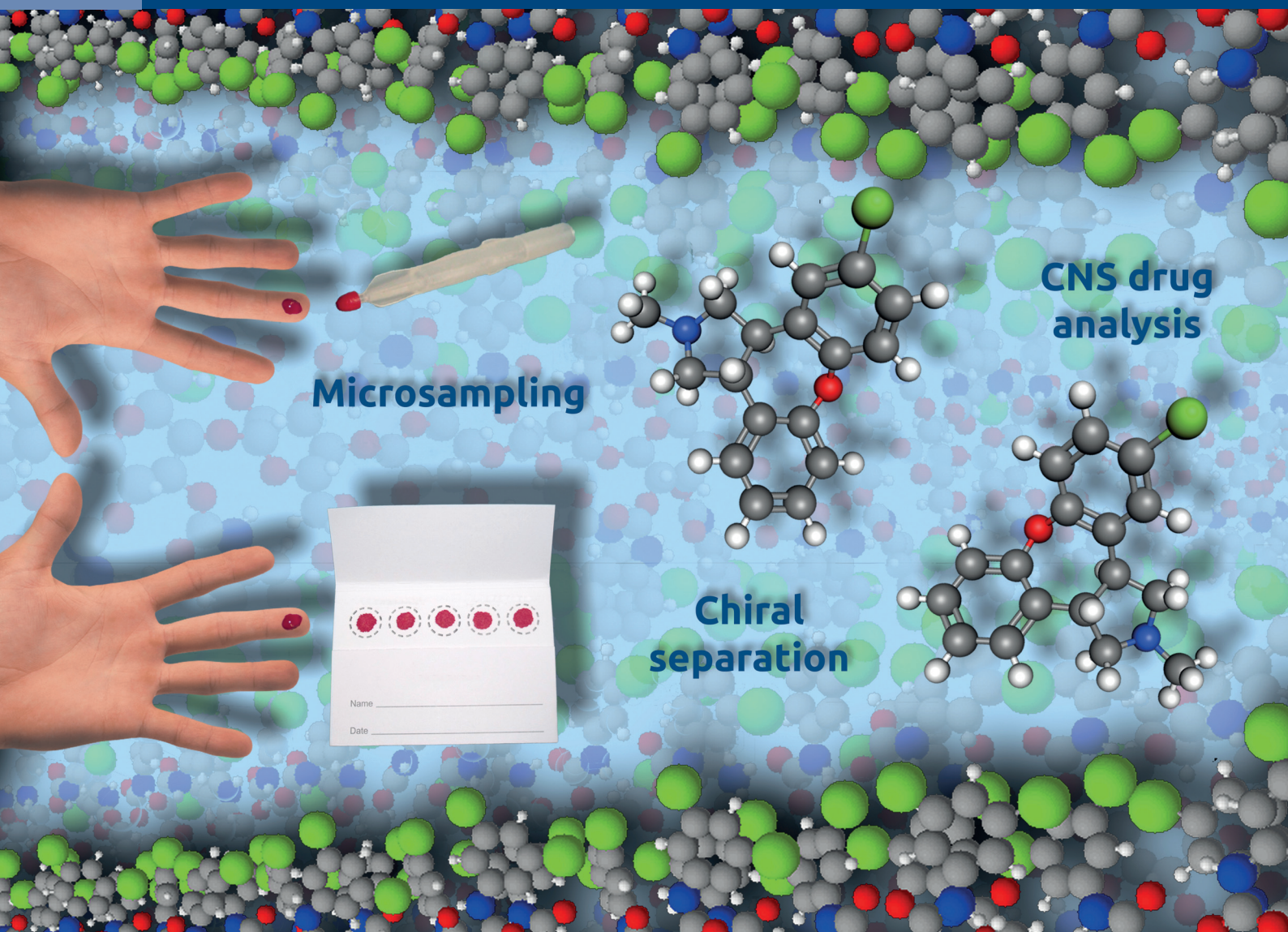


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RESEARCH ARTICLE

Effect of different immobilization strategies on chiral recognition properties of *Cinchona*-based anion exchangers

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In the enantiomeric separation of highly polar compounds, a traditionally challenging task for high-performance liquid chromatography, ion-exchange chiral stationary phases have found the main field of application. In this contribution, we present a series of novel anion-exchange-type chiral stationary phases for enantiomer separation of protected amino phosphonates and N-protected amino acids. Two of the prepared selectors possessed a double and triple bond within a single molecule. Thus, they were immobilized onto silica support employing either a thiol-ene (radical) or an azide-yne (copper(I)-catalyzed) click reaction. We evaluated the selectivity and the effect of immobilization proceeding either by the double bond of the *Cinchona* alkaloid or a triple bond of the carbamoyl moiety on the chromatographic performance of the chiral stationary phases using analytes with protecting groups of different size, flexibility, and π -acidity. The previously observed preference toward protecting groups possessing π -acidic units, which is a typical feature of *Cinchona*-based chiral stationary phases, was preserved. In addition, increasing the bulkiness of the selectors' carbamoyl units leads to significantly reduced retention times, while very high selectivity toward the tested analytes is retained.

KEYWORDS

chiral anion exchange, chiral stationary phases, click-chemistry immobilization, enantiomer separation, N-protected amino acids

1 | INTRODUCTION

The systematic advancement of novel chiral selectors has provided access to a wide range of chiral stationary phases (CSPs), which enable the separation of almost any racemic mixture of interest [1]. The enantiomer separation of highly polar compounds, a particularly challenging task for LC, is the main field of application for CSPs operating on ion exchange mechanism [2]. Ion exchangers are brush-type CSPs, which

are based on small chiral organic molecules (chiral selectors) chemically linked to the surface of a solid support (usually silica). Chiral recognition of a pair of enantiomers by a chiral selector is modulated by the present physicochemical properties of the solid support and the employed immobilization chemistry. Highly polar compounds may interact not only with the selector and the silica surface, but also with the linker part of the selector. Such interactions may lead to increased retention and decreased enantioselectivity, if a high degree of nonenantioselective interaction occurs in the selector-analyte diastereomeric complex formation [3,4].

The effect of the selector linker on the chromatographic performance of a brush-type CSP has been extensively studied. For example, it was shown that surface proximity as well as sophisticated linker chemistry may significantly improve

Abbreviations: APA, aminophosphonic acid; CSP, chiral stationary phase; DCL, dichlorophenyl linker; *n*Pr, *n*-propyl; Prg, propargyl; QD, quinidine; QN, quinine; Tam, triazolomethylene

Conflict of interest: The authors declare no potential conflict of interest.

enantioselectivity [4–7]. Since the development of *Cinchona* carbamate type anion exchangers [8], several studies focused on the systematic optimization of the selector structure have been performed [9–15], including research focused on different anchoring strategies [4,16,17]. Importantly, longer retention times and slightly enhanced enantioselectivity were achieved by attaching the selector by the carbamoyl unit double bond, in comparison to the selector of a similar structure bonded by the *cinchonan* double bond. This clearly indicated stronger interaction of both enantiomers with the silica surface as well as the linker [4]. Therefore, controlled selector loading, enabled by azide-yne click reaction [16,18], can be used to tune the chromatographic parameters of carbamoyl unit-immobilized *Cinchona*-based CSPs.

In this contribution, we report on the differences in chiral recognition properties of selectors immobilized by either radical thiol-ene or copper(I) catalyzed azide-yne approaches. These immobilization strategies create linker units with different steric demands. Consequently, the size of the binding cavity formed by the selectors on the final CSP is expected to vary. To evaluate the effect of the immobilization strategy on enantioselectivity, we analyzed two sets of acids bearing various protecting groups. We show that for certain analytes, the site of immobilization and the size of the linker formed upon immobilization play a crucial role in the chiral recognition process. Our findings will help to create highly selective CSPs for specific analytes.

2 | MATERIALS AND METHODS

Technical grade and HPLC grade solvents were purchased from VWR (Vienna, Austria), Carl Roth (Karlsruhe, Germany), and LachNer (Neratovice, Czech Republic). Dichloromethane used for synthesis was dried by distillation from phosphorous pentoxide before use. Chemicals used for synthesis and mobile phase additives were obtained from Sigma–Aldrich or Fluka (Vienna, Austria). Silica gel for flash chromatography was purchased from Merck (Darmstadt, Germany). HPLC silica (spherical fully porous particles: 5 μm size, 12 nm porosity) was purchased from Daiso Fine Chem (Düsseldorf, Germany) and 3-mercaptopropyl- or 3-azidopropyl-modified in-house. NMR spectroscopy was performed on an Agilent 400-MR DDR2 spectrometer operating at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C . Chemical shifts are referenced internally to the residual nondeuterated solvent. 2D NMR spectra were obtained using standard sequences as supplied by the manufacturer. The selector coverage of the modified silica gel was determined by elemental analysis using a Perkin-Elmer 2400 instrument. Selector coverages were calculated based on nitrogen content.

Chromatographic measurements were carried out using an 1100 Series HPLC system from Agilent Technologies

equipped with an autosampler, a binary pump, degasser, solvent tray, multiple wavelength detector, and switching valve for six columns. Chromatographic data were acquired and processed with ChemStation (Agilent Technologies), and evaluation was carried out with Microsoft Excel 2010. The flow rate was 1 mL/min and as a detector signal the wavelength of 254 nm was chosen. The void volume was determined by injection of methanolic solution of acetone. The sample injection volume was set to 5 μL and sample concentration was 1–2 mg/mL in methanol (MeOH). Elution was performed in isocratic mode using a mobile phase composed of MeOH/AcOH (98:2 v/v) and ammonium acetate (0.5 wt%). Racemic analytes used for the evaluation of the novel AX-type CSPs were either commercially available (Sigma–Aldrich, Bachem) or synthesized in-house according to published procedures.

2.1 | Synthesis of the selectors

Selectors for the studied CSPs (Figure 1) were prepared by two different synthetic pathways. First, the respective alkaloid was activated with 4-nitrophenylchloroformate and subsequently reacted with the appropriate amine. Second, the

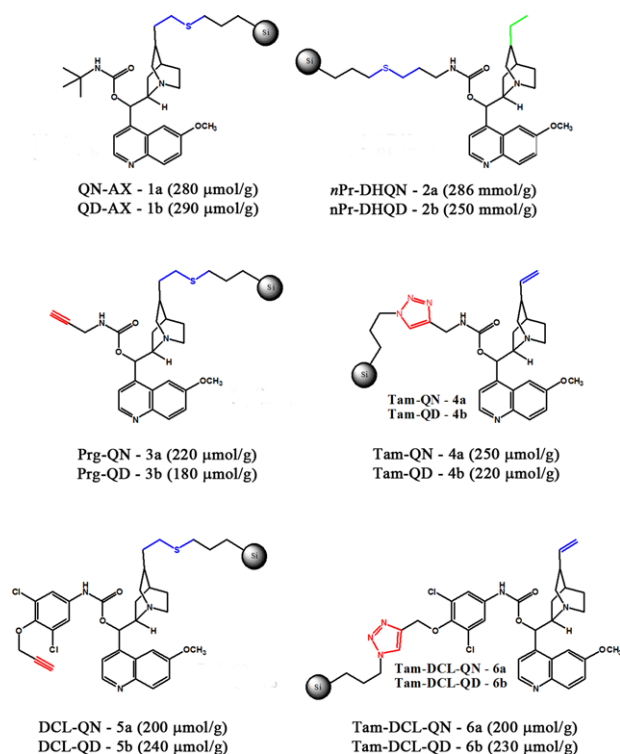


FIGURE 1 List of chiral stationary phases used in the study. Quinine-based stationary phases are denoted as QN, quinidine-based stationary phases are denoted as QD, and DH stands for dihydroquinidine. Prefixes stand for structural parts connected to quinine/quinidine scaffold: *n*Pr, *n*-propyl; Prg, propargyl; Tam, triazolomethylene; DCL, dichlorophenyl linker. Selector coverage of the respective chiral stationary phase is given in parentheses

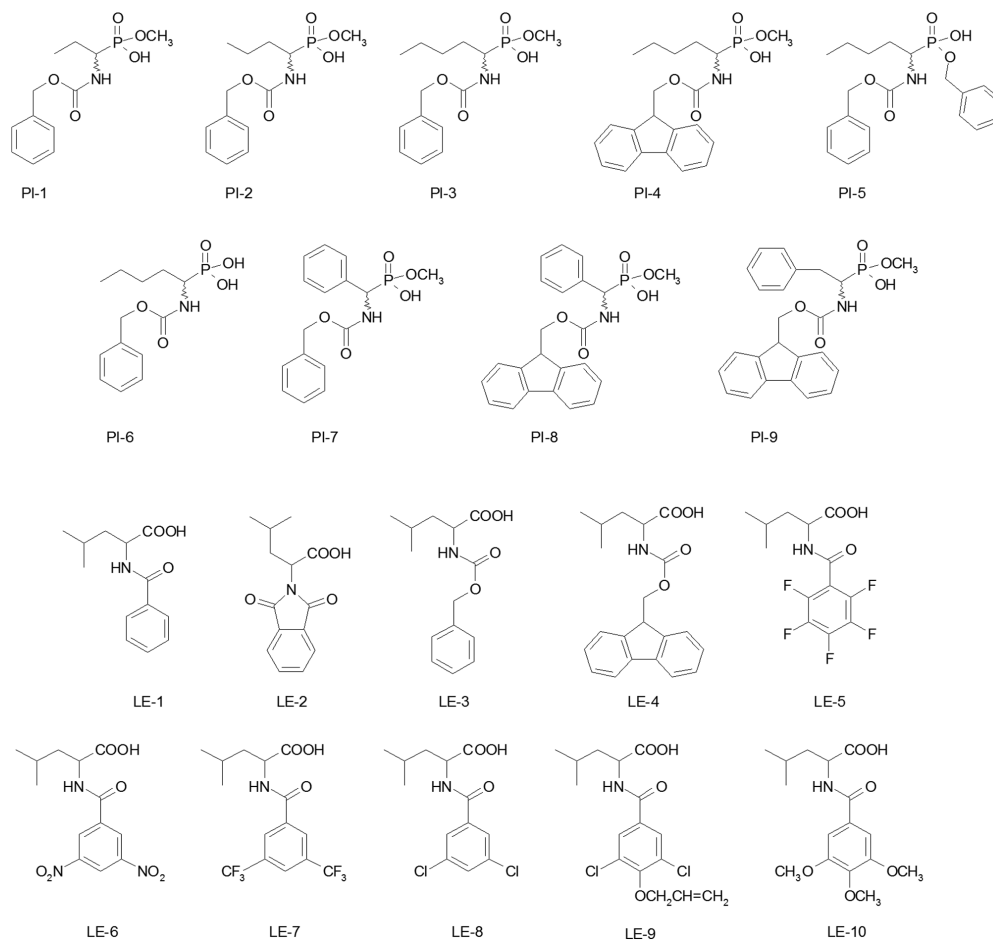


FIGURE 2 Analytes used to evaluate the stereodiscriminatory properties of the library of CSPs. PI-1–PI-9: aminophosphonic acid derivatives; LE-1–LE-10: leucine derivatives

respective alkaloid was reacted directly with the alkylisocyanate to form the target selectors (for details, please see Supporting Information). Commercially available CSPs Chiralpak QN-AX (where QN is quinine) (**1a**) and Chiralpak QD-AX (where QD is quinidine) (**1b**) bearing QN- and QD-based selector, respectively, were used as reference.

2.2 | Immobilization of selectors

To evaluate the rate of immobilization, we used selectors possessing a double and triple bond (**IVb**), only the triple bond in the carbamate part (**Vb**), only the *Cinchona* double bond (**VIb**), and no multiple bond (**VIIb**). The synthesis of selectors and details on immobilization procedures are given in the Supporting Information.

2.3 | Analytes used in the chromatographic evaluation of CSPs

Since the CSPs are chiral anion exchangers, racemic acids are ideal analytes to test chiral recognition properties. Specifically, we determined chromatographic

properties of monomethyl/monobenzyl esters of N-protected aminophosphonic acids (APAs, series PI in Figure 2) and N-protected leucine derivatives (series LE in Figure 2).

3 | RESULTS AND DISCUSSION

3.1 | Synthetic aspects: Immobilization of selectors with different multiple bonds

Since some of the selectors possess both a double and a triple bond, it was necessary to determine if immobilization procedures are specific enough to yield only the intended CSPs (Figure 1). For immobilization by the triple bond, we envisioned a Huisgen 1,3-dipolar cycloaddition. This azide-yne reaction is considered to be selective and tolerant to other functional groups. However, it has already been shown that a double bond can also react with an azide group forming a triazoline ring [19–21]. On the other hand, the improved copper(I)-catalyzed azide-yne click reaction proceeds selectively between an azide group and a terminal triple bond, while it leaves other functional groups intact [22,23]. A

radical reaction is more difficult to control and not only the double bond, but also the triple bond of the selectors can react with a thiol group [24,25]. We determined the rate of immobilization of each type of multiple bond to confirm that double bond immobilization is fast and efficient enough to provide the CSPs with free triple bond (**3a,b** and **5a,b**, Figure 1).

If we assume the constant concentration of the formed radical species, immobilization adopts the first-order reaction kinetics. Thus, the decrease in selector concentration is nonlinear. Moreover, the saturation of the silica support surface with the selector leads to steric hindrance and slower immobilization of next selector molecules. It is therefore reasonable to assume that the determination of concentration decrease in a selector bearing a double bond or a triple bond, both a double and triple bond or no multiple bonds, provides good estimation of the radical reaction selectivity (see Supporting Information, section 3.1). The results indicate that CSPs obtained by the radical immobilization of selectors possessing both multiple bonds comprise molecules bonded exclusively by the double bond, while the triple bond appears to be less prone to engage in the radical reaction. It is conceivable that the triple bond starts to react in the later stages of the immobilization reaction, but the decrease in selector concentration is difficult to follow at the later stages of the reaction due to excessive solvent evaporation. Therefore, the differences observed in chromatographic behavior of the studied CSPs are discussed throughout the paper referring to radical immobilization by double bond exclusively.

3.2 | Chromatographic performance of chiral anion exchangers

All screened CSPs are chiral anion exchangers in nature and are typically operated under polar-organic mobile phase conditions. Since the newly prepared materials are analogues of commercially available Chiralpak QN-AX, previously optimized mobile phase conditions for the enantioseparation of acidic analytes were adopted [8]. Thus, the screening was performed in a mobile phase consisting of MeOH/AcOH (98:2, v/v) mixture with ammonium acetate (0.5 wt%). Such conditions ensure protonation of the more basic quinuclidine nitrogen of the chiral *Cinchona* part of the selectors, while the analytes are either fully deprotonated (series PI, $pK_a \sim 1$) or reversibly deprotonated (series LE, $pK_a \sim 4.5$).

In polar-organic mobile phase conditions, chiral separation is driven by the ion-exchange mechanism. The analytes primarily interact with selectors by electrostatic attraction, which is responsible for retention of analytes. Chiral recognition is realized by additional interactions, such as π - π , steric, and hydrophobic interactions. Hydrogen bonding also plays a part in the recognition process. Note that this interaction is slightly

disfavored due to the protic environment of the mobile phase used in this study.

3.3 | Enantioseparation of N-protected APAs derivatives

APAs represent important building blocks for phosphopeptidomimetics, which are intensively studied compounds with high potential in treatment of various diseases including cancer [26,27]. Thus, the production of enantiomerically pure building blocks is of high interest for the synthesis of various drug candidates. To evaluate the effect of different protecting groups on chiral separation of APAs (Table 1), we used benzyloxycarbonyl (Z) and fluorenylmethoxycarbonyl (Fmoc) N-protecting groups as well as methyl and benzyl ester modifications. One analyte (PI-6) possessed an unprotected phosphonic acid unit. A free, strong acid group could be detrimental to enantioseparation because two acidic groups would compete for one protonated group within the selector.

3.3.1 | Enantioseparation of N-protected APAs derivatives on QN- and QD-based CSPs

Depending on the overall structure of the selector and its spatial organization, the analyte may or may not fit into its binding pocket. Thus, very straightforward conclusions can be drawn from the interaction between variously protected analytes and differently immobilized selectors. Since the QN- and QD-based CSPs usually exhibit pseudo-enantiomeric behavior, trends in the enantioseparation of APAs are similar for both families of CSPs. However, small differences caused by different spatial arrangements of the respective *Cinchona* alkaloids can be seen in Table 1.

The tested APAs can be divided to aliphatic (PI-1 to PI-6) and aromatic (PI-7 to PI-9) derivatives. In the case of the commercially available reference CSP (Chiralpak QN-AX), increasing length of the aliphatic chain disrupted interactions between the selector and the respective analyte. Retention times became shorter and we observed decreasing selectivity and resolution for C-5 compared to C-4 and C-3, resulting in coelution of enantiomers in case of PI-3 (five carbon atoms). However, replacing the N-protecting Z group with the Fmoc group led to a dramatic increase in selectivity and resolution in case of PI-4 compared to PI-3. Since the Fmoc group possesses a larger aromatic unit, it is assumed that enhanced π - π interactions with the selector take place. Due to the highly polar mobile, π - π interactions are, in particular, strengthened. However, the steric effect should also be considered. A minor improvement of enantioseparation was also observed upon the introduction of a benzyl ester unit instead of a methyl ester group. As expected, PI-6 with two free hydroxy groups was only partially separated. Aromatic APAs derivatives (PI-7 to PI-9) showed better retention as well as selectivity and resolution values than aliphatic

TABLE 1 Enantioseparation of N-protected aminophosphonic acids derivatives on quinine-based chiral stationary phases under polar organic mode conditions

CSP Analyte	QN-AX			<i>n</i> Pr-DHQN			Prg-QN			Tam-QN			DCL-QN			Tam-DCL-QN		
	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>
PI-1	4.12	1.13	1.69	2.17	1.30	5.42	0.95	1.16	2.31	0.80	1.09	1.10	0.83	1.38	4.10	1.18	1.30	2.34
PI-2	1.41	1.07	0.97	2.11	1.27	4.98	0.95	1.17	2.42	0.74	1.09	1.07	0.87	1.36	3.78	1.16	1.26	2.04
PI-3	n.d.	1.00	0.00	2.07	1.17	2.48	0.96	1.17	2.48	0.70	1.09	1.08	0.95	1.38	3.80	1.21	1.27	2.16
PI-4	2.65	1.92	9.90	3.04	1.24	4.85	1.41	1.21	3.66	0.96	1.19	2.16	1.36	1.25	3.49	1.89	1.32	2.82
PI-5	2.16	1.17	2.50	3.74	1.45	8.36	1.65	1.34	5.33	0.71	1.10	1.06	0.95	1.38	4.43	2.07	1.26	2.45
PI-6	3.43	1.16	1.26	4.46	1.27	5.50	1.62	1.07	1.06	2.27	1.34	4.22	1.64	1.22	3.66	3.54	1.37	2.98
PI-7	6.73	1.28	4.44	4.88	1.26	5.87	2.03	1.13	2.58	1.75	1.15	2.76	1.43	1.09	1.53	0.50	1.31	0.82
PI-8	6.16	2.20	14.15	7.56	1.29	6.64	3.15	1.25	5.06	2.32	1.21	3.87	2.88	1.41	7.46	4.57	1.46	4.28
PI-9	5.98	1.36	4.98	3.80	1.03	0.83	1.41	1.21	3.66	1.26	1.13	1.73	n.d.	1.00	0.00	n.d.	1.00	0.00
CSP Analyte	QD-AX			<i>n</i> Pr-DHQD			Prg-QD			Tam-QD			DCL-QD			Tam-DCL-QD		
	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>	<i>k</i> ₂	α	<i>R</i>
PI-1	2.23	1.12	2.30	2.06	1.20	4.31	n.d.	1.00	0.00	1.23	1.16	2.62	1.03	1.21	2.42	1.24	1.45	2.80
PI-2	2.14	1.11	2.07	2.02	1.13	3.02	n.d.	1.00	0.00	1.13	1.15	2.41	1.10	1.16	2.03	1.22	1.40	2.59
PI-3	2.12	1.08	1.48	1.98	1.11	2.49	n.d.	1.00	0.00	1.06	1.14	2.20	1.20	1.16	1.99	1.27	1.41	2.70
PI-4	4.28	1.89	12.91	3.44	1.53	11.06	2.26	1.47	4.61	1.54	1.27	4.77	2.47	1.54	7.39	2.11	1.50	3.57
PI-5	n.d.	1.00	0.00	3.51	1.24	5.72	2.01	1.07	0.71	1.59	1.20	3.50	2.23	1.21	3.18	2.16	1.44	3.39
PI-6	5.64	1.07	0.76	3.37	1.08	2.07	2.23	1.06	0.63	n.d.	1.00	0.00	n.d.	1.00	0.00	2.34	1.16	1.21
PI-7	5.37	1.26	5.36	4.92	1.16	4.29	n.d.	1.00	0.00	3.37	1.25	5.30	2.08	1.06	1.00	2.19	1.08	0.64
PI-8	10.88	2.01	15.86	8.81	1.53	12.55	5.91	1.48	5.73	4.72	1.28	5.75	5.44	1.70	10.37	5.32	1.68	5.17
PI-9	4.77	1.34	6.16	4.07	1.14	3.58	2.83	1.29	3.30	1.54	1.27	4.77	2.64	1.24	3.76	2.33	1.07	0.61

*k*₂, retention factor of second eluting enantiomer; α, enantioselectivity coefficient; *R*, resolution; n.d. = not determined, the respective analyte was not enantioseparated.

APAs. As observed earlier for PI-4, the introduction of Fmoc protecting group instead of the Z protecting group increased retention, enantioselectivity, and resolution (see PI-7 and PI-8, Table 1). However, with increasing flexibility of the APA, selectivity and resolution values decreased (compare PI-8 and PI-9, Table 1). Thus, it can be concluded that substances with flexible groups bind less effectively in the cavity of the *tert*-butylcarbamoyl quinine selector. This is probably due to less preferred conformational states of the analytes. In comparison to Chiralpak QN-AX, the Chiralpak QD-AX column was better for the enantioseparation of alkylphosphonic acids PI-1–PI-3. Slightly higher resolution of Fmoc-protected APAs on QD-AX was also observed. On the other hand, acid PI-5 was not resolved which can be caused by the nonideal steric fit, when the flexible benzyl unit is connected directly to the chiral center.

The second stationary phase based on allylcarbamoyl quinine (*n*Pr-DHQN, where *n*Pr and DHQN are *n*-propyl and dihydroquinine, respectively) was efficient for the enantioseparation of all samples, except for PI-9. Also in this case, gradually reduced selectivity and resolution were observed for analytes PI-1–PI-3, respectively. The effect of Fmoc protecting group was not as pronounced as in case of Chiralpak QN-AX. The replacement of the methyl ester group with the benzyl unit proved more beneficial (compare PI-4

and PI-5, Table 1). Interestingly, PI-6 with free phosphonic acid unit was very well resolved. The aromatic APAs PI-7 and PI-8 were also well separated, while PI-9 was only partially resolved. These results indicate that the binding cavity of the selector is located between the aromatic plane of the selector and the silica solid surface. Thus, the analytes that fulfill steric requirements interact similarly with the selector (*R* > 4), while PI-9, which is too large and flexible, is only partially separated. The above-mentioned small difference in spatial arrangement of QD in *n*Pr-DHQD (in comparison to respective QN-based selector, where DHQD is dihydroquinidine) led to the enantioseparation of all APAs. Additionally, in this case, the introduction of the Fmocprotecting group caused a significant enhancement of selectivity and resolution of PI-4 and PI-8 and facilitated the chiral resolution of PI-9, which was not fully resolved with the QN analogue.

For Prg-QN (where Prg is propargyl), all analytes except for PI-6 were baseline separated with short retention times (*k* < 2) for most of them. This hints at enhanced steric repulsion between the selector and the analytes, while the main enantioselective forces within the QN scaffold remain unchanged. It is interesting to note that, similarly to *n*Pr-QN, the largest improvement in selectivity and resolution was achieved by the replacement of methyl with benzyl in the ester group. On the other hand, the Prg-QD column was not very efficient and only

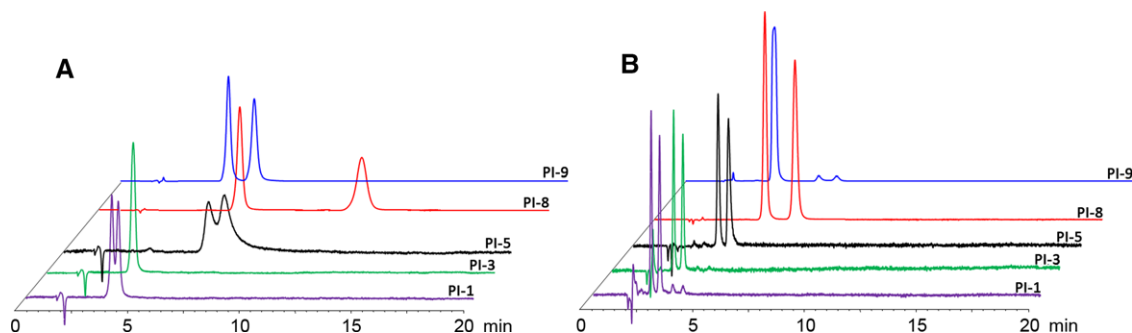


FIGURE 3 Enantioseparation of selected analytes from PI series: (A) using Chiralpak QN-AX; (B) using DCL-QN-5a in polar organic mode (mobile phase MeOH/AcOH, 98:2 v/v, with ammonium acetate 0.5% (w/v)). Flow rate was set to 1 mL/min and temperature to 25°C

three analytes were baseline separated, all of which possessed the Fmoc protecting group. Partial resolution was observed for PI-5 (benzyl ester group) and PI-6 (both hydroxy units free). This effect cannot be ascribed to low coverage (220 $\mu\text{mol/g}$) or bad packing of the stationary phase, since the same column performed very well in the separation of protected leucines (*vide infra*). It seems that the orientation of the respective selector (in particular its propargylcarbamoyl unit) is not ideal for the enantioseparation of APA monomethyl esters (PI1–3 and PI5–7).

Immobilizing the selector of Prg-QN by the triple bond instead of double bond gave rise to Tam-QN CSP (where Tam is triazolomethylene; **4a** in Figure 1). The performance of this CSP was poor for aliphatic APAs, while it slightly improved for aromatic APAs. Similar to other CSPs, the introduction of Fmoc or benzyl ester groups led to an improvement of chromatographic performance. Interestingly, the best results were obtained for PI-6 ($\alpha = 1.34$, $R_s = 4.22$), for which also long retention time was observed. This indicates that both hydroxy groups in the free phosphonic unit engage in attractive interaction with the selector. The difference in the spatial arrangement of QN- and QD-based selectors is evident also in this case. While the aliphatic APAs were well resolved ($\alpha \sim 1.15$, $R \sim 2.40$), the acid PI-6 was not separated. Moreover, retention times of the analytes were longer than for Tam-QN, which indicates that Tam-QD interacts more strongly with the majority of the tested APAs.

Direct comparison of the aforementioned steric effects imposed by different immobilization strategies can be made for the last two CSPs (**6a** and **6b** in Figure 1). Both DCL-QN (**5a** and **5b**) (where DCL is dichlorophenyllinker) and Tam-DCL-QN possess carbamoyl parts of similar size. While DCL-QN can freely rotate, Tam-DCL-QN is a part of the linker to the silica support and thus cannot move freely. These conformational constraints are reflected in retention factors of the analytes, which are higher on Tam-DCL-QN. This is most probably due to insertion of the analytes into the binding cavity blocked at one side by the silica support. Generally, the short retention times and good selectivity values obtained

on DCL-QN CSP enable its use in fast screening of APAs. Importantly, this CSP was efficient in the separation of minor impurities present in the samples, which was not achieved with the commercial column (Figure 3). For both selectors, analyte size is a crucial parameter since PI-9 with the spacious Fmoc protecting group and flexible benzyl group of the arylphosphonic acid was not separated (Figure 3B, Table 1). Similar results were achieved with DCL-QD and Tam-DCL-QD. However, the chromatographic parameters were less uniform than in case of QN-based selectors. As in the previous cases, the introduction of the bulky Fmoc group led to the significant increase in selectivity and resolution values leading to baseline and partial separation of PI-9 on DCL-QD and Tam-DCL-QD, respectively. The worst-resolved analyte was again PI-6, which was probably due to insertion between the aromatic plane of the selector and the silica support, partially resolved on Tam-DCL-QD.

Overall, QN- and dihydroquinine-based selectors showed highly similar chromatographic behavior for the separation of the given set of APAs. On the other hand, QD- and dihydroquinidine-based selectors sometimes offered higher selectivity and resolution values (e.g., PI-4 or PI-8) and were capable of separating analytes not resolved on the QN-analogue (e.g., PI-9 on DCL-QD).

3.4 | Enantioseparation of N-protected leucine derivatives

The remarkable selectivity of *Cinchona* alkaloid-based CSPs toward N-protected leucines (in particular 3, 5-dinitrobenzoylleucine) has been reported previously, for example, in Ref. 28. Based on this knowledge, we prepared a series of different N-protected leucine derivatives and tested the chiral recognition properties of the novel selectors for these analytes (Table 2).

In agreement with the previous findings, the commercial CSPs Chiralpak QN-AX and Chiralpak QD-AX exhibited remarkable selectivity and resolution values throughout the LE series. Generally, higher selectivity values were obtained

TABLE 2 Enantioseparation of variously protected N-protected leucine derivatives on the quinine and quinidine-based chiral stationary phases

CSP Analyte	QN-AX			nPr-DHQN			Prg-QN			Tam-QN			DCL-QN			Tam-DCL-QN		
	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R
LE-1	2.08	2.68	15.21	2.23	1.69	11.00	0.96	1.62	8.27	0.64	1.28	3.01	0.69	1.37	4.12	1.00	1.35	4.99
LE-2	19.24	3.98	22.67	16.51	2.27	19.27	4.61	2.05	14.90	3.35	1.61	8.60	3.00	1.83	11.46	7.27	2.15	14.56
LE-3	1.21	1.33	4.04	1.61	1.09	1.72	0.75	1.13	1.60	n.d.	1.00	0.00	n.d.	1.00	0.00	n.d.	1.00	0.00
LE-4	2.66	1.99	11.37	3.18	1.38	7.13	1.36	1.33	5.47	0.89	1.17	2.06	1.18	1.24	3.42	1.64	1.21	3.52
LE-5	1.44	1.98	9.18	1.46	1.25	4.06	0.61	1.14	1.67	n.d.	1.00	0.00	n.d.	1.00	0.00	n.d.	1.00	0.00
LE-6	23.56	15.92	41.23	24.70	8.90	45.27	6.93	5.77	36.44	3.52	3.39	21.83	3.43	3.06	22.42	5.77	3.14	23.37
LE-7	5.42	9.99	30.53	3.86	4.41	27.21	1.58	3.90	20.30	0.60	2.16	7.52	0.79	2.79	11.35	0.90	2.31	2.24
LE-8	10.54	9.03	34.28	8.69	4.49	32.75	2.78	3.36	23.64	1.30	2.06	11.14	1.55	2.15	13.66	2.17	2.11	14.10
LE-9	10.77	10.06	36.74	8.99	4.82	33.63	3.07	3.69	26.11	1.33	2.20	11.83	1.75	2.35	15.16	2.43	2.28	16.77
LE-10	5.08	6.18	27.23	5.14	3.97	26.25	2.42	3.64	23.03	1.21	2.23	11.47	1.53	2.63	15.77	2.15	2.53	15.87
Analyte	QD-AX			nPr-DHQD			Prg-QD			Tam-QD			DCL-QD			Tam-DCL-QD		
	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R	k ₂	α	R
LE-1	2.84	2.14	17.27	1.98	1.90	14.17	1.33	1.86	2.46	0.90	1.37	5.64	0.88	1.56	5.55	0.97	1.51	1.14
LE-2	11.53	2.01	15.82	13.23	2.85	27.17	8.45	3.20	23.29	5.53	1.87	13.52	5.24	2.49	15.84	4.40	1.54	7.18
LE-3	1.85	1.27	4.56	1.29	1.16	3.00	0.92	1.19	2.85	n.d.	1.00	0.00	0.75	1.15	1.52	0.76	1.08	0.89
LE-4	3.55	1.63	11.56	2.45	1.49	6.50	1.76	1.43	7.47	1.27	1.19	3.21	1.59	1.37	5.01	1.54	1.31	4.43
LE-5	1.84	1.55	8.96	1.25	1.34	5.74	0.81	1.31	3.99	n.d.	1.00	0.00	0.62	1.17	1.49	0.59	1.08	0.87
LE-6	19.92	8.88	44.84	19.07	10.21	53.12	10.64	7.56	42.07	5.69	4.10	30.51	4.63	3.40	22.83	5.77	3.76	21.98
LE-7	5.07	5.96	33.94	3.65	5.29	33.07	2.29	5.11	26.75	0.87	2.48	11.80	1.19	3.62	13.71	0.89	2.89	11.19
LE-8	10.01	5.35	37.26	7.34	5.22	38.74	4.06	4.19	29.57	2.01	2.45	17.16	2.19	2.50	14.51	2.15	2.47	14.29
LE-9	12.06	6.65	39.78	7.72	5.70	39.68	4.74	4.89	32.97	2.01	2.57	17.80	2.39	2.82	19.08	2.43	2.73	16.72
LE-10	5.22	4.12	29.33	5.14	5.03	35.63	3.18	4.46	29.17	1.83	2.73	17.37	2.23	3.41	17.54	2.13	3.10	16.07

Chromatographic conditions: flow rate 1 mL/min, temperature 25°C.

k₂, retention factor of second eluting enantiomer; α, enantioselectivity coefficient; R, resolution.

for QN-AX in comparison to QD-AX, while higher resolution was in most cases found for the QD-AX column. The highest selectivity values were found for analytes with electron-withdrawing substituents on the aromatic unit of their protective groups (LE-6–LE-9). Reduced chromatographic performance was observed for LE-10 with three electron-donating methoxy groups. It is likely that not only electronic effect, but also steric hindrance of the methoxy groups plays a significant role in the interaction with the selectors. Additional reduction of selectivity and resolution was observed for phthalimide-protected LE-2.

Considerable reduction of retention, selectivity, and resolution was observed for LE-1 and LE-3 through LE-5. There are two factors to which this behavior can be ascribed. First, higher flexibility and longer distance of the aromatic unit from the chiral center in case of Z- and Fmoc-protecting groups (LE-3 and LE-4, respectively) probably lead to reduced π – π interactions. Second, the nonsubstituted benzoyl unit (LE-1) most probably does not offer efficient π – π interactions with the selectors compared to the substituted aromatic units. In addition, the steric effect of this group is also reduced. On the other hand, pentafluorophenyl is a highly electron-deficient aromatic unit (LE-5). Thus, its

interaction with the *Cinchona* aromatic moiety should be preferred. The reduction of chromatographic parameters observed in our experiments can be explained by the fluorophilic effect of this protecting group [29], which probably diminishes the strength of concerted enantioselective interactions.

The effects of various protecting groups introduced above for the commercial columns can be found also for the novel CSPs. The best results were again achieved for leucines bearing substituted benzoyl units (LE-5 through LE-10). The commercial column Chiralpak QN-AX showed higher selectivity than QD-AX for all studied analytes from LE series but the opposite is true for the rest of the studied CSPs. All QD-based CSPs outperformed their QN-based analogues in the LE separations. This phenomenon can be ascribed to the slight difference in the spatial arrangement of the respective diastereoisomers (QN and QD) with respect to their carbamoyl units. While they can be regarded as (pseudo-) enantiomeric for most purposes, which is important for broad applicability, certain applications (both the PI and LE series) reveal their diastereomeric relationship through significant differences in the chromatographic performance, in particular the enantioselectivity.

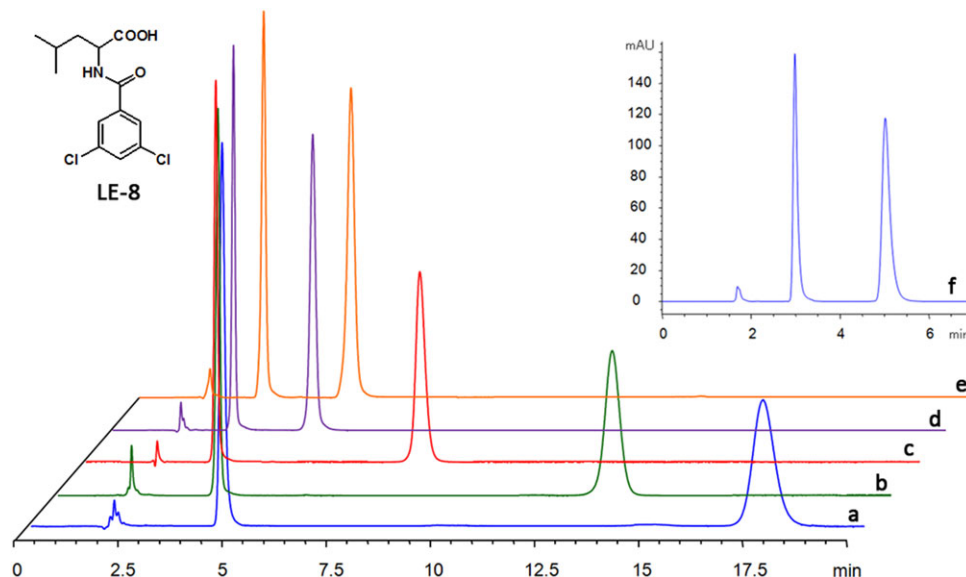


FIGURE 4 Enantioseparation of 3,5-dichlorobenzoylleucine (LE-8) on different CSPs: (a) Chiralpak QD-AX, (b) *nPr*-QD, (c) Prg-QD, (d) Tam-QD, (e) DCL-QD, and (f) Tam-DCL-QD inset due to shorter method time

Generally, shorter retention times were achieved with selectors possessing bigger carbamoyl units in comparison to the commercial columns. Since the QD-based CSPs were more efficient in the enantioseparation of the analytes, such CSPs could be used for fast screening purposes (Figure 4).

3.5 | Elution order

Since many of the leucine derivatives were synthesized in our laboratory from commercially available enantiomerically pure D- and L-leucine, it was possible to investigate the elution order of the respective enantiomers. Therefore, in case of LE-7, an L-enantiomer-enriched mixture was prepared, whereas in the case of LE-9, the elution order was verified by injecting single D-enantiomer. It was found that D enantiomers elute first on DCL-QN, whereas on DCL-QD the L enantiomers elute first (Figure 5). The same elution order was observed throughout the series of CSPs for all LE analytes, independent of the immobilization procedure employed. These results are consistent with previous results for QN- and QD-based ion exchangers and provide further proof for the pseudoenantiomeric behavior of the CSPs based on QN and QD [8].

3.6 | Influence of selector loading on chromatographic parameters

Since *nPr*-QN and *nPr*-QD CSPs performed similarly to the commercial materials and even outperformed them for the PI series, we decided to study the effect of reduced selector loading. We showed in an earlier publication that for selectors immobilized by the carbamate unit using *click*-chemistry, the optimum selector loading is approximately 300 $\mu\text{mol/g}$ and higher loading leads to reduced chromatographic

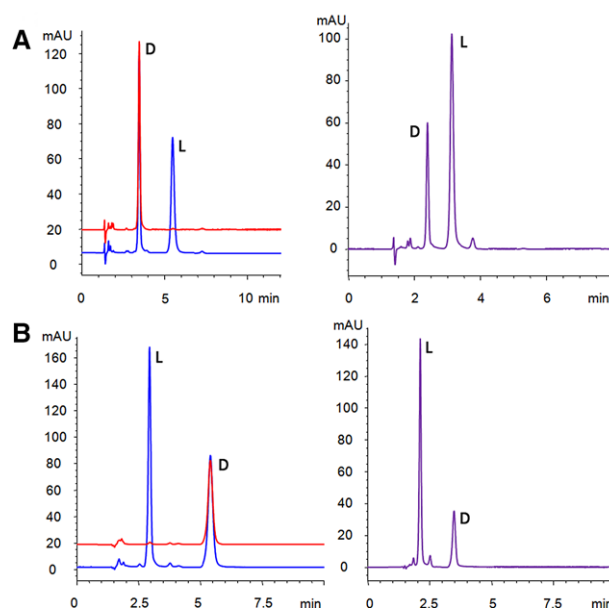


FIGURE 5 Enantioseparation of LE-9 and LE-7 on (A) DCL-QD and (B) DCL-QN. Sample concentration was 2 mg/mL, flow rate was set to 1 mL/min, temperature to 25°C. Detection wavelength of 280 nm was used

performance [16]. It is reasonable to investigate CSPs with rather low selector coverage, as low selector loading reduces the overall production costs of the respective CSP. Therefore, we prepared an “*nPr*-QN_2” CSP with the selector loading of 157 $\mu\text{mol/g}$, and compared its performance with the original *nPr*-QN (loading 286 $\mu\text{mol/g}$). To gain a broader view of the effect of an analyte structure on the chromatographic performance of the CSPs, a different analyte set was used (see ESI, Supporting Information Figure S6).

As expected, a direct comparison of retention times showed that on the *nPr-QN_2* the analytes are generally less retained. The ratio of retention times *nPr-QN/nPr-QN_2* was determined to be 1.63 ± 0.41 , which corresponds well with a selector loading ratio of 1.82. Selectivity was generally higher for the low-loaded phase (see Supporting Information Figure S7), while for resolution the trend was not that pronounced (see Supporting Information Figure S8). The higher efficiency of the low-loaded CSP was further documented by smaller theoretical plate height for the majority of the analytes (see Supporting Information Table S1). Based on these results, it can be concluded that for *Cinchona*-based selectors immobilized by the carbamate unit using the thiol-ene process, lower selector coverage is preferable.

4 | CONCLUDING REMARKS

While researchers can choose from a wide variety of commercially available CSPs, the demand for novel, highly specific CSPs remains strong. The pharmaceutical industry is a major driving force due to strict regulations regarding the stereochemical purity of drugs and drug precursors of interest. In this study, we demonstrated that novel, chiral anion exchangers structurally related to commercial QN-AX and QD-AX are capable of resolving certain chiral APAs with higher selectivity than the benchmark CSPs. The performance of the novel CSPs can be easily tuned by the type of immobilization chemistry that affords bonded selectors with different overall flexibility. It should be noted that the immobilization chemistry does not influence the elution order of protected acidic analytes, which is driven by the *Cinchona* scaffold. In addition, immobilization by the carbamoyl unit leads to CSPs that are highly efficient despite low selector coverage. Immobilization of selectors bearing two or more multiple bonds also offers new strategies for the synthesis of cross-linked CSPs. All these aspects need to be taken in account, when designing novel *Cinchona* anion exchangers that are envisioned to be used for enantioseparation (analytical or preparative) of a specific analyte.


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ANIMAL AND HUMAN RIGHTS

This study did not utilize samples or procedures requiring approval by an institutional review board.

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REFERENCES

1. Lämmerhofer, M., Chiral recognition by enantioselective liquid chromatography: mechanisms and modern chiral stationary phases. *J. Chromatogr. A* 2010, *1217*, 814–856.
2. Lämmerhofer, M., Liquid chromatographic enantiomer separation with special focus on zwitterionic chiral ion-exchangers. *Anal. Bioanal. Chem.* 2014, *406*, 6095–6103.
3. Levkin, P., Maier, N. M., Lindner, W., Schurig, V., A practical method for the quantitative assessment of non-enantioselective versus enantioselective interactions encountered in liquid chromatography on brush-type chiral stationary phase. *J. Chromatogr. A* 2012, *1269*, 270–278.
4. Krawinkler, K. H., Gavioli, E., Maier, N. M., Lindner, W., Evaluation of different surface-anchoring strategies for *Cinchona* carbamate type selectors. *Chromatographia* 2003, *58*, 555–564.
5. Pirkle, W. H., Welch, C. J., An improved chiral stationary phase for the chromatographic separation of underivatized naproxen enantiomers. *J. Liq. Chromatogr.* 1992, *15*, 1947–1955.
6. Ling, F. H., Lu, V., Svec, F., Fréchet, J. M. J., Effect of multivalency on the performance of enantioselective separation media for chiral HPLC prepared by linking multiple selectors to a porous polymer support via aliphatic dendrons. *J. Org. Chem.* 2002, *67*, 1993–2002.
7. Wolrab, D., Frühauf, P., Kohout, M., Lindner, W., Click chemistry immobilization strategies in the development of strong cation exchanger chiral stationary phases for HPLC. *J. Sep. Sci.* 2013, *36*, 2826–2837.
8. Lämmerhofer, M., Lindner, W., Quinine and quinidine derivatives as chiral selectors I. Brush type chiral stationary phases for high-performance liquid chromatography based on cinchonan carbamates and their application as chiral anion exchangers. *J. Chromatogr. A* 1996, *741*, 33–48.
9. Gyimesi-Forrás, K., Akasaka, K., Lämmerhofer, M., Maier, N. M., Fujita, T., Watanabe, M., Harada, N., Lindner, W., Enantiomer separation of a powerful chiral auxiliary, 2-methoxy-2-propionic acid by liquid chromatography using chiral anion exchanger-type stationary phases in polar-organic mode; investigation of molecular recognition aspects. *Chirality* 2005, *17*, S134–S142.
10. Lämmerhofer, M., Franco, P., Lindner, W., Quinine carbamate chiral stationary phases: Systematic optimization of steric selector-selectand binding increments and enantioselectivity by quantitative structure-enantioselectivity relationship studies. *J. Sep. Sci.* 2006, *29*, 1486–1496.
11. Hoffmann, C. V., Reischl, R., Maier, N. M., Lämmerhofer, M., Lindner, W., Stationary phase-related investigations of quinine-based zwitterionic chiral stationary phases operated in anion-, cation-, and zwitterion-exchange modes. *J. Chromatogr. A* 2009, *1216*, 1147–1156.
12. Wernisch, S., Bisi, F., Cazzato, A. S., Kohout, M., Lindner, W., 2-Acyl-dimmedones as UV active protective agents for chiral amino acids: Enantiomer separations of the derivatives on chiral anion exchangers. *Anal. Bioanal. Chem.* 2013, *405*, 8011–8026.

13. Sardella, R., Lisanti, A., Carotti, A., Blasi, P., Lindner, W., Natalini, B., Ketoprofen enantioseparation with a *Cinchona* alkaloid based stationary phase: Enantioselective recognition mechanisms and release studies. *J. Sep. Sci.* 2014, *37*, 2696–2703.
14. Zhao, J., Wu, H., Wang, D., Wu, H., Cheng, L., Jin, Y., Ke, Y., Liang, X., Improvement of chiral stationary phases based on *Cinchona* alkaloids bonded to crown ethers by chiral modification. *J. Sep. Sci.* 2015, *38*, 3884–3890.
15. Bajtai, A., Fekete, B., Palkó, M., Fülöp, F., Lindner, W., Kohout, M., Ilisz, I., Antal, P., Comparative study on the liquid chromatographic enantioseparation of cyclic β -amino acids and the related cyclic β -aminohydroxamic acids on *Cinchona* alkaloid-based zwitterionic chiral stationary phases. *J. Sep. Sci.* 2017, <https://doi.org/10.1002/jssc.201701190>.
16. Hettegger, H., Kohout, M., Mimini, V., Lindner, W., Novel carbamoyl type quinine and quinidine based chiral anion exchangers implementing alkyne–azide cycloaddition immobilization chemistry. *J. Chromatogr. A* 2014, *1337*, 85–94.
17. Kacprzak, K. M., Maier, N. M., Lindner, W., Triazolo-linked *Cinchona* alkaloid carbamate anion exchange-type chiral stationary phases: Synthesis by click chemistry and evaluation. *J. Chromatogr. A* 2011, *1218*, 1452–1460.
18. Sardella, R., Carotti, A., Gioiello, A., Lisanti, A., Ianni, F., Lindner, W., Natalini, B., Chromatographic separation of free dafachronic acid epimers with a novel triazole click quinidine-based chiral stationary phases. *J. Chromatogr. A* 2014, *1339*, 96–102.
19. Basak, A., Roy, S. K., Das, S., Hazra, A. B., Ghosh, S. C., Jha, S., Chemistry of enediyne azides: Activation through a novel pathway. *Chem. Commun.* 2007, 622–624.
20. Kacprzak, K., Gierczyk, B., Clickable 9-azido-(9-deoxy)-*Cinchona* alkaloids: Synthesis and conformation. *Tetrahedron* 2010, *21*, 2740–2745.
21. Padwa, A., Ku, A., Ku, H., Mazzu, A., Intramolecular cycloaddition reactions of vinyl azides bearing alkenyl and alkynyl groups. *J. Org. Chem.* 1978, *43*, 66–72.
22. Kolb, H. C., Finn, M. G., Sharpless, K. B., Click chemistry: Diverse 447 chemical function from a few good reactions. *Angew. Chem. Int. Ed. Engl.* 2001, *40*, 2004–2021.
23. Hoogenboom, R., Thiol–Yne chemistry: A powerful tool for creating highly functional materials. *Angew. Chem. Int. Ed.* 2010, *49*, 3415–3417.
24. Bader, H., Cross, L. C., Heilbron, I., Jones, E. R. H., 132. Researches on acetylenic compounds. Part XVIII. The addition of thiolacetic acid to acetylenic hydrocarbons. The conversion of monosubstituted acetylenes into aldehydes and 1: 2-dithiols. *J. Chem. Soc.* 1949, 619–623.
25. Fairbanks, B. D., Scott, T. F., Kloxin, C. J., Anseth, K. S., Bowman, C. N., Thiol–Yne Photopolymerizations: novel mechanism, kinetics, and step-growth formation of highly cross-linked networks. *Macromolecules* 2009, *42*, 211–217.
26. Naydenova, E. D., Todorov, P. T., Troev, K. D., Recent synthesis of aminophosphonic acids as potential biological importance. *Amino Acids* 2010, *38*, 23–30.
27. Gargano, A. F. G., Buchinger, S., Kohout, M., Lindner, W., Lämmerhofer, M., Single-step Ugi multicomponent reaction for the synthesis of phosphopeptidomimetics. *J. Org. Chem.* 2013, *78*, 10077–10087.
28. Maier, N. M., Schefzick, S., Lombardo, G. M., Feliz, M., Rissanen, K., Lindner, W., Lipkowitz, B. K., Elucidation of the chiral recognition mechanism of *Cinchona* alkaloid carbamate-type receptors for 3,5-dinitrobenzoyl amino acids. *J. Am. Chem. Soc.* 2002, *124*, 8611–8629.
29. Kohout, M., Kählig, H., Wolrab, D., Roller, A., Lindner, W., Novel chiral selector based on Mefloquine—A comparative NMR study to elucidate intermolecular interactions with acidic chiral selectands. *Chirality* 2012, *24*, 936–943.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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