase composition, amides were less retained than lactones,

suggesting that the lactone unit significantly contributed to retention. However, the interactions formed in the presence of the lactone unit were mostly non-selective, rarely leading to higher enantioselectivities. No change in EEO has been observed due to the variation in the *n*-hexane/2-PrOH ratio.

In a set of experiments, the Lux Cellulose-1 column (with the same DMPC selector but linked to cellulose backbone) applying a mobile phase composed of *n*-hexane and 2-PrOH (80/20 *v/v*) was studied to gather information about the possible effects of the polysaccharide backbone. Under the applied conditions, significantly lower enantioselectivities were obtained, and only one analyte (**6**) could be baseline resolved (data not shown). Compared to the Lux Amylose-1, no reversal in EEO was observed.

Immobilization of the selector offers a much broader application range and the choice of utilizing non-standard eluents for the enantioseparations, however, it may have a marked influence on the chiral recognition ability [19,25–27]. A set of experiments was carried out to compare the separation performances of covalently immobilized (Lux i-Amylose-1) and coated (Lux Amylose-1) ADMPC-based CSPs. Under NP conditions applying *n*-hexane/2-PrOH (80/20 *v/v*) eluent, twelve and eleven out of thirteen analytes could be baseline resolved using coated and immobilized ADMPC selectors, respectively (Table S4). In most cases, the retention factors of the first peaks were lower, while the enantioselectivities and resolutions were higher on the coated-type column. When comparing the so-called success rates (number of efficient separations) achieved with coated and immobilized CSPs, similar results to ours were mentioned in several publications, that is, no striking differences were reported between the coated and immobilized phases [27,28]. However, it is important to note that, in these studies, unlike our approach, only a generic mobile phase is usually applied without optimization for the studied separation. Regarding the retention behavior and enantioselectivity observed in our experiments, Thunberg et al. reported similar results, suggesting that the higher degree of achiral interactions led to longer retentions and lower enantioselectivities on the immobilized phase [29]. Reversed EEO is often reported when an immobilized selector is applied instead of a coated one [19,27], however, in our case, the same EEO was observed on both CSPs. (Analyte **6** could not be resolved on the coated phase, however, it was partially separated on the immobilized phase employing a mobile phase composed of *n*-hexane and 2-PrOH 80/20 *v/v*.)

3.2. Study of solvent choice and selector immobilization in the polar-organic mode

The choice of solvent influences, on the one hand, the available interactions and, on the other hand, the higher-order structure of the polysaccharide chains, thus the enantiorecognition ability of the polysaccharide-based CSPs [23,24,30,31]. Compared to traditional NP applications, polar organic solvents can offer shorter analysis times with different selectivities when polysaccharide-based CSPs are applied [32–34]. To investigate the influence of the nature of the eluent on the chromatographic parameters in PO mode, five different neat solvents, MeCN, MeOH, EtOH, 1-PrOH, and 2-PrOH were used. The results obtained with the Lux Amylose-1 column are presented in Table 1.

Taking into account the number of baseline separations, the best results were obtained with MeCN and EtOH; both solvents offered twelve enantioseparations with $R_s > 1.5$ of thirteen analytes. Comparing the results obtained with different alcohols, in most cases, retention factors of the first peaks reduced in the order EtOH > MeOH > 1-PrOH > 2-PrOH. Considering the relative polarity of the alcohols (0.546, 0.617, 0.654, 0.762 for 2-PrOH, 1-PrOH, EtOH, and MeOH, respectively [35]), it seems that the higher the polarity, the longer the retentions are, except for EtOH. EtOH provided the longest retentions among the studied alcohols. Typically, better separations were achieved for lactones applying MeOH, while amides could be separated with higher enantioselectivities and resolutions applying EtOH or 1-PrOH. These findings suggest that the nature of alcohol affects the interactions formed between the selector and the analyte not only through its polarity. Compared to NP conditions, EEOs were the same in EtOH and different for analyte **10** in MeCN, analyte **5** in MeOH and 2-PrOH, and analytes **1** and **3** in 1-PrOH. As mentioned above, a change in the nature or concentration of alcohols was found to lead to conformational changes in polysaccharide chains [23,31]. These changes will affect the observed chiral selectivity of the CSP, depending on the size and structure of the analyte, so generalization is difficult to make.

Polysaccharide-based CSPs are very frequently applied in the NP mode, and quite a few studies compared coated and immobilized phases

Table 1
Effects of polar organic solvents on the chromatographic parameters in the separation of azole analogs of monoterpene lactones and amides applying Amylose-1 CSP.

Analyte	k_1, α, R_S	Mobile phase				
		MeCN	MeOH	EtOH	1-PrOH	2-PrOH
1	k_1	1.30	0.57	0.66	0.49	0.20
	α	2.31	4.00	1.99	1.17	1.77
	R_S	6.20	8.05	7.73	1.24	2.72
	EEO	A < B	A < B	A < B	B < A	A < B
2	k_1	0.59	1.59	1.52	0.87	0.22
	α	2.10	1.55	1.85	1.67	2.65
	R_S	4.46	7.17	9.81	6.53	5.94
	EEO	A < B	A < B	A < B	A < B	A < B
3	k_1	0.77	3.20	4.10	1.77	0.46
	α	3.40	5.20	2.68	1.03	1.35
	R_S	10.56	28.84	18.78	0.34	2.75
	EEO	A < B	A < B	A < B	B < A	A < B
4	k_1	1.33	2.32	2.87	1.37	0.37
	α	1.71	2.35	1.57	1.18	1.69
	R_S	3.98	14.82	8.04	2.45	3.87
	EEO	A < B	A < B	A < B	A < B	A < B
5	k_1	1.04	1.16	2.14	0.91	0.69
	α	3.02	1.17	1.00	1.00	1.10
	R_S	6.76	1.13	0.00	0.00	0.70
	EEO	A < BA < B	B < A	–	–	B < A
6	k_1	0.53	1.40	1.64	0.83	0.39
	α	1.14	1.06	1.32	1.48	1.18
	R_S	0.55	0.84	4.18	4.84	1.12
	EEO	A < B	B < A	B < A	B < A	A < B
7	k_1	0.58	2.07	3.07	1.79	0.54
	α	9.79	18.19	5.66	1.98	2.12
	R_S	14.38	29.20	26.62	11.04	7.16
	EEO	A < B	A < B	A < B	A < B	A < B
8	k_1	3.30	0.13	0.37	0.14	0.06
	α	1.80	2.39	5.08	4.87	3.36
	R_S	2.36	0.97	4.77	6.35	1.99
	EEO	B < A	B < A	B < A	B < A	B < A
9	k_1	2.16	0.26	1.00	0.34	0.15
	α	1.56	4.99	9.11	44.85	3.27
	R_S	2.35	2.50	10.95	10.07	4.91
	EEO	B < A	B < A	B < A	B < A	B < A
10	k_1	2.51	0.51	1.61	1.30	0.33
	α	2.15	4.70	3.49	1.00	1.00
	R_S	3.05	2.76	9.55	0.00	0.00
	EEO	B < A	A < B	A < B	A < B	–
11	k_1	1.80	0.23	0.92	0.32	0.12
	α	1.96	6.01	6.08	5.38	1.50
	R_S	3.08	2.86	9.93	10.60	1.16
	EEO	B < A	B < A	B < A	B < A	B < A
12	k_1	2.45	0.28	0.59	0.26	0.07
	α	1.99	1.00	1.87	2.75	2.86
	R_S	2.53	0.00	1.92	4.68	2.09
	EEO	B < A	–	B < A	B < A	B < A
13	k_1	2.00	0.74	1.89	0.94	0.19
	α	2.96	1.63	4.66	14.79	3.32
	R_S	3.73	0.77	7.47	9.24	5.56
	EEO	B < A	B < A	B < A	B < A	B < A

Chromatographic conditions: column, Phenomenex Lux Amylose-1; mobile phase, 100 % MeCN, MeOH, EtOH, 1-PrOH, 2-PrOH, flow rate, 1.0 ml min^{-1} (0.5 ml min^{-1} in 100 % 1-PrOH and 2-PrOH); detection, 205–215 nm; temperature, 25 °C.

in the PO mode [11,16,28,29,36]. To explore the possible similarities and differences of coated and immobilized ADMPC-based CSPs in PO mode, experiments were carried out with five neat solvents (MeCN, MeOH, EtOH, 1-PrOH, and 2-PrOH) and a reduced set of analytes **5, 6, 8**, and **9**, representing all the typical structural characteristics of the studied compounds (amide vs. lactone, azole vs. benzoazole). The results obtained are presented in Table S5. Compared to the NP mode (Table S4), much higher differences can be seen, however, these differences are strongly dependent on the nature of the polar solvent. In the case of MeCN, values of k_1 are drastically reduced on the immobilized phase, and the enantiorecognition ability of the immobilized ADMPC-based selector completely disappeared in three of four cases. Despite these findings, in the case of analyte **9**, slightly higher selectivity and resolution were obtained with the immobilized selector. In the case of MeOH, higher enantioselectivities and resolutions were obtained with the immobilized phase three times out of four, while k_1 was markedly reduced for all the analytes studied. Interestingly, for analyte **5**, a reversal of EEO was also observed. In the case of EtOH, retention factors of the first peaks were markedly reduced on the immobilized selector. In most cases, the coated CSP offered better performance. Still, at least partial separation could be achieved for all the studied analytes with the immobilized CSP. Regarding the retention behavior, the results obtained in the presence of 1-PrOH or 2-PrOH were significantly different. In all cases, higher k_1 was obtained on the immobilized selector, however, no general trend could be seen when comparing the enantioselectivities and resolutions. The nature of the polar solvent affects retention and enantiorecognition in several ways, resulting in pronounced differences, even in the case of structurally related compounds.

In a similar set of experiments, as discussed in the previous chapter, the Lux Cellulose-1 column was studied with 100 % MeCN, MeOH, EtOH, 1-PrOH, and 2-PrOH to explore how the polysaccharide backbone affects the chiral recognition ability. Like under NP conditions, when PO mode was applied, markedly reduced enantiorecognition ability was obtained with the cellulose-based CSP, and none of the studied analytes could be baseline separated in any of the five polar organic solvents (data not shown). Based on these results, it can be concluded that the amylose-based CSP offers a much better fit for the studied analytes than the cellulose-based one, independently of the applied chromatography mode.

3.3. Effects of additives in the polar-organic mode

Based on results discussed in earlier papers, additives were expected to affect chiral recognition more markedly in PO than in NP mode [37,38]. To investigate the possible effects of additives in the PO mode, measurements were carried out with the same acid and base additives as in NPM (FA and DEA) using neat EtOH or MeCN as the eluent. In HPLC experiments, additives are most frequently added to the bulk eluent in volume percentage. Comparing the results might be difficult in these cases since the molar concentrations will differ. To make the results easier to compare in this series of experiments, 1:1 and 2:1 molar ratios of FA and DEA were applied. (The molar concentration of DEA was set at 9.71 mM, which corresponds to 0.1 *v*%, while the 2:1 molar ratio of FA and DEA approximately corresponds to 0.1/0.1 *v*/*v*% .)

Contrary to expectations, the data obtained with MeCN (Table S6) and EtOH (Table S7) as bulk solvents showed only a slight additive effect on the chromatographic parameters. In most cases, the applied additives did not significantly affect either retention or enantioselectivity. Interestingly, adding FA in the cases of analytes **1** and **8** resulted in poor peak shapes and drastically reduced resolutions in both eluents. As discussed earlier, structurally strongly related analytes **1** and **8** have the highest pK_a values (Table S3), and the formation of ion-pairs was suggested to describe the chromatographic behavior under NP conditions. With polar organic solvents instead of the apolar *n*-hexane, the formation of protonated ions (instead of ion-pairs) can be expected, which can be responsible for the reduced retention and poor peak shape, although the influence of other structure-related effects cannot be excluded. The application of additives did not cause a change in EEO in any of the eluents studied.

3.4. Study of hysteresis

Utilizing mobile phases based on binary solvent systems is the most frequently applied approach for fine-tuning retention and selectivity in liquid chromatography. When changing the composition, typically, it is not expected that, in the case of a given composition, the retention and selectivity could depend on the direction from which the given composition is approached. However, this seems to be the case for polysaccharide-based selectors under certain chromatographic conditions. The eluent history-dependent (hysteretic) behavior of polysaccharide-based CSPs was first reported by Horváth and Németh [11]. Based on the results published so far, the hysteresis phenomenon appears to be a characteristic behavior of amylose-based CSPs [11–13,16,17], while in the case of cellulose-based CSPs, no [16,17] or negligible [11] hysteresis was found. Since the phenomenon is thought to be associated with the higher-order structure of the polysaccharide chains, the way of immobilization of the polysaccharide selectors is expected to influence the hysteresis, as it was reported for a few compounds [11].

Following the protocol described by Horváth et al. [12], a washing procedure with the use of a 2-PrOH/EtOH 50/50 (*v*/*v*) mixture was applied in three column volumes to ensure the column was properly reset to its ``original'' state. Chromatographic parameters with mobile phase compositions of 100/0, 80/20, 60/40, 40/60, 20/80, and 0/100 were measured to create the hysteresis loops. Applying Lux Amylose-1 column in NP mode *n*-hexane/2-PrOH, in PO mode 2-PrOH/1-PrOH, 2-PrOH/MeOH, MeOH/MeCN, and MeOH/EtOH mobile phase systems were investigated. The Lux i-Amylose-1 column was utilized in PO mode with 2-PrOH/MeOH eluents to gather information about the possible effects of selector immobilization. To evaluate the impact of the cellulose backbone on the hysteretic behavior, a cellulose-based column with the same DMPC selector would be the best choice. Unfortunately, Lux Cellulose-1 in PO mode was inefficient in the enantioseparation of the studied analytes, so another cellulose-based column, the Lux Cellulose-4 was employed for this purpose. (Unfortunately, there is no Lux Amylose column available with the same *tris*-(4-chloro-3-methylphenylcarbamate) selector, so the results obtained are compared to the results collected with the ADMPC-based column.)

The hysteretic behavior causes changes in retention times independently for each enantiomer. Since both the degree and the direction of the change in retention time can be different, enantioselectivity may stay constant or the hysteresis of the enantioselectivity also happens. To illustrate all the possible consequences of the phenomenon an example is shown in Fig. 3. Applying Lux Amylose-1 column with a 2-PrOH/1-PrOH mobile phase system the hysteretic behavior resulted not only in remarkable changes in enantioselectivity but also reversed EEO in the case of analyte **3**. To illustrate the phenomenon, instead of chromatograms, a hysteresis curve is generally applied, where k_1, k_2, or α is plotted against the solvent composition. The hysteresis curve is a good solution for visualization of the changes but has some severe limitations. On the one hand, it does not provide information for the single enantiomers about the precise extent and direction of the changes. On the other hand, it does not allow direct comparison of results obtained in different systems. For the quantitative description of the phenomenon, hystereticity (in analogy to selectivity) was introduced very recently by Horváth et al. [14]. Hystereticity factor (ν) was defined as the ratio of the two retention factors determined for the same enantiomer under the same conditions but with two different antecedents, where the larger value is the numerator, and the smaller is the denominator. Here, we suggest a slightly modified definition. Instead of elution order, hystereticity factor is defined based on the direction from which the given composition is approached:

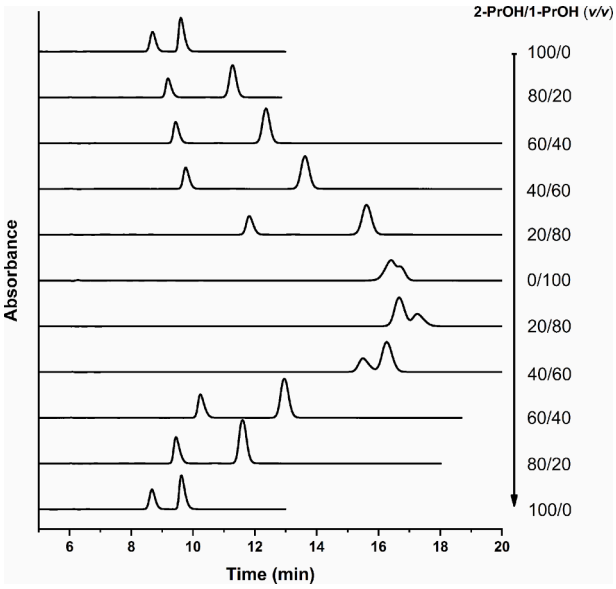

Fig. 3. Chromatographic representation of the hysteretic behavior of the Lux Amylose-1 CSP observed with analyte 3.
Chromatographic conditions: column, Phenomenex Lux Amylose-1; mobile phase, 2-PrOH/1-PrOH (100/0–0/100 *v/v*); flow rate, 0.5 ml min^{-1}; detection, 205–215 nm; temperature, 25 °C.

$$v_A = k_{A_d}/k_{A_f} \quad (1)$$

and

$$v_B = k_{B_b}/k_{B_f} \quad (2)$$

where k_{Af} is the retention factor of enantiomer *A* measured at a certain mobile phase composition from the forward direction (0/100), while k_{Ab} is the retention factor of the same enantiomer *A* measured at the same mobile phase composition from the backward (opposite) direction (from 100/0). Analogously ν_B can be defined for the enantiomer *B* (Eq. (2)). Applying this approach, information on how each enantiomer is affected can be extracted; the higher the deviation of hystereticity from one, the stronger the hysteretic effect is.

In Fig. 4, hysteresis curves of retention factors and enantioselectivities together with hystereticity measured in NPM and POM with Lux Amylose-1 are presented. For a more precise evaluation of whether the deviation of hystereticity from one is significant, statistical evaluation is needed. Relative standard deviations were calculated from three parallel consecutive measurements of three different analytes in three different mobile phase compositions. Confidence intervals of 95 % probability were calculated, while also taking the propagation of error into account. Based on these results (Table S8), the highest value of the RSD (0.8 %) was used for all further calculations, and a confidence interval of 1 ± 0.028 was applied for all presented values. To help evaluation, for the representation of the confidence interval auxiliary lines were drawn in Fig. 4. (All the points out of the confidence interval were considered as significant deviation, i.e. justified hystereticity.) As all the presented curves illustrate, hysteretic behavior was observed for several analytes under different chromatographic conditions. In other words, depending on the structure of the analyte and employed mobile phase in all the studied eluent systems examples for the hysteretic behavior of ADMPC-based selector were found. The simplest way to characterize hystereticity is the calculation of its deviation from one. Since either positive or negative deviation is possible, in the following we apply the absolute value for the representation ($\Delta\nu = |\nu - 1|$). The highest hystereticity ($\Delta\nu > 1$) was observed in PO mode in the PrOH-containing systems, while in the presence of MeOH, EtOH, and MeCN typically modest ($\Delta\nu \geq 0.3$) or low ($\Delta\nu < 0.3$) hystereticity was found. In NP mode (mostly at higher 2-PrOH content) low or insignificant hystereticity was observed. We think this approach is easily applicable to characterize hystereticity from a chromatographic point of view. However, from a mathematical point of view, it suffers from some limitations; since it is based on a division, the scale obtained will be between zero and infinity, with one in the middle. Further refinement of this approach is also possible by the utilization of a logarithmic scale. Using the logarithmic approach results in a symmetrical scale and information can be extracted about the percentage contribution of each enantiomer to the hysteresis. As illustrated in Table S9, the mobile phase composition has a marked effect on the contribution of each enantiomer to the hysteresis. In most cases, one of the enantiomers has a significantly higher contribution to the hysteresis. It is worth noting, that in the 2-PrOH/MeOH eluent at a composition of 40/60 (*v/v*), lactones have positive, while amides have negative log (ν_A/ν_B) values suggesting that structurally strongly related compounds can behave uniformly from a hysteretic point of view.

Hysteresis is thought to be related to the higher-order structure of the polysaccharide chains, so the „freedom" of the polysaccharide chains to arrange themselves in an ordered structure can be expected to affect their hysteretic properties. The potential influence of immobilization on the hysteresis was evaluated with the Lux i-Amylose-1 column and 2-PrOH/MeOH eluent system. As illustrated for analyte **9** in Fig. 5(A) (compared to Fig. 4), the immobilization of the selector has a marked effect on the hystereticity. Compared to the results obtained with the coated phase markedly lower hystereticity ($\Delta\nu < 0.2$) was found. Not only the values of $\Delta\nu$ were lower, but also the mobile phase composition range where the hysteresis occurred was narrower; significant hystereticity was only observed at higher MeOH contents.

Since efficient baseline resolutions could not be achieved with Lux Cellulose-1 to gather information about the possible effects of the polysaccharide backbone Lux Cellulose-4 was employed. In earlier studies, no or negligible hysteresis was found when applying cellulose-based CSPs [11,16,17]. Oppositely to these results, in the case of analyte **5**, we found significant hysteresis, as illustrated in Fig. 5(B). It is worth noting, that in a wider composition range applying a 2-PrOH/-MeOH mobile phase system, the hysteretic behavior was only observed in the case of analyte **5** of the studied analytes. Based on the presented data we think the hysteretic behavior is also characteristic of the cellulose-based CSPs, however, the more rigid structure of the carbamoylated cellulose results in lower sensibility to the eluent history experienced by the column.

4. Conclusions

Amylose and cellulose-based *tris*-(phenylcarbamate) selectors were utilized to study the enantioseparation of azole analogs of monoterpene lactones and amides. The amylose *tris*-(3,5-dimethylphenylcarbamate) selector provided superior enantioseparations compared to the cellulose-based selectors under all the studied chromatographic conditions. The applied acid and base additives in most cases were found to affect peak symmetry without significantly altering the enantiorecognition capability of the amylose-based selector independently from the applied mode of chromatography.

The structurally strongly related analytes showed uniform chromatographic behavior in the normal phase. Retention times of the analytes decreased with increased 2-propanol content, while enantioselectivity was not affected significantly. This chromatographic behavior suggests that H-bonding and non-selective interactions are markedly reduced with increased eluent polarity. The lactone unit (when present in the structure) markedly contributed to retention, mostly through non-selective interactions. Regarding the influence of selector immobilization (coated vs. covalently immobilized), it can be stated that enantioselectivities were higher in most cases with the coated phase, but no remarkable difference was obtained in the number of efficient separations.

Much higher differences were observed in the chromatographic

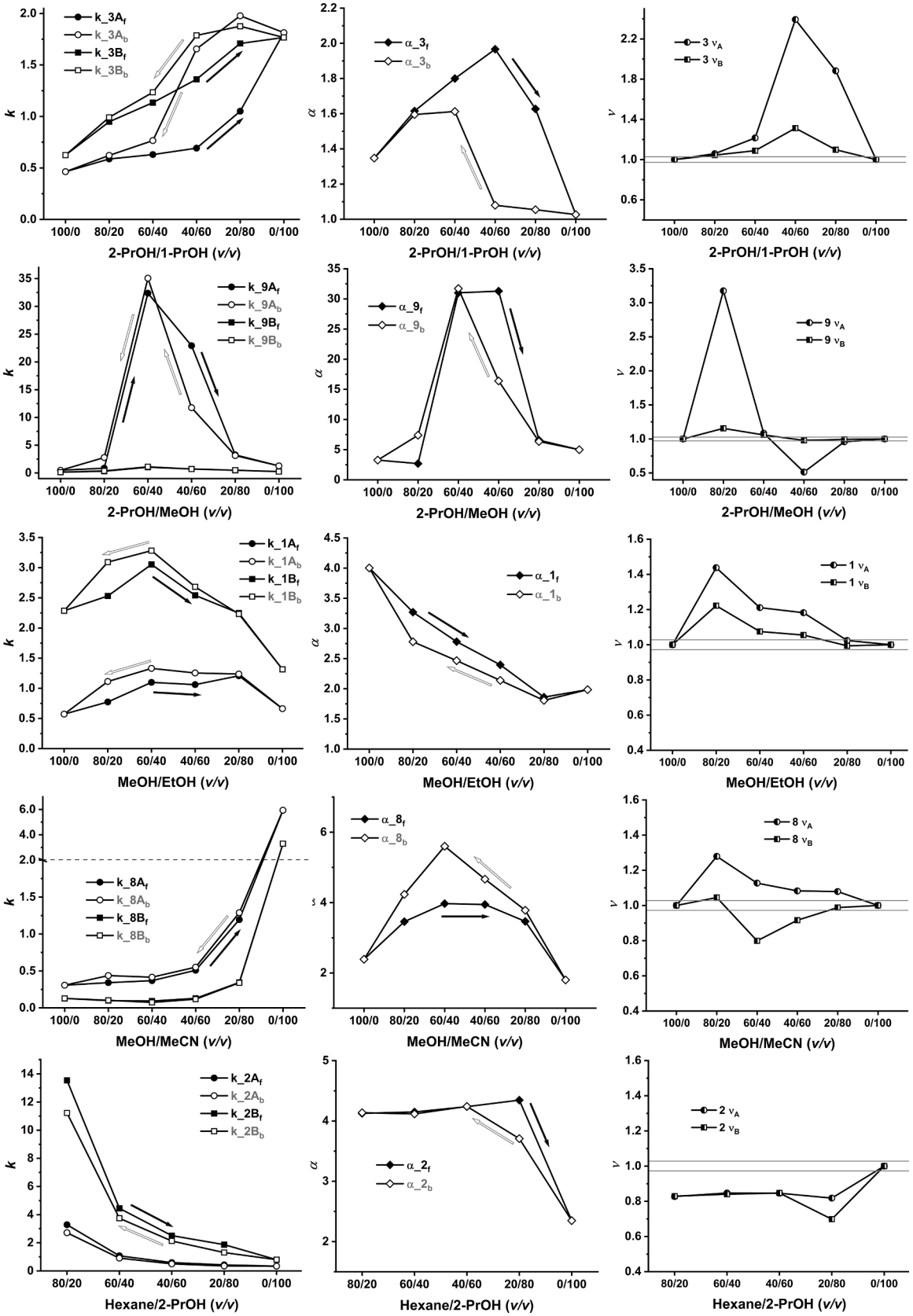

Fig. 4. Hysteresis curves of azole analogs of monoterpene lactones and amides on Lux Amylose-1 CSP.
Chromatographic conditions: column, Phenomenex Lux Amylose-1; mobile phases, 2-PrOH/1-PrOH; 2-PrOH/MeOH; MeOH/EtOH; MeOH/MeCN; hexane/2-PrOH; flow rate, 1.0 ml min^{-1} or 0.5 ml min^{-1} in case of 2-PrOH/1-PrOH and 2-PrOH/MeOH eluent systems; detection, 205–215 nm; temperature, 25 °C.

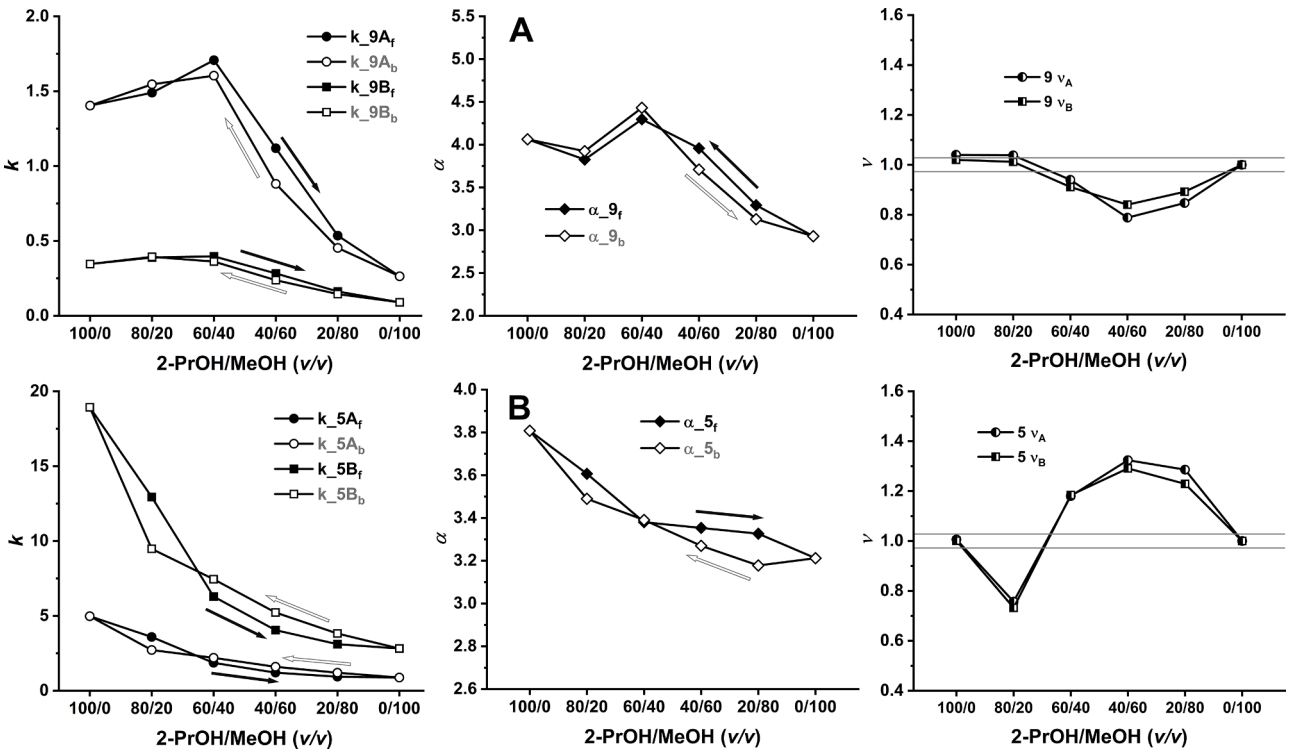

Fig. 5. Hysteresis curves of analyte 9 on Lux i-Amylose-1 CSP (A) and analyte 5 on Lux Cellulose-4 CSP (B).
Chromatographic conditions: mobile phase, 2-PrOH/MeOH (100/0–0/100 *v/v*); flow rate, 0.5 ml min^{-1}; detection, 205–215 nm; temperature, 25 °C.

properties of the analytes in polar organic mode. Neat solvents (acetonitrile, methanol, ethanol, 1-propanol, and 2-propanol) were applied as eluent, and acetonitrile and ethanol offered the highest number of baseline separation in the case of Lux Amylose-1 column. Except for ethanol, the higher the polarity of the alcohol, the longer the retention time was for all the studied azole analogs. Compared to the normal phase, the enantiomeric elution order was reversed in several cases. Immobilization of the amylose *tris*-(3,5-dimethylphenylcarbamate) selector led to reduced retention times for most of the studied analytes when applying acetonitrile, methanol, or ethanol, while 1- and 2-propanol behaved differently. Depending on the structure of the analyte and selector the nature of the polar organic solvent was found to alter the observed chromatographic properties in several different ways, so enantioseparation characteristics are difficult to predict in polar organic mode.

Several new examples were found for the eluent hystory-dependent behavior of the amylose *tris*-(3,5-dimethylphenylcarbamate) selector. For the quantitative characterization of the hystereticity, a simple statistical approach is suggested, offering a reliable evaluation and the possibility to compare the results obtained at different chromatographic systems. As expected, immobilization of the amylose *tris*-(3,5-dimethylphenylcarbamate) selector was found to reduce hystereticity significantly. Contrary to earlier findings, an example was also found for the hysteretic behavior of a cellulose-based selector. However, compared to the amylose-based selector, the cellulose-based selector showed significantly lower sensibility to the eluent history experienced by the column.

CRediT authorship contribution statement

Gábor Németi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Róbert Berkecz:** Writing – original draft, Methodology, Conceptualization. **Tam Minh Le:** Writing – original draft, Resources. **Zsolt Szakonyi:** Writing – original draft, Resources. **Antal Péter:** Writing – original draft, Methodology. **István Ilisz:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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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.2024.464660.

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