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RESEARCH PAPER

Enantioseparation of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate tagged amino acids and other zwitterionic compounds on cinchona-based chiral stationary phases

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Abstract The fluorescent tag 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC; AccQ Fluor reagent kit from Waters) is a commercial N-terminal label for proteinogenic amino acids (AAs), designed for reversed-phase separation and quantification of the AA racemates. The applicability of AQC-tagged AAs and AA-type zwitterionic compounds was tested for enantiomer separation on the *tert*-butyl carbamate modified quinine and quinidine based chiral stationary phases, QN-AX and QD-AX employing polar-organic elution conditions. The investigated test analytes included the enantiomers of the positional isomers of isoleucine (Ile), threonine, homoserine, and 4-hydroxyproline. Furthermore, β-AAs, cyclic, and heterocyclic AAs including trans-2-amino-cyclohexane carboxylic acid and trans-2-aminocyclohexyl sulfonic acid, phenylalanine derivatives substituted with halides with increasing electronegativity and 3,4-dihydroxyphenylalanine, cysteine-related derivatives including homocysteic acid, methionine sulfone, cysteine-*S*-acetic acid, and cysteine-*S*-acetamide as well as a small range of aminophosphonic acids were enantioseparated. A mechanistic interaction study of AQC-AAs in comparison with fluoresceine isothiocyanate-labeled AAs was performed. The chiral and chemoselective recognition processes involved in enantiomer separation and retention was systematically discussed. Special emphasis was set on the influential factors exhibited by the chemistry, branching position, and spatial properties of the investigated zwitterionic analytes. The general interest to separate and distinguish between different types of branched-chained AAs and metabolic side products thereof lies in the toxicity of some of these compounds, which makes for instance allo–Ile an attractive candidate in disease-related biomarker research.

Keywords Enantiomer separation · D-Amino acid · 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate · Fluoresceine isothiocyanate · Quinine

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Introduction

L-Amino acids (AA) are the basic building blocks of proteins and are also important biologically active compounds. The zwitterionic nature of AAs, combined with their structural diversity and well-defined stereochemistry, has a fundamental influence on the inherent chiral nature of all living systems. Many scientists in the fields of chemistry, biochemistry, biology, pharmacy, agriculture, and food chemistry are striving to gain a better understanding of the simple, yet complex chiral nature of AAs. The occurrence of D-amino acids (D-AAs), the underlying mechanism of D-AA formation, and their physiological function in biological systems remain interesting and challenges D-AA research, necessitating accurate, sensitive, and reproducible qualitative as well as quantitative analytical concepts.

In the past, D-AAs, the optical antipodes of the naturally occurring L-AAs were considered to be "unnatural," and their existence was rationalized as simple by-products of chemical reactions or impurities introduced by bacterial contamination. Recently, naturally occurring D-AAs were found in biological matrices such as bacterial cell walls [1], mammalian tissues, or body fluids [2–5] as well as food products [6, 7]. In food chemistry, for example, D-AAs are frequently used as an adulteration indicator to confirm the authenticity of honey [6]. The amount and composition of D-AAs in sake was explored by Gogami [7] and 15 D-AAs were attributed

to the exotic taste and health benefits exhibited by this Japanese wine. Recently, the origin, presence, distribution and physiological function of D-AAs in microorganisms, plants, and fermented food products [8] as well as the utilization of free D-AAs present in soil by soil microbes and plants [9] were summarized in review articles.

In addition, recent research has established, D-AAs as potential biomarkers to identify diagnostic health issues [5, 10]. In fact, the optical antipodes of the naturally occurring AAs have important influence on biological active substances such as neurotransmitters or hormones [10] or are in some cases themselves physiologically active such as the glial neurotransmitter D-serine [11]. Significant amounts of D-leucine were found in tissues of rates and their physiological fluids, such as liver, kidney, cerebrum, cerebellum and urine. Besides D-leucine, also significant amounts of D-allo-isoleucine (aIle), D-valine, D-isoleucine and D-serine were detected in urine [2, 12]. Note that branched-chained amino acids (BCAA) [13] as well as D-Pro [14] have neurotoxic effects. BCAA can lead to diseases with early-stage metabolic disorders, which can induce severe brain-damages, if left untreated. Elevated levels of aIle compared with Phe in plasma samples of maple syrup urine disease (MSUD) patients were identified as a possible indicator for inter-current episodes of MSUD [15]. Another application of aIle detection is based on the time-dependent epimerization of isoleucine (Ile) to aIle, which is used for geochronical dating [16]. Furthermore, D-Aspartic acid appears to be involved in the formation of nerve synapses, found in several neuroendocrine and endocrine tissues of healthy individuals [17]. D-alanine is a structural element of opioid peptides in frog skin [18]. Recently, the origin, presence, distribution and physiological function of D-AAs in microbes, plants and fermented food products [8] and specifically, the diagnostic values of certain D-AAs in mammals [10, 19] were summarized in review articles.

For the unambiguous establishment of D-AAs as potential biomarkers, an accurate quantification of D-AAs in the presence of a vast excess of L-AAs is mandatory [19]. The classical approach for D-AA analysis involves chiral gas chromatography of derivatized AAs combined with enantiomers labeling and mass spectrometric detection [20]. A comparison of different AA derivatization methods for GC-MS separation shows that the resolution values of methyl chloroformate/methanol (MCF/MeOH) derivatives separated on a Rt-DEXsa column are superior to those of penta-fluoropropionic anhydride/hepta-fluorobutanol (PFPA/HFP) derivatized AAs on Chirasil valine (Val. On the other hand, the latter provides higher reproducibility and sensitivity, while some AAs such as asparagines (Asn), Asp, Glu and Gln cannot be accurately quantified due to the degradation of Asn and Gln to Asn and Glu [21]. Since liquid chromatographic (LC) separation is performed under fairly mild conditions, chiral LC methods gain increasing attractiveness. For the chiral LC separation of L- and D-AAs, two distinct approaches are described in literature. AAs can be labeled with chiral derivatization reagents (CDR) such as o-phthalaldehyde (OPA) [22], OPA with N-isobutyryl-L-cysteine (IBLC) [23] or Marfey reagent [24], followed by the separation of the so-formed diastereomeric AA derivatives on a reversed-phase (RP) column combined with ultraviolet (UV) or MS detection. The other, more convenient approach involves the derivatization of AAs with achiral tags such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [25–27], fluorescein isothiocyanate (FITC) [28, 29], 9-fluorenylmethyl chloroformate (FMOC-Cl) and N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD) [30], followed by enantiomer separation on a chiral stationary phase (CSP). Note that these AA-tags introduce additional, aromatic and fluorescent active moieties, facilitating the AA-derivative with UV/VIS as well as fluorescence detection modes [19].

The main limitation of the CDR functionalization strategy is that these CDR-reagents must possess high enantiomeric purity. Furthermore, some CDRs fail to derivatize all AAs. OPA, for instance, does not derivatize proline (Pro) and hydroxyproline [22]. The actual advantage of the CDR approach is the high theoretical plate numbers that is provided by RP-separation on C18-columns.

The disadvantage of the use of CSPs lies in the deviation of separation efficiency induced by different CSP types for AAs of differing polarity. For instance, quinine (QN)- and quinidine (QD)-type CSPs work best for hydrophilic AAs, while Pirkle type CSPs [31] are ideal for hydrophobic AAs [3]. In addition, the choice of tagging derivative may also contribute or reduce separation efficiency, depending on the chemistry and sterical properties of the AAs and the CSP of choice.

In this study, the direct approach, using AQC for AA-labeling [32–34] and employing the two cinchona-derived, *tert*-butyl-QN based weak anion exchange type CSPs, QX-AX and QD-AX for enantioselective AQC-AA separation is described. This selector type was introduced by Lindner et al. [35–38] and was successfully applied for the separation of a broad variety of *N*-protected AAs, drugs and other chiral acids. In this context, the *N*-terminal labeling of AAs is a widely used technique for the direct enantioseparation via liquid chromatography, since it circumvents the zwitterionic character of AAs [36] and introduces a potent chromophore which increases the sensitivity for analyte detection. However, the remaining chromophore or fluorophore bearing reagent, even when present in low quantities may hinder analyte peak identification or quantification.

The fluorescence label AQC, which has been introduced by Cohen in 1993 [25] resolves several of these problems. The *N*-hydroxyl succinimidyl activated carbamate of AQC reacts within seconds with the primary and secondary amines of AAs under alkaline condition, while the excess reagent

hydrolyzes to 6-aminoquinoline and N-hydroxy-succinimide. In addition, the AQC-AA derivatives exhibit a strong blue shift compared with the decomposition products of AQC. Besides, the separation of AQC-AAs on RP-columns, also AQC derivatized AAs and peptides were reported using native or modified cyclodextrine bonded chiral stationary phase in liquid chromatography [4, 39, 40] or in capillary electrophoresis [41]. On QN-AX and QD-AX CSPs, only the separation of AQC-tagged valine, leucine (Leu), and Ile [2] as well as Pro and hydroxyproline [14] were described so far.

In the here presented article, AQC was used for the derivatization of all proteinogenic AAs as well as a set of other amino-acid type compounds, which also included aminosulfonic acid and aminophosphonic acid analogs. As a reference method, all proteinogenic AAs were also derivatized with FITC. Enantiomer separation of fluorescent-tagged AAs was performed on the pseudo-enantiomeric thus diastereomeric cinchona-type CSPs, the QN-AX and the QD-AX columns, employing polar-organic mobile phase conditions with addition of acetonitrile in case of FITC-tagged AAs. All other zwitterionic test compounds were only labeled with AQC and investigated under identical mobile phase conditions. Hence the influence of side-chain variation such as a change in position, polarity and sterical hindrance was visualized and discussed.

Experimental

Chemicals

The AccQ Fluor reagent kit was purchased from Waters (Milford, MA, USA) and contained AQC, borate buffer and acetonitrile. Acetic acid (AcOH), formic acid (FA), ammonium acetate (NH_4OAc), ammonium formate (NH_4FA), all of analytical grade and FITC Isomer 1 with 90 % chemical purity, dimethylformamide (DMF), sodium borate and sodium hydroxide pellets (NaOH) were purchased from Sigma-Aldrich (Vienna, Austria).

The racemates and the L-AAs of alanine (Ala), aspartic acid (Asp), Asn, cysteine (Cys), histidine (His), Ile, Leu, lysine (Lys), methionine (Met), serine (Ser), threonine (Thr), allo-threonine (*a*Thr), tryptophane (Trp), tyrosine (Tyr), Val, phenylalanine (Phe), 3,4-dihydroxyphenylalanine (DOPA), and methioninesulfone (Mso); the racemates of Cys, homocysteic acid, allo-isoleucine (*a*Ile), norvaline (Nva), isoserine (Ise), homoserine (Hse), Pro, phospholeucine (pLeu), para-fluoro-Phe, *p*-chloro-Phe, and ortho-fluoro-Phe; the D-AA and the L-AA of glutamine (Gln), trans-4-hydroxyproline, and *cis*-4-hydroxyproline; the L-arginine (Arg), glycine (Gly), trans-2-amino-cyclohexylsulfonic acid (ACHS), 2-amino butyric acid (AABA), trans-2-amino-cyclohexanecarboxylic acid, iodoacetamide, and iodoacetate were from Sigma-Aldrich GmbH (Vienna, Austria). The racemate of Arg, D-Pro, D-*a*Ile, and L-*a*Ile were from Bachem (Bubendorf, Switzerland). DL-p-Br-Phe was purchased from Loba Chem. (Fischamend, Austria). Methanol and acetonitrile were of HPLC gradient grade, purchased from VWR (Vienna, Austria). Water was in-house purified using a millipore purification system. 1-amino-propylphosphonic (APP), 1-amino-1-cyclohexylmethyl phosphonic acid (ACHMP), and 1-amino-2-hydroxy-2-phenyl-ethyl-phosphonic acid (AHPEP) were in-house prepared [42]. Cysteine-*S*-acetic amide (Cys-*S*-AcAm) and cysteine-*S*-acetic acid (Cys-*S*-AcAC) were prepared by reacting Cys with iodoacetamide and iodoacetate [12].

Instruments

An Agilent 1200 Series HPLC system equipped with a quaternary pump, degasser, column compartment, and autosampler tray with temperature control, diode array detector, fluorescence detector, and Chemstation Rev B.04.03 software were from Agilent Technologies (Waldbronn, Germany). The elemental analysis of modified silica gels was performed with an EA 1108 CHNS-O from Carlo Erba (Milan, Italy). A thermomixer-compact from Eppendorf (Hamburg, Germany) was used for AA derivatization with FITC.

Chromatographic columns and separation conditions

The chiral separation columns, QN-AX and QD-AX contained the *tert*-butyl carbamate-modified QN- and QD-based selectors, immobilized onto 3-mercaptopropyl-modified (SH content, 680 μmol/g) and silanol endcapped silica gel. Crude silica gel, Daisogel 120-5P, with a particle size of 5 μm and a pore size of 120 Å was from Daiso Co., LtD (Düsseldorf, Germany). Selector densities were calculated on the basis of nitrogen content, determined by elemental analysis, and they were 346 mmol QD-AX/g silica gel (C, 14.66; H, 2.29; N, 1.454; S, 1.77 wt.%) and 412 mmol QN-AX/g silica gel (C, 15.77; H, 2.37; N, 1.730; S, 1.67 wt.%). The modified silica gels were in-house slurry packed into 150×5.0 mm I.D. stainless steel columns from Bischoff Chromatography GmbH (Leonberg, Germany). Procedures for selector synthesis, selector immobilization and column packing are described elsewhere in more detail [38]. Note that the here described CSP columns are identical to the commercially available Chiralpak QN-AX and QD-AX columns from Chiral Technologies (Illkirch, France).

All experiments were performed, if not stated otherwise, at a flow rate of 1 ml/min in isocratic mode at 25 °C, employing 10 % (*v*/*v*) acetone in the corresponding mobile phase as void volume marker. The void marker was injected separately at the beginning and the end of each sequence.

Adjustment of mobile phase pH was performed with acetic acid, formic acid, or 10 % sodium hydroxide solution.

Note that the stated pH values for the reversed-phase eluents resemble the apparent pH values defined by the pH of the buffer solution. The mobile phase composition for the separation of AQC-tagged AAs in polar-organic mode was MeOH with 360 mM AcOH and 65 mM NH_4OAc. For FITC-labeled AA derivatives the mobile phase was MeOH/ACN 80/20 with 150 mM FA and 75 mM NH_4FA. For the chiral separation using the reversed-phase mode the mobile phase MeOH/20 mM NH_4OAc (apparent pH 6.0) 80/20 (*v*/*v*) was used (data shown in the Electronic supplementary material (ESM)).

Experimental values, which are herein presented, include the retention factor (k) and the selectivity factor alpha (α). The retention factor is defined as $(t_R - t_0)/t_0$, whereas t_R is the retention time of the test analyte and t_0 is the retention time of the void volume marker. The selectivity factor is defined as k_2/k_1, whereas k_2 is the retention factor of the second eluting analyte.

Derivatization procedures for AAs and zwitter ionic compounds

AA tag AQC

The here described pre-column derivatization procedure follows mostly the derivatization protocol established by Cohen [43]. In order to ensure reproducible derivatization yields, AQC was used in 10-fold molar excess to the analyte. AA stock solutions containing 50 mM of the corresponding analyte in 0.1 N HCl were stored at 4 °C and diluted to 1 mM with Millipore water prior to use. Aliquots of 10 μL analyte solution were pipetted directly into 100 μL glass inserts, positioned in crimp-caped HPLC vials. After addition of 70 μL borate buffer and 20 μL AQC reagent solution (3 mg/mL or 10 mM), the derivatization solution was incubated for 10 min at 55 °C in a water bath. Note that no difference in derivatization efficiency was noticed, if the heating step was performed in an oven or a water bath, nor if high recovery vials from Sigma-Aldrich (Vienna, Austria) or ordinary crimp-cap HPLC vials with glass inserts were used (data not shown). However, the heating step reduced the occurrence of a minor derivatization side product of Tyr [43]. The AQC-AA derivatives were stable for at least one week, if stored at 4 °C. The derivatization scheme is provided in Fig. S1 in the ESM.

AA tag fluoresceine isothiocyanate

Aliquots of 100 μL 20 mM borate buffer (pH 9.8) were pipetted into amber-colored Eppendorf® reaction tubes (Hamburg, Germany), followed by addition of 10 μL of 10 mM FITC solution in DMF and 10 μL of the corresponding 1.1 mM AA solution. The AAs were used in 1.1 molar excess compared with the FITC reagent. The reaction solution was incubated on a thermo shaker at 60 °C for 2 h. For AAs with two reactive groups, such as Lys, a 2:1 ratio of reagent to AA was employed in order to address both amine moieties (Fig. S2 in the ESM).

Results and discussion

General aspects

Chromatographic conditions were chosen to facilitate the enantioseparation of a broad range of *N*-derivatized zwitterionic compounds, including proteinogenic AAs and non-proteinogenic AAs with amino-acid-type motive. The amino group is positioned at the α-C or the β-C atom and the acidic moiety may be carboxylic acid, sulfonic, acid or phosphonic acid or a combination thereof. Furthermore, positional isomers with increasing hydrophobicity and sterical hindrance as well as halogen-substituted Phe derivatives and positional isomers of Tyr are compared.

The aim of this work was primarily to establish a mechanistic analyte–ligand interaction study. Since the generated data had to be comparable in terms of retention time and enantioseparation performance, the optimization of separation conditions was omitted. Hence, the mobile phase conditions were not ideal and may leave room for further optimization work. Besides constant flow rates and column temperatures, enantioseparation of AAs were conducted using either the polar-organic or the reversed-phase (RP) elution mode (data provided in the supporting information only). In previous studies, MeOH with 350 mM AcOH and 65 mM NH_4OAc was determined being the most suited condition for the chiral separation of chemically diverse analytes using cinchona-based anion exchange-type chiral stationary phases (CSPs) [35]. The QN/QD type columns (QN/QD-AX) exhibit weak anion-exchange characteristics, for which the chiral recognition mechanism was extensively studied [35, 36, 38]. The main driving force for acidic analyte retention is the anion-exchange site of the quinuclidine ring. Analyte retention follows the stoichiometric displacement model [35] and can be manipulated by addition of acidic additives to the mobile phase. The choice of organic modifier, the acid to base ratio and the column temperature can be varied to adjust analyte retention and enantioselectivity.

In case of N-terminal AA-tags, chiral recognition and analyte retention also correlate with other additional interaction forces, such as hydrogen bonding between the carbamate group of the CSP and the urea-group of the AQC-tag or the thiourea-group of the FITC-tag, π–π stacking between the aromatic moieties of the CSP and the aromatic AA-tag, van-der-Waals interaction, and steric hindrance induced by the *tert*-butyl group of the CSP and the variable rest of the analyte (Fig. 1). It is important to mention that the N-terminal

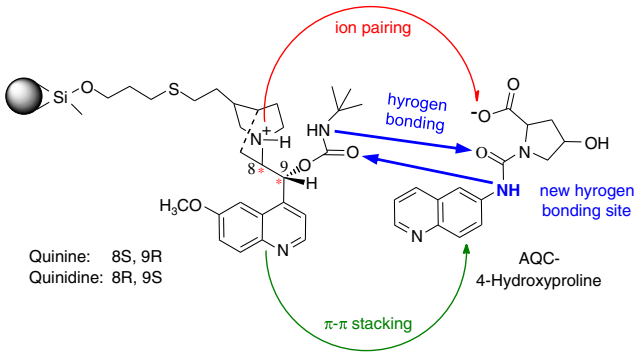

Fig. 1 Selector–selectand interaction exemplified by the diastereomeric *tert*-butyl carbamate QN- and QD-type selector (QN-AX and QD-AX) and AQC-tagged 4-hydroxy-proline

urea-linkage of AQC provides for primary as well as secondary AAs an additional hydrogen bond donor site. The latter would be absent for secondary AAs, in the case of the amide-linkage tags *N*-2,4-dinitrophenyl (DNP) [44] and 6-methoxyquinoline [45], as well as the amino-linkage tag *N*-3,5-dinitrobenzoyl (DNB) [37]. Mandl et al. has demonstrated a significant decrease in chiral recognition for *N*-methyl-substituted DNB-AAs and N-DNP-AAs compared with N-DNB-AAs on *tert*-butyl carbamoylated QN CSPs. This proves the importance of such an additional hydrogen bond donor group for enantiomer separation [37].

QN and QD are diastereoisomers, which are often described as "pseudo-enantiomers," due to the inverse orientation of the two key chiral centers at positions C8 and C9 (Fig. 1). Analytes were separated using single injection of the racemic AA mixtures followed by identification with one of the two pure enantiomers, if available. Although these selectors are inversed in their enantiomer selectivity, the combination of differences in retention strength induced by sterical hindrance combined with enantiomer separation can provide unique separation results for complex mixtures on one or the other of these chiral QN-AX/QD-AX columns.

Enantioseparation of proteinogenic AAs

Separation of N-6-aminoquinolyl-carbonyl AA derivatives

The enantioseparation performance of *N*-6-aminoquinolyl-carbonyl AA (AQC) derivatives of 19 chiral and 1 achiral proteinogenic AA is illustrated in Fig. 2a, b. The raw data are summarized in Table S1 in the ESM. The comparison of the actual contribution of the AA side chain to the entire retention (Fig. 2a) and enantioseparation (Fig. 2b) performance of the AQC-AAs provides the basis for the systematic mechanistic discussion of analyte–chiral selector interaction. For this purpose, AAs have been merged into groups with similar interaction modes, such as hydrophilic, nucleophilic, aromatic, amidic, basic, and acidic AAs. In general on QN-AX, the D-enantiomers and on QD-AX, the L-enantiomers are the first eluting solutes, with exception of Pro and Asp, where the elution order is reversed (Table S1 in the ESM).

Furthermore, the discriminated enantiomers shows in all cases and for both chiral columns lower retention factors (k_1) compared with Gly with exception of Cys, Trp, Asp, and glutamic acid (Glu). Also, the k_2 values for Ala, Leu, Pro, Gln, Arg, and His were lower than k of Gly, for both selector types, respectively. Taking the three-dimensional orientation of the chiral analyte towards the chiral selector into account, it becomes obvious that the discriminating increment that is active for one antipode can be reduced or become ineffective for the other, if sterical hindrance is involved. As depicted in Fig. 1, enantiorecognition and chemoselectivity is dependent on a variety of different interaction modes including hydrogen bonding, ion pairing, π–π interaction and sterical hindrance. On the other hand, AA residues with high π–electron density can interact with the aromatic quinoline ring system of neighboring CSP molecules on the stationary phase via π–π [46], carbonyl–π [47], sulfanyl–π [48], or sulfonyl–π interaction [49]. Hence, enantioseparation and retention strength are influenced by different interaction types, which have to be viewed separately. Note that interaction factors which induce enantioseparation can also increase retention and vice versa for the one or the other selector type or for both.

Most AA-AQC derivatives are separated with a separation factor above 1.5, with exception of Ala, Pro, Gln, Asp, and Glu on CSP QN-AX and Asp, Glu, and Gln on CSP QD-AX. Best enantioseparation is observed for Thr, Ile, Asn, Val, and Trp on CSP QN-AX and Ile, Val, Thr, Cys, and Phe on CSP QD-AX (Fig. 2b). Strongest retention, however, is exhibited by the acidic AAs, Asp and Glu, aromatic and amidic AAs, Trp, Asn, and Tyr, followed by Cys, Thr, and Phe. The origin of the overall observed trend that retention times are highest for the QN-AX column (Fig. 2a) lies in the higher selector density of 66 mmol/g bound to QN-AX CSP.

Aliphatic and hydrophobic AAs The enantioseparation of aliphatic AAs (Fig. 2b) are mainly driven by discriminating effects, exhibited by the bulkiness of the AA-side chain rather than by hydrophobic interactions. The least bulky AAs, Ala, Leu, and Met show the lowest separation factors among hydrophobic AAs. It is apparent that the positioning of a β-methyl group in the side chain of Val and Ile induces steric hindrance strongest for the discriminated enantiomer during selector–selectand complex formation, compared with Leu, possessing a γ-methyl branch. The substitution of a methylene group by a sulfanyl group does not influence chiral separation since Leu and Met possess the same number of chain units and similar separation factors. The influence of sterical factors is best observed for the secondary

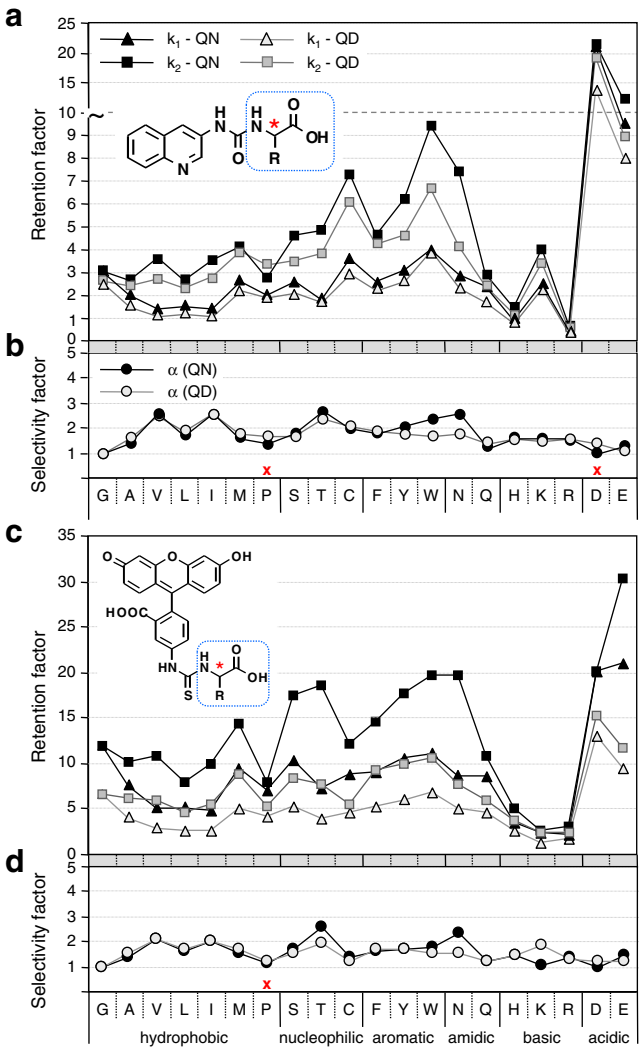

Fig. 2 (**a**) Retention factors and (**b**) separation factors for AQC-tagged proteinogenic AAs on QN-AX and QD-AX CSPs, compared with (**c**) retention factors and (**d**) separation factors obtained for the corresponding FITC-tagged AAs. A reversal in elution order is indicated by an *asterisk*. Chromatographic conditions: mobile phase, MeOH with 360 mM AcOH and 65 mM NH_4OAc; column temperature, 25 °C; flow rate, 1 mL/min; and fluorescence detection, $\lambda_{(ex)}$=248 nm and $\lambda_{(em)}$=395 nm. Trp was only detected at 280 nm because of fluorescence quenching

AA, Pro, where a significant decrease in separation efficiency is seen for QN-AX and QD-AX compared with all other hydrophobic AAs. In addition, the lack of a hydrogen donor site at the linkage point to Pro (Fig. 1) is a feature, known to be responsible for the reversal in elution order of Pro. This effect combined with an additional hydrogen bond donor site, facilitated by the new urea-linking group of the AQC-tag, might also account for the observed increase in separation efficiency of AQC-Pro compared wiht NBD-Pro on QN-AX and QD-AX [14].

Concerning the influence of the AA side chain on retention, basically the same trend is observed for the retention factor k_1 as earlier described for the separation factors. The only deviation is seen for Met (Fig. 2a). For this AA the unexpectedly high retention time is most probably induced by additional electron donor acceptor interaction between the sulfanyl group of Met and the positively charged quinuclidine ring of the selector (Fig. 1).

Nucleophilic AAs The nucleophilic AAs Ser, Thr and Cys follow the same trend as described for hydrophilic AAs, namely a higher separation factor for β-methylated and β-hydroxylated Thr, compared with Ser (Fig. 2b). Thr also shows a strong deviation in separation factors for the two antipodes with more distinct enantioseparation on QN-AX compared with QD-AX. Regarding separation factor (Fig. 2a), Cys provides similar results as previously shown for Met, but a comparison of retention factors shows a strong contribution of hydrogen bonding between the strong nucleophilic thiol moiety of Cys, and the carbamate group of both selector types. For Ser and Thr, identical retention behavior was observed for the D-enantiomers on QD-AX, while on QN-AX a significant increase in retention is observed for L-Thr. The first eluting antipode of Thr, however provided the lowest retention factors in this group of AAs.

Aromatic AAs The three different aromatic moieties Phe, Tyr and Trp exhibit only a slight declination of enantioselectivity on QD-AX, while on QN-AX a clear increase in α from 1.81 (Phe) to 2.36 (Trp) is observed (Fig. 2b). Note that the "pseudo" enantiomeric nature of the QN and QD selector may account for this observation. Taking the previous results into consideration, it can be expected that the π–π interaction as well as the hydrogen donor sites of Tyr and Trp support chiral recognition by offering additional interaction possibilities for selector–selectand complex formation compared with Phe. These strong non-stereoselective interactions with the selector are responsible for the elevated retention times for both antipodes of Tyr and Trp and lead to k_1 values above those of Gly (Fig. 2a). In general, the retention factors rise in the order Phe, Tyr, and Trp for both enantiomers, respectively, which is also the order of increasing p-electron density within the aromatic ring system.

Amidic AAs The main difference between the two amide group containing AAs Asn and Gln, lies in an additional methylene group in the side chain of Gln. This seemingly small difference has a strong influence in their sterical structures, since the amide group in Asn is in β-position and in Gln in γ-position. As previously discussed for the hydrophobic AAs, Leu and Ile, the position of the substituent is important in terms of chiral recognition as well as analyte retention. Therefore the observed trend that separation factors as well as retention factors are higher for Asn compared with Gln is not unexpected. It is however, surprising that the

k_2 values of Asn on QN-AX (7.38) is comparable to that of Cys (7.27), which may indicate that both side chains function as hydrogen donor during hydrogen bond interaction between selector and selectand.

Basic AAs The basic AAs Lys, His and Arg exhibit almost identical selectivity factors on both CSPs. Since the reagent AQC reacts with both primary amino groups of Lys, this AA possesses no basic moiety, but carries two aromatic quinoline groups in their stead. Therefore Lys observes a much stronger retention compared with His and Arg, which exhibit the lowest retention factors of all investigated proteinogenic AAs. In actual fact, the first eluting enantiomers of Arg and His are excluded from retention, by repulsion of their basic group by the weak anion exchange moiety of the selector.

Acidic AAs In comparison to the amidic AAs, Asn, and Gln, their acidic counterparts Asp and Glu show poor separation on both CSPs. In particular, the enantiomers of Asp cannot be separated on QN-AX and those of Glu are not resolved on QD-AX. In contrast to the basic AAs, the acidic AAs are very strongly retained. This result can be expected because of ion pairing interaction between the acidic AA side chain and the anion exchange moieties of both selectors. The additional acidic group in β- and γ-position of Asp (p*K* 3.65) and Glu (p*K* 4.25) is in actual fact a competitor to the α-carboxylic acid group for ion pairing interaction with the quinuclidine amine. Therefore, additional inter-molecular interaction with neighboring selector molecules in terms of ion pairing or acid–π interaction [49] has to be considered. Furthermore, all interaction modes, which would otherwise support enantioselective separation are here overruled and partially nullified by this strong interaction. Because of the exceptionally strong retention, with separation factors of 1.29 for Glu on QN-AX and 1.38 for Asp on QD-AX, and no separation for the other selector type, an influence in the diastereomeric character of the QN and QD selectors seems apparent. The observed reversal in the elution order of the enantiomers of Asp may be the result of such an overruling of retention inducing interaction versus enantioseparation. As earlier mentioned for Val and Ile, Thr and Cys, as well as Asn, AA side chain residues with substituents in the β-position interact strongest with both QN/QD-type CSPs.

Comparison with of polar-organic and the RP elution mode In addition to the polar-organic elution mode, also RP conditions employing a mobile phase with MeOH/aqueous 20 mM NH_4OAc (pH 6.0) in 80/20 ratio were tested (data not shown). However, only the secondary AAs exhibited better separation performance in RP mode compared with polar organic mode, while aromatic, basis and acidic AAs, such as Phe, Tyr, Trp, and Asp were not separated at all under RP condition.

Separation of fluoresceine thiourenyl derivatives

FITC reacts under alkaline conditions with amines forming fluoresceine thiourenyl derivatives (Fig. S2 in the ESM). The obtained separation performance for the proteinogenic AAs tagged with FITC is illustrated in Fig. 2c, d and all retention and separation factors are summarized in Table S2 in the ESM. The thiourea linking group of FITC-AA derivatives stands in structural analogy to the urea bridge of the corresponding AQC-AAs. Although the thiourea linker of FITC is slightly more acidic than the urea group of AQC, the addition of acetonitrile to the polar-organic mobile phase used for FITC-tagged AAs should reduce the acidity of thiourea and facilitate similar interaction properties for both linker groups with the respective selector site. Concerning the chemical structure of the FITC-tag, it is not only much bigger in size than the AQC-tag, but it also possesses three aromatic rings with a carboxylic acid group as well as a quinone and a hydrochinone moiety. Due to the more hydrophobic and aromatic nature of FITC, 20 % acetonitrile had to be added to the methanolic mobile phase. Acetonitrile combined with formic acid and ammonium formate was described in literature as additives which reduced π–π interaction and enhances the elution strength of the polar-organic mobile phase for separation on QN-type CSPs [35, 36].

Due to strong π–π interaction between the large FITC-tag and the quinoline ring of the CSP, already the simple AA, Gly is strongly retained on both CSPs, QN-AX and QD-AX, with retention times of 11.9 and 6.55 min, respectively. As earlier mentioned, QN-AX possesses a slightly higher selector density than its counterpart, which may lead to the observed deviation in retention strength of QN-AX and QD-AX. The general trend however is the same as observed for AQC-AAs. Retention times are higher for separation on QN-AX compared with QD-AX. The main deviations are observed for Cys, Lys, Asp, and Glu. On the QN-AX column, Asp and Glu are the only AAs, whose retention factors are both higher than that of Gly. The retention factors of both enantiomers of Ala, Val, Leu, Ile, Pro, Gln, His, Lys, and Arg are below that of Gly. The enantiomers of Trp, Asp and Glu eluted after Gly-FITC using QD-AX, while Ala, Val, Leu, Ile, Pro, Cys, Gln, His, Lys, and Arg elute before Gly. A comparison of retention and separation behavior of AA-FITC derivatives provide evidence that retention inducing interactions are dominating the retention factor diagram in Fig. 2c, while the FITC-tag does not improve chiral separation (Fig. 2d), compared with the corresponding diagrams of the AA-AQC derivatives (Fig. 2a, b). The following AAs are separated with separation factor below 1.5: Ala, Pro, Cys, Gln, Lys, His, Arg, Asp, and Glu on the QN-AX column, and Ala, Pro, Cys, Gln, Lys, Arg, and Asp on the QD-AX column.

Hydrophobic, nucleophilic, and acidic AAs Independent of the fluorescent tag used, the methylene group in β-position

of Val and Ile has a beneficial influence on retention and enantioselectivity of aliphatic AAs. The exceptionally strong retention of Met on both CSPs may be the cause of a combined effect of enhanced retention due to π–π interaction between the aromatic moieties of the FITC-tag and the quinoline ring system of the selector and electron donor/acceptor interaction between the sulfanyl group of Met and the positively charged nitrogen of the QN/QD selector. Also, the same switch in elution order is observed for the secondary AA, Pro-FITC, as observed for Pro-AQC.

The nucleophilic AAs Ser and Thr possesses separation factors higher than 1.5, as observed for the corresponding AQC-AAs. It is however, surprising that Cys-FITC shows such a strong reduction in retention as well as enantiomer separation compared with Cys-AQC. A possible explanation may lay in the increase of the pK_a values of acidic groups in aprotic media. The addition of 20 % ACN to the methanolic mobile phase may be sufficient to increase the pK_a of the thiol-group of Cys, which reduces ion pair interaction and leads to a reduction of retention factors for Cys, compared with Ser and Thr, which possess a less acidic hydroxyl group. A comparison of the retention behavior of the Cys enantiomers in Fig. 2c (Table S2 in the ESM) shows that L-Cys-FITC exhibits reduced retention on QN-AX and D-Cys-FITC on QD-AX, compared with the corresponding derivatives of Ser. The same reduction in retention factors can be seen for the acidic AA Asp on both CSPs. In accordance to Asp-AQC, no separation of enantiomers is performed on QN-AX and only weak separation on QD-AX. No reversal in elution order is detected for the enantiomers of Asp-FITC on QD-AX. Since Glu has its second carboxylic acid group in γ-position, retention factors are lower than for Asp.

Amidic, basic and aromatic AAs The amidic AAs, Asn and Gln as well as the basic AAs, His and Arg follow the same trend for retention and separation (Fig. 2c) as previously shown for their AQC-labeled counterparts. However, due the strong π–π interaction exhibited by the FITC-tag, His and Arg do not observe an exclusion effect, but ionic repulsion of the basic groups of AA by the positively charged selector are surely responsible for their low retention times compared with hydrophobic AAs. In comparison to AQC-Lys, FITC-Lys exhibits a strong reduction in retention for both enantiomers on both CSPs, combined with an increase in separation performance on QD-AX. The reason for this effect is unclear. Since the derivatization reagent was added in 10-fold excess, a double labeling of both primary amino-groups of Lys can be expected. The high aromaticity of $(FITC)_2$-Lys ought to lead to higher retention times than here observed even in the presence of some acetonitrile, but the sterical bulkiness of $(FITC)_2$-Lys may reduce the interaction possibilities of this AA with the selector. Intra-molecular π–π interaction between the two FITC-moieties may also lead to a preferred sterical conformation of the molecule, which could explain the strong deviation in selectivity observed for $(FITC)_2$-Lys on the two diastereomeric CSPs.

Because of the additional π–π interaction and hydrophobic interaction exhibited by the large aromatic FITC tag (Fig. 2c), aromatic AAs show more pronounced retention factors compared with their counterparts carrying the AQC-tag (Fig. 2a), with exception of Trp. FITC-Trp shows in relation to the other aromatic AAs, slightly shorter retention times compared with AQC-Trp. In general, the reduction of selectivity factors (Fig. 2d) for aromatic AAs indicates that acetonitrile not only reduces π–π interaction, but also quenches other molecular interactions which are essential for chiral recognition and enantioseparation.

Comparison of AQC and FITC for AA separation

The structural difference of the fluorophore FITC compared with AQC lies in the much higher π–electron density of its large aromatic ring system comprising two phenol rings and one benzene ring with a carboxylic acid group which can undergo ring-closure into a spiro-lactone. The increase in π–π interaction property leads to elevated retention times, which necessitate the addition of acetonitrile to the methanolic mobile phase in order to quench electron donor–acceptor interactions. Unfortunately, the latter does not only reduce analyte retention, but also reduces the separation factors of all FITC-AAs with exception of Ala, Ile, Gln, and Glu on QN-AX as well as Ser, Lys, and Glu on QD-AX, for which a slight increase in α values are seen.

Separation of other AQC-tagged AA-type compounds

The AQC label provided sufficient enantioseparation performance and retention behavior for proteinogenic AAs. Further investigations were assigned to provide more in-depth insight into selector–selectand interaction and extent the application field towards enantioseparation of AQC-tagged AA-type compounds with different isomeric side chains, including secondary amines, bulky ring systems as well as sulfonic acid and phosphonic acid analogs, on cinchona based weak anion exchange type columns. An overview of investigated AA-type test analytes is shown in Fig. 3. The position of functional substituents and the branching of alkyl chains is high-lighted in a separate line at the lower part of Fig. 4.

Branching of aliphatic AAs and their isomers

By comparison of separation factors obtained for AQC-tagged AAs of Ala, Val, Leu, and Ile with their non-proteinogenic isomers, Nva, AABA, and *a*Ile (Fig. 3a), the positional influence of an additional alkyl-type substituent on enantioseparation becomes obvious (Fig. 4b). However,

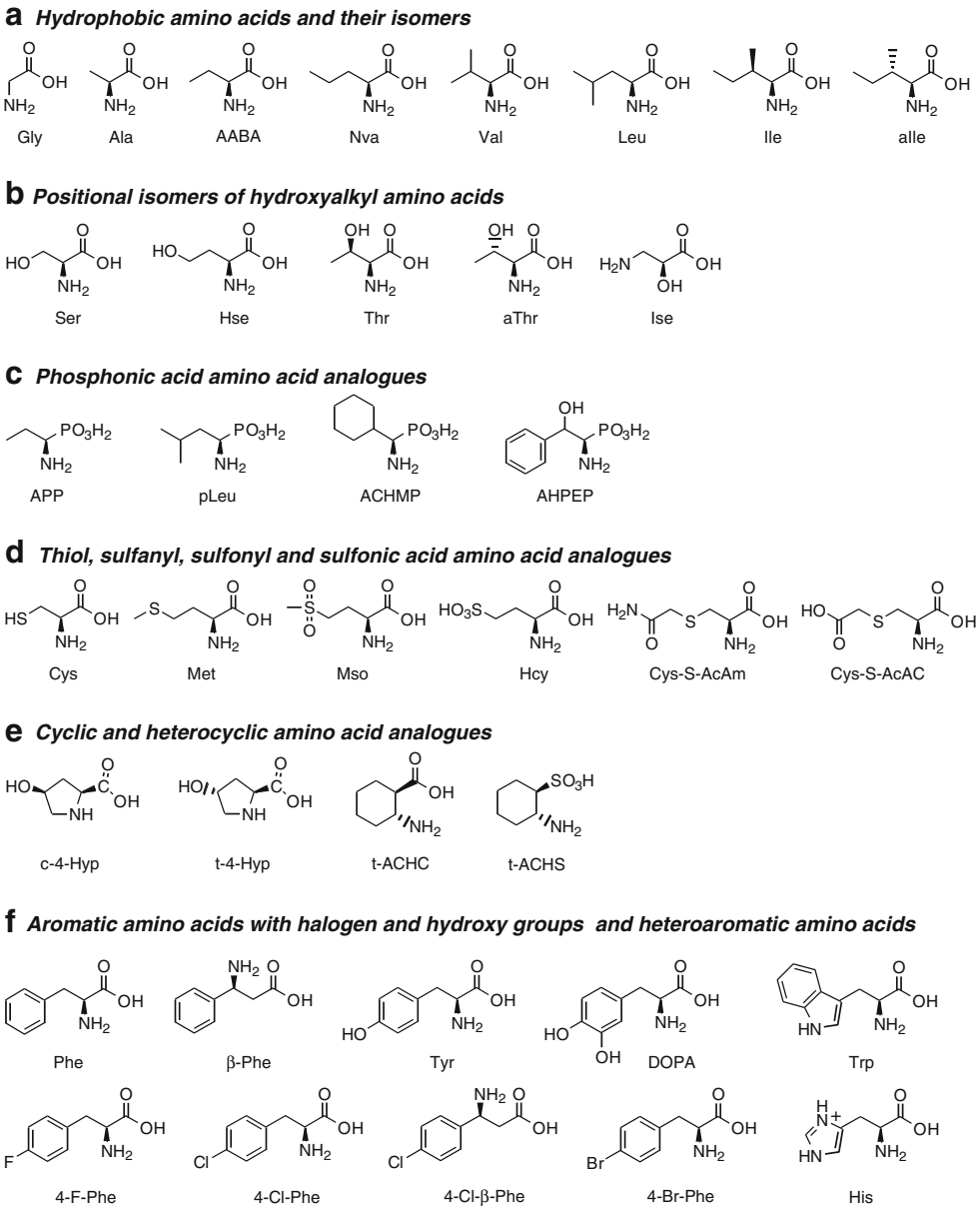

Fig. 3 Overview of investigated AA-type compounds for discussion of specific selector–selectand interactions. If not otherwise stated, all structures are shown in L-conformation

only for the short-chained compounds Gly, Ala, and AABA that an increase in selectivity is observed for increasing *n*-alkyl chain length. Leu contains one additional carbon chain unit compared with Nva, but because of its position on the γ-C, this methylene group does not contribute to selectivity. As for the β-methyl substituted AAs Val, Ile, and *a*Ile, they all show elevated selectivity factors compared with the former but do not vary from one another. Note that a list of retention and separation factors is provided in the supporting information in Table S3 in the ESM.

The retention factors (Fig. 4a) of the second eluting enantiomers decrease with increasing length of the *n*-alkyl chain or as soon as a substituent in γ-position is present (e.g., Leu) but increase with the addition of a methylene group in β-position (e.g., Val). This trend is seen for Ala compared with AABA as well as Leu compared with Val, Ile, and *a*Ile. It can be stated that an increase in sterical hindrance caused by a substituent in β-position, directly beside the chiral center (α-position) of the AA leads to a reduction in accessibility of one enantiomer, which observes a fast elution. The other antipode finds a better fit into the selector binding pocket and is therefore the favored binding partner, which elutes last.

As shown in Fig. 5 (Table S4 in the ESM), the AQC-derivatives of L-Ile and L-*a*Ile as well as D-Ile and D-*a*Ile coelute on QD-AX, while on QN-AX, L-Ile and L-*a*Ile can be partially separated. This effect is most probably the result of the higher selector coverage on the QN-AX CSP. However, a stronger preference towards selector–selectand interaction cannot be ruled out. On the contrary, the second stereogenic center of Ile is localized in β-position, which interacts with the

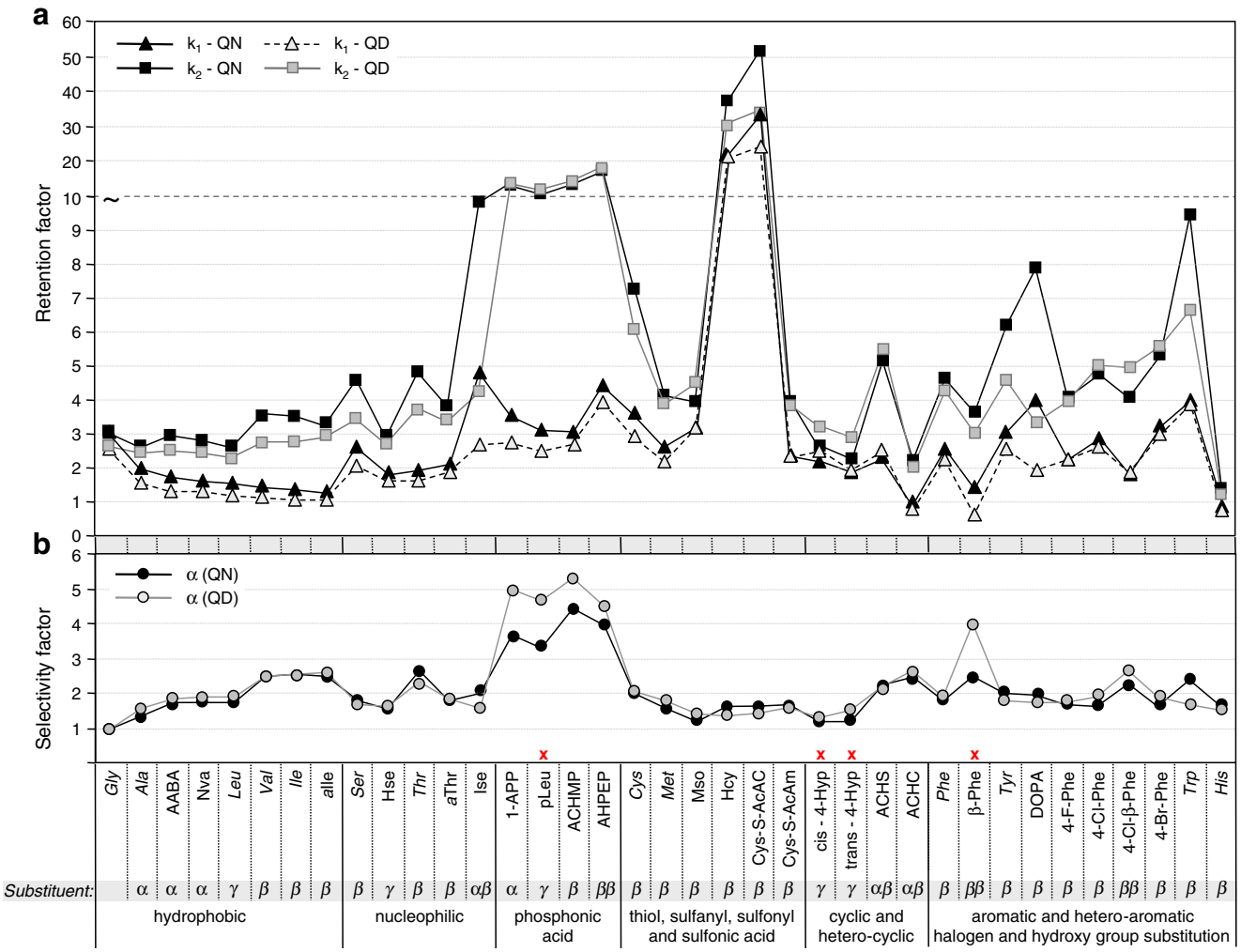

Fig. 4 (**a**) Retention factors and (**b**) separation factors obtained for the separation of AQC-tagged zwitterionic compounds on QN-AX and QD-AX. A reversal in elution order is indicated by an *asterisk*. Chromatographic conditions: mobile phase, MeOH with 360 mM AcOH and 65 mM NH_4OAc; column temperature, 25 °C; flow rate, 1 mL/min; and fluorescence detection, $\lambda_{(ex)}$=248 nm and $\lambda_{(em)}$=395 nm. Trp was only detected at 280 nm because of fluorescence quenching

selector either by hydrophobic interactions or by different steric demands during complex formation. It is reported, that hydrophobic interaction provides only negligible contribution to chiral recognition [50], while the relevance of steric hindrance during complex formation of cinchona based selectors and aliphatic AAs has been shown for DNB-(S)-Leu by X-ray spectroscopic analysis [38] and by quantitative structure property related studies [5]. Steric hindrances of the *tert*-butyl residue and the Ile side chain might facilitate enantiodiscrimination but does not influence diastereoselectivity.

Note that Zahradnickova et al. has used OPA in combination with 1-isoindolyl-(1-thioglycosides) derived from 1-thio-b-L-fucose for pre-column chiral derivatization of homoisoleucine (HIle) epimers and enantiomers. Separation was performed via RP-chromatography leading also to co-elution of L-enantiomers of HIle and allo-HIle as well as co-elution of the corresponding D-enantiomers [51].

Positional isomers of hydroxyalkyl AAs

In this group of positional isomers, Ser, Thr, and *a*Thr carry a hydroxy group in β-position, while Hse has an additional methylene group in its side chain and a hydroxyl group in γ-position. In case of isoserine (Ise), the amino group and the hydroxyl group are switched in their position compared with Ser (Fig. 3). Concerning selectivity, no difference in separation factor was observed for β-methyl substituted AABA or β-hydroxy substituted Ser (Fig. 4b) on QN-AX. A comparison of retention factors in Fig. 4a (Table S3 in the ESM), however, provides evidence that strong hydrogen bonding plus steric factors are involved in the separation of Ser, Thr, and Ise. For the γ-methyl and γ-hydroxyl analogs, Nva and Hse, it is apparent that the hydroxyl-substitution in γ-position leads to a reduction in selectivity, with little influence on analyte retention. In the case of Val, Thr and *a*Thr, the β-methyl plus β-hydroxy

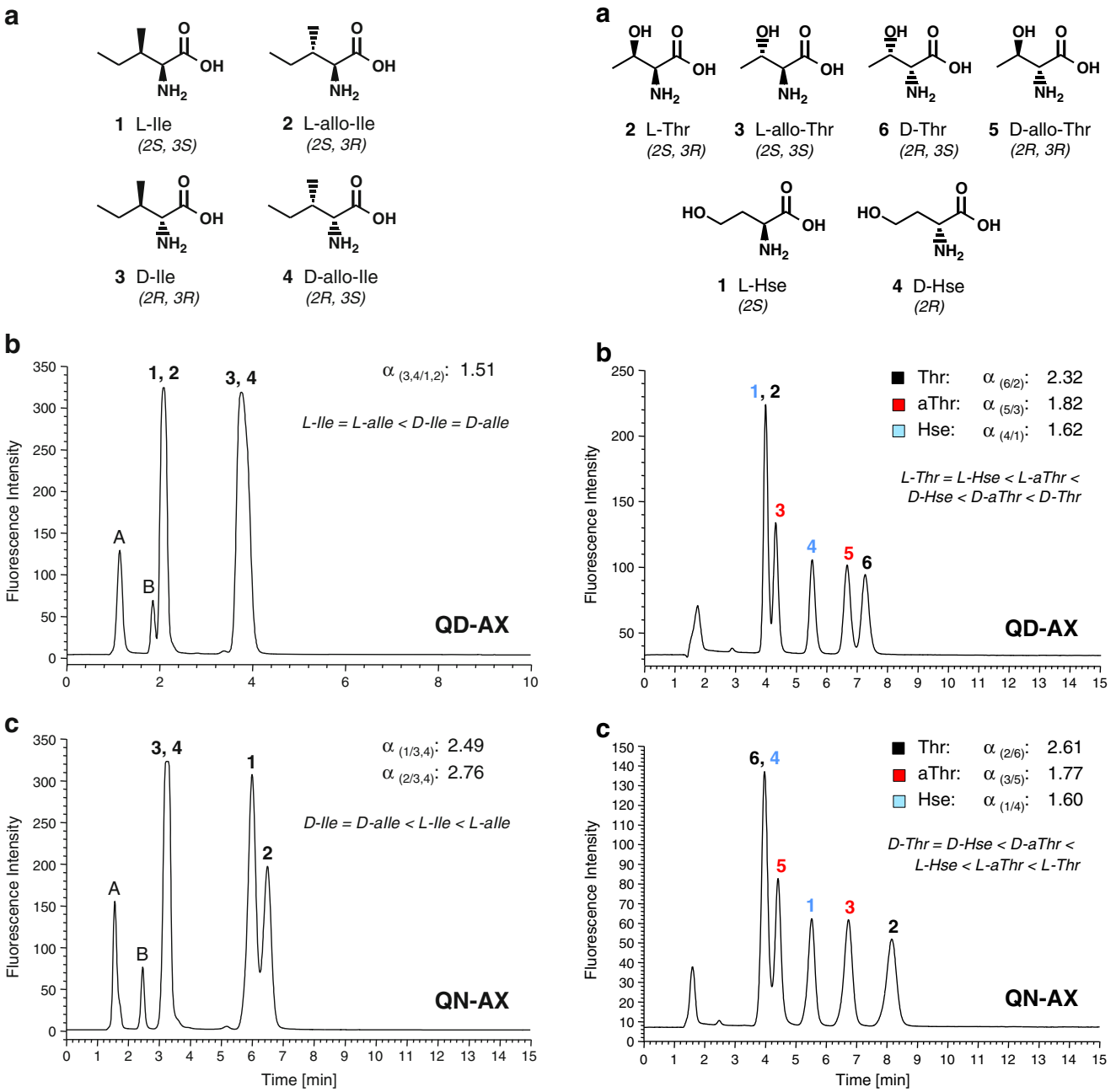

Fig. 5 (**a**) Structural overview of stereoisomers of isoleucine and chromatograms of AQC-tagged L,D, allo-L, and allo-D isoleucine on (**b**) QD-AX and (**c**) QN-AX. A, B AQC-related reagent peaks. Chromatographic conditions: mobile phase, MeOH with 360 mM AcOH and 65 mM NH_4OAc; column temperature, 25 °C; flow rate, 1 mL/min; and fluorescence detection, $\lambda_{(ex)}$=248 nm and $\lambda_{(em)}$=395 nm

Fig. 6 (**a**) Structural overview of stereoisomers of threonine including homo-serine and chromatogram of AQC-tagged L,D, allo-L, and allo-D threonine as well as L- and D-homo-serine on (**b**) QD-AX and (**c**) QN-AX. A, B AQC-related reagent peaks. Chromatographic conditions: mobile phase, MeOH with 360 mM AcOH and 65 mM NH_4OAc; column temperature, 25 °C; flow rate, 1 mL/min; and fluorescence detection, $\lambda_{(ex)}$=248 nm and $\lambda_{(em)}$=395 nm

substitution of Thr and *a*Thr exhibits a substantial variation in selectivity and retention. The second chiral centre in β-position leads to a difference in sterical orientation of the β-hydroxy group in L-Thr (2S, 3R), L-*a*Thr (2S, 3S), D-Thr (2R, 3S), and D-*a*Thr (2R, 3R). Since β-positional substituents influence chiral discrimination strongest, Thr and *a*Thr can be nicely separated on QD-AX as well as QN-AX (Fig. 6; Table S5 in the ESM).

Hereby, a 3S orientation in β-position is favored over 3R on QD-AX since L-*a*Thr is stronger retained than L-Thr and D-Thr elutes after D-*a*Thr. In addition, L-Thr co-elutes with L-Hse on QD-AX and D-Thr co-elute with D-Hse on QN-AX. This result indicates that the hydroxyl group in the 3R configuration of L-Thr does not participate in any kind of chemoselective recognition on QD-AX. The same accounts

for its mirror image D-Thr on QN-AX. In case of the β-AA Ise, the hydroxyl group is in α-position to the carboxylic acid group, while the amino group is in β-position. However, in order to compare N-terminal α- and β-AA derivatives, one has to view the position of all functional groups from the perspective of the amino group and not from the carboxylic acid group. For AQC-Ise, the hydroxyl and the carboxylic groups are now in 2(ß)-position to the urea-linking group. The two hydrogen bond donor/acceptor groups in 2(β)-position lead to strongly elevated retention times, facilitating selectivity factors between 1.59 and 2.02 for QD-AX and QN-AX. As will be later discussed in more detail, β-AAs such as β-Phe observe a higher degree of enantioseparation compared with their α-AA counterparts.

Aminophosphonic acid

With α values ranging from three to five, the aminophosphonic acid test group provides the highest selectivity factors of all here investigated zwitterionic compounds (Fig. 4; Table S3 in the ESM). A comparison of the carboxylic acid and phosphonic acid analogs, Leu and pLeu yield strongly increased retention factors for the second eluting enantiomer on both investigated CSPs as well as a switch in elution order for pLeu. As earlier mentioned for Val, Ile, Ser, and Thr, β-substituted AA-type compounds such as ACHMP and AHPEP observe better separation compared with γ-substituted compounds such as pLeu. Furthermore, the comparison of the side chain influence of aminophosphonic acids reveals an additional retention increment exhibited by the aromatic moiety of AHPEP in contrast to the cycloalkane ring of ACHMP. Unfortunately, only the racemic mixtures of APP, ACHMP, and AHPEP were available and therefore no elution order could be determined for these compounds.

Thiol, sulfanyl, sulfonyl, and sulfonic AA analogs

The partially higher acidity of the thiol group in β-position of Cys provides a slight increase in retention and selectivity compared with its analog Ser (Fig. 4; Table S3 in the ESM). As expected from earlier discussions, the methyl-sulfanyl group in γ-position of Met leads to a decrease in retention as well as selectivity compared with Cys. However, the substitution of a methylene group by a sulfanyl group introduces additional electron donor/acceptor properties, which is clearly visualized by a slight increase in retention factors compared with its alkyl-type AA analogs, Nva or Ile. This type of molecular interaction, which may include sulfanyl–π and sulfonyl–π interactions in case of Mso, can be classified as being non-enantioselective selector/selectand interaction. The further oxidation of the sulfur group to a sulfonic acid group in γ-position of homocysteine (Hcy) provides a rather surprising elevation in retention, considering the position of the sulfonic acid group. The further increase in retention observed for Cys-*S*-AcAC, which possesses a sulfanyl group in β-position and a carboxylic acid group in δ-position, leads to the suggestion that i) retention decreases with increasing distance of the branching group or the substituent, which includes acidic moieties, and ii) increases for strong acidic substituents in γ-position as well as weak acidic substituents in δ-position. Since the corresponding amido-compound, Cys-*S*-AcAm exhibits retention properties similar to Met, Mso, or Gln, ion pair interaction between quinuclidine of the selector and carboxylic acid of Hcy can be expected. Nonetheless, as known from literature, electron donor acceptor interaction between a carboxylic acid or sulfonic acid group and a poly-aromatic selector can provide strong retention [49]. Therefore an electron donor/acceptor interaction of the acidic moiety in γ-position (Hcy) or δ-position (Cys-*S*-AcAC) with the amino-quinoline ring system of a neighboring selector molecule has to be considered and cannot be ruled out. Nonetheless, the elevated distance of the acidic moiety from the AQC-tag compared with Glu may provide an increase in accessibility for an intra-molecular ion-exchange interaction through a bypass of the bulky *tert*-butyl group of the selector.

For this investigated group of compounds, it can be concluded that the affinity of the thiol, sulfanyl, sulfonyl, sulfonic, acid and carboxylic acid containing selectand to the selector are mainly non-enantioselective and only induce a variation in retention strength.

Cyclic and hetero cyclic AAs analogs

Among the proteinogenic AAs, only Pro possesses a secondary amine group, which is part of a five-membered pyrrolidine ring. As earlier discussed, Pro as well as its 4-hydroxy substituted analogs, 4-hydroxy-proline (4-Hyp) exhibit a conversion in elution order compared with the other investigated AAs (Table S1 in the ESM). Since Hyp has two chiral centers, all together four stereoisomers are expected, namely the enantiomers pairs of the diastereomers cis-4-Hyp and trans-4-Hyp (Fig. 7a). Although selectivity factors are lower than observed for Ile and *a*Ile (Fig. 5) as well as Thr and *a*Thr (Fig. 6), which can be expected for substitution in γ-position, the separation efficiency for all four AQC-tagged 4-Hyp-isomers is surprisingly good on QD-AX with almost equi-distant peaks in the elution order trans-D, *cis*-D, *trans*-L, and *cis*-L 4-Hyp. The reason for the nice enantioseparation on QD-AX in Fig. 7b compared with the partial co-elution of 4-Hyp isomers on QN-AX in Fig. 7c lies in diastereomeric character of the QN and the QD-selector and in the conformational stability of the pyrrolidine ring of 4-Hyp. Although the pucker of the pyrrolidone ring of 4-Hyp can be in exo or endo position, intra-molecular hydrogen bonding

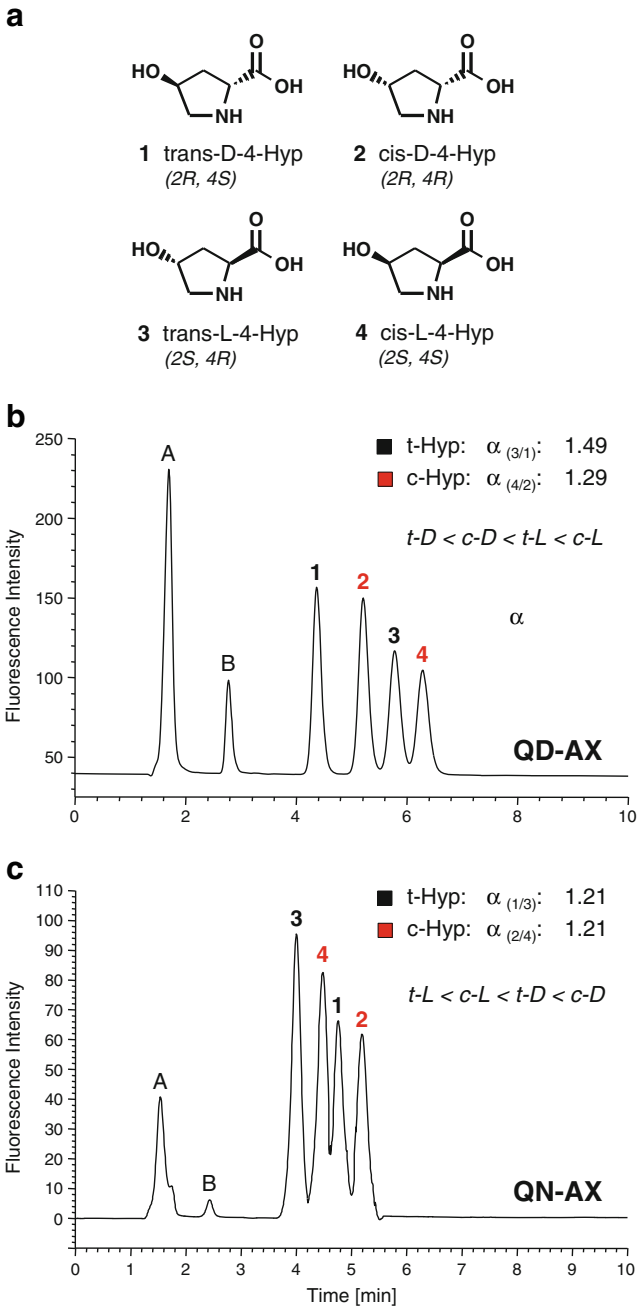

Fig. 7 (**a**) Structural overview of stereoisomers of cis/trans-4-hydroxyproline and chromatograms of their AQC-tagged derivatives on (**b**) QD-AX and (**c**) QN-AX. A, B AQC-related reagent peaks. Chromatographic conditions: mobile phase, MeOH with 360 mM AcOH and 65 mM NH_4OAc; column temperature, 25 °C; flow rate, 1 mL/min; and fluorescence detection, $\lambda_{(ex)}$=248 nm and $\lambda_{(em)}$=395 nm

between the carbonyl group and the hydroxyl group in cis-position stabilizes 4-Hyp into the endo ring pucker position [52], which leads to a stronger retention of *cis*-L-Hyp (2S, 4S) on QD-AX and *cis*-D-Hyp (2R, 4R) on QN-AX.

Besides 4-Hyp, two other cyclic pseudo-AAs, namely trans-2-amino cyclohexane sulfonic acid (t-ACHS) and trans-2-amino cyclohexane carbonic acid (t-ACHC) were investigated. In both cases, the acid moiety is in α-position and the amino group in β-position (Fig. 3e). As predicted for β-substituted compounds, separation factors for t-ACHS and t-ACHC are almost doubled compared with 4-Hyp (Fig. 4; Table S3 in the ESM) with slightly higher α values for t-ACHC. However, due to the higher acidity of the sulfonic acid moiety, t-ACHS observes stronger retention compared with its t-ACHC counterpart.

Aromatic AAs with halogen and hydroxyl group substitution

As shown in Fig. 4, halide substituents on the phenyl group (Fig. 3f) influences separation performance of the AQC derivatives to a minor degree (Fig. 4) since enantio-recognition is not dependent on a slight shift in electron density of the phenyl-ring of Phe. Concerning retention behavior, retention times of the corresponding compounds increase with decreasing electronegativity of the substituent, in the order F>Cl>Br. However, a shift of the amino group from the α-position to the β-position is of greater importance and leads to a decrease in retention combined with an increase in enantiomer separation for β-Phe and 4-Cl-β-Phe. In case of β-Phe, an increase of 2.14 in α value is obtained compared with α-Phe on QD-AX. Furthermore, the enantiomer elution order is reversed for β-Phe. For 4-Cl-β-Phe, 4-F-Phe, and 4-Br-Phe, only racemic mixtures were analyzed; therefore, no elution order is applicable.

A comparison of DOPA with Tyr, leads to similar selectivity factors combined with an increase in retention for QN-AX and a decrease in retention on QD-AX. This result exemplifies the fact that the QN and the QD selector are diastereomers and not enantiomers, and can therefore interact quite differently with the same enantiomer pair. In case of the hetero-aromatic AA Trp, an exceptionally high α value of 2.36 is determined on QN-AX, while an α value of only 1.68 is obtained on QD-AX. Retention factors of Trp are comparable for the first eluting peak on both CSPs, but the second eluting peak observes a 30 % reduction in retention on QD-AX compared with its counterpart. In general, the stronger retention of Trp compared with Phe and halide substituted Phe derivatives indicate the involvement of an additional electron donor–acceptor interaction event besides the hydrogen bond property exhibited by the indole ring. Although, with decreasing electronegativity of the substituent bound to the aromatic ring, the negative inductive effect is reduced (F>Cl>Br>I>OH>H) and the electron density of the ring is increased. The latter is due to the dominating electron pushing resonance effect of the lone electron pair of halides. The increase in electron density within the benzene ring favors electron-donor/acceptor interaction with the positively charged quinuclidine ring of the selector (nucleophile), and possibly also allows additional π–π interaction with the quinoline ring of neighboring selector molecules to take place.

Hence, retention increases in the stated order from 4-F-Phe to 4-Cl-Phe and Tyr to DOPA. In case of His, repulsion of the positively charged His by the equally charged quinuclidine nitrogen leads to reduced retention and reduced α values on QN-AX as well as QD-AX.

Conclusions

Enantioselectivity of proteinogenic AQC-tagged AAs is strongly dependent on the chemistry and stereochemistry of the AA side chain. The incorporation of a N-terminal urea bond by the AQC-tag compared with an amido-group introduced, e.g., DNB, facilitate for primary as well as secondary AAs an additional hydrogen bond donor site, which is known to be essential for chiral recognition [37]. Hence, better separation performances were obtained for the enantiomers and epimers of AQC-Pro and AQC-Hyp. Furthermore, retention and enantiomer discrimination increase for β-substituted AA type compounds compared with their α- and γ-substituted analogs. Retention times increase mostly in the order of increasing interaction properties of the β-substituent: e.g., alkyl<sulfanyl<hydroxyl<sulfhydryl<amide<aromate< carboxylic acid<phosphonic acid<sulfonic acid. Strongest retention has been observed for the di-acids, Cys-*S*-AcAC and Hcy, followed by the amino-phosphonic acids, AHPEP and ACHMP. Selectivity factors are highest for amino phosphonic acids, β-substituted AAs and β-AAs such as β-Phe, Ile, *a*Ile, Thr, *a*Thr, t-ACHS, t-ACHC, Tyr, and Ise. In general, the separation efficiency for structural isomers of AQC-tagged AA-type compounds on QN-AX- and QD-AX-type CSPs increase significantly from the β-methyl substituted Ile and *a*Ile to β-hydroxy substituted Thr and *a*Thr isomers. An increase in selectivity was also observed by Péter et al. for DNP α-substituted Pro, in the order α-methyl, β-phenyl, and β-naphthyl [44]. Furthermore, additional intra-molecular hydrogen bonding can increase the conformational stability of non-aromatic cyclic compounds such as 4-Hyp, for which a nice separation of all four structural isomers on QD-AX was observed. Since the electronegativity of halide substituents in para-position of Phe influence analyte retention, it has been suggested that besides hydrogen bonding and ion pairing interactions, also electron donor/acceptor interactions between the aromatic AA side chain and the positively charged quinuclidine ring may contribute to analyte retention and also to a smaller extent to enantioselective and chemoselective separation. In addition, also π–π interaction as well as carbonyl–π, carboxylic acid–π, sulfonic acid–π, or phosphonic acid–π interaction with the aromatic quinolinic moieties of adjacent selector molecules is considered to provide some contribution to retention. Note that electron donor/acceptor interactions can be reduced and modulated by addition of acetonitrile to the polar-organic mobile phase as it was shown for FITC-tagged AAs. Unfortunately also, a slight reduction in separation efficiency is observed, since ion pairing is involved in enantiomer recognition.

From the practical point of view, special emphasis has been set on the separation of branched AAs, Ile, *a*Ile, Thr, and *a*Thr as well as the secondary AAs, Pro, and Hyp. In general, only few analytical methods are described in literature dealing with the separation of Leu, Ile, *a*Ile, Thr, and *a*The on the epimer as well as the enantiomer level. These methods include chiral GC-MS of N-alkoxycarbonyl alkylamide [53] and heptafluorobutyl chloroformate derivatized AAs [54] on Chirasil-L-Val, RP separation with OPA/IBLC pre-column derivatization [55] and 2-dimensional RP/CSP separation employing 4-fluoro-7-nitro-2,1,3-benzoxadiazole [2, 56]. The latter method has also been employed for Pro and Hyp, isolated from collagen of mice, which is known to be very rich in Pro and Hyp.

The attractiveness in generating a one-dimensional chiral HPLC method employing a commercially available derivatization kit such as AQC for the separation of all proteinogenic and some biologically interesting unnatural AAs lies in the simplicity and reproducibility of such an analytical method, which is a prerequisite for routine analysis in biomarker research and drug analysis. Furthermore, only a solid and thorough understanding of the underlying interaction mechanisms, which are responsible for analyte retention and enantiomer separation allows the future design of tailor-made CSPs with well-defined interaction properties.

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