State-of-the-art enantioseparations of natural and unnatural amino acids by high-performance liquid chromatography

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Abbreviations

AA, amino acid; Ac, acetyl; AcOH, acetic acid; ADAM, 1-aminoadamantane; AQC (AccQ-Tag), 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate; BA, butylamine; *t*-Boc, *t*-butyloxycarbonyl; BSA, bovine serum albumin-based CSP; Bz, benzoyl; CAD, corona discharge detector; CBZ (Z), benzyloxycarbonyl; CD, cyclodextrin; CLEC, chiral ligand-exchange chromatography; CSP, chiral stationary phase; *R*(-)-DBD-PyNCS, *R*(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; DCT, dichloro-*s*-triazine; 3,5-DCIB, 3,5-dichlorobenzoyl; DEA, diethyl amine; 2D-HPLC, two dimensional HPLC; 3,5-DNB, 3,5-dinitrobenzoyl; DNP, 2,4-dinitrophenyl; Dns, dansyl; EA, ethylamine; ELSD, evaporative light scattering detector; Exc/Em, excitation/emission wavelength; EtOH, ethanol; FA, formic acid; FI, fluorescence detection; FLEC, (+)-1-(9-fluorenyl)ethyl chloroformate; FMOC, 9-fluorenylmethyl-chloroformate; FP, succinimidyl ferrocenyl propionate; GITC, 2,3,4,6-*tetra-O*-acetyl- β -D-glycopyranosil isothiocyanate; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; IBLC, *N*-isobutyryl-L-cysteine; LOD, limit of detection; LOQ, limit of quantitation; Marfey reagent (FDAA, MR, FDNP-L-Ala-NH₂), 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; MBIC, (*R*)-methyl benzyl isothiocyanate; MeCN, acetonitrile; MCT, monochloro-*s*-triazine; MQD, *O*-9-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine; MS, mass-spectrometry; NAC, *N*-acetyl-L-cysteine; Nap-Btz, (*S*)-1-[H-benzo(d) (1,2,3)triazol-1-yl]-2-[6-methoxynaphthalen-2-yl-propan-1-one]; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitrobenzofurazan; NEIC, (*S*)-1-(1-naphthyl) ethyl isothiocyanate; (*S*)-NIFE, (*S*)-*N*-(4-Nitrophenoxy-carbonyl)phenylalanine methoxyethyl ester; *N*-NPTH, *N*-naphthoyl; (*S*)-OBS, *O*-benzyl-(*S*)-serine; OPA, *o*-phthalaldehyde; L-PGA-OSu, L-pyrogutamic acid succinimidyl ester; L-PGA[d(5)]-OSu, isotopic variant of L-pyrogutamic acid

succinimidyl ester; Phg, phenylglycine; PA, propylamine; *N*-PTH, *N*-phthaloyl; QD, quinidine; QN, quinine; QN-AX and QD-AX, *t*-butyl carbamoylated QN and QD CSPs; L-STC, (*S*)-trityl-L-cysteine; TAHS, *p*-*N,N,N*-trimethylammonioanilyl *N'*-hydroxysuccinimidyl carbamate iodide; TBA, tributylamine; TEA, trimethylamine; TEAA, triethylammonium acetate; *N*-TCPHT, *N*-tetrachlorophthaloyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TPA, tripropylamine; UHPLC, ultra-high-performance liquid chromatography; ZWIX(+)TM, sulfocyclohexyl carbamoylated QN-based zwitterionic CSP; ZWIX(-)TM, sulfocyclohexyl carbamoylated QD-based zwitterionic CSP

Abstract

This review discusses recent publications on the separation and analysis of natural and unnatural amino acid enantiomers by liquid chromatography. Focus is placed on methodological aspects relating chiral stationary phases and chiral columns which can cope with the challenge. Conceptually, amino acids can be enantioseparated in free form, which refers to the resolution of polar ampholytes, or as *N*-protected amino acids, which can be regarded as acidic commodities. Such synthons are used, for instance, in peptide synthesis protocols. Amino groups are frequently tagged with highly fluorescent or MS/MS active labels in order to generate sensitive and simultaneously stereoselective assays of diverse amino acids in complex matrices. It is our intention to present the state of the art of enantioselective amino acid analysis by HPLC concepts and to pinpoint practical aspects.

Introduction

Chirality caused by molecular asymmetry has special effects on physical, chemical, biological and pharmacological properties appearing at a molecular level. Amino acids (AAs), and carbohydrates (sugars), and the peptides, proteins, glycoproteins, polysaccharides *etc.* composed of such chiral building blocks are stereochemically defined. In biological systems, chiral entities (*e.g.* receptors) often display different biologically relevant responses to the enantiomers of chiral compounds such as drugs, agrochemicals, food additives, fragrances, *etc.*, and also natural compounds including the D- and L-enantiomers of proteinogenic and non-proteinogenic ~~amino~~ acids (AAs).

As a result of the awareness of the importance of stereoselective bioactivity phenomena such as pharmacodynamics and pharmacokinetics, a strong demand has arisen in life and pharmaceutical sciences for efficient tools with which to separate the individual stereoisomers of a large body of chiral compounds *per se*, but also in trace amounts in biological matrices.

At an analytical level, liquid-phase separation techniques [liquid chromatography (LC) and capillary electrophoresis (CE)] are currently most widely applied; high-performance LC (HPLC) and supercritical fluid chromatography (SFC) have become very popular, though CE is also very powerful analytical technology [1,2]. For preparative enantiomer separations, HPLC and particularly SFC are the methods of choice [3]. Gas chromatography (GC) provides highly efficient enantioselective analysis systems for compounds which fulfill specific chemical requirements. Very useful GC separation systems exist for fully derivatized AAs [4]. However, the subject of this review rather a critical overview of publications of the last 5 years dealing with LC methodologies for the stereoselective resolution of AAs and some peptides.

Various strategies have been developed for the differentiation of enantiomers by chromatography: (i) *indirect methods* involving derivatization of a mixture of target enantiomers

by a chiral derivatizing agent (CDA) and the generation of diastereomeric compounds which are physicochemically different and should therefore be chromatographically resolvable on achiral chromatographic columns; (ii) *direct methods* in which chiral mobile phase additives are used together with standard achiral stationary phases; and (iii) *direct methods* in which chiral separations are achieved by using a chiral stationary phase (CSP). The direct methods are based on the intermediate formation of diastereomeric associates between the chiral selector (SO) moiety and the two enantiomers of the chiral analytes, the selectands (SAs). When the stability constants of the two diastereomeric associates, symbolized by $[(R)\text{-SO} \leftrightarrow (R)\text{-SA}]$ and $[(R)\text{-SO} \leftrightarrow (S)\text{-SA}]$, are sufficiently different from each other, direct enantioseparation can be achieved. In order to stabilize such associates, multiple intermolecular interactions between the SO and the SAs must come into play.

Over the years, HPLC and SFC with diverse types of CSPs have become the preferred methodologies to resolve mixtures of enantiomers and racemates. Where applicable, GC remains a powerful analytical tool for chiral separations due to the exceptionally high efficiency. For preparative enantiomer separations, HPLC and SFC are increasingly more often the method of choice thanks to a number of convincing arguments often associated with “green chemistry” [3,5].

This review discusses only the status and recent developments in the analytical separation of enantiomers of free and *N*-derivatized (tagged) natural and unnatural AAs, focusing on the direct HPLC methods. A recent review concentrated on indirect AAs-enantioseparations [4].

As depicted in Scheme 1, the structural variability of AAs is quite high, in view of (i) α -, β - and γ -AAs, (ii) cyclic AAs, (iii) constraint AAs and (iv) structural isomers, including the insertion of an additional chiral center besides the one to which the amino group is attached to. Further, it

should be emphasized that AAs are ampholytes bearing at least one basic amino group and one acidic carboxylic group. For more detailed chemical structural information, we refer to the specific literature, which is massive.

Due to the intrinsically available free primary and/or secondary amino group and the carboxyl group of free AAs, these functionalities can be targeted by specific derivatization, labeling or tagging reactions. In peptide chemistry, *N*-protected AAs are most often dealt with *t*-butyloxycarbonyl (Boc), 9-fluorenylmethyl-chloroformate (Fmoc) and benzyloxycarbonyl (Cbz or Z) as the most popular protecting groups. Under specific conditions, these protecting groups can be cleaved off without affecting the chemical structure of the peptide backbone. For analytical purposes, the Fmoc group and other tagging reagents and groups are commonly employed, some of which are listed in Scheme 2. In this context, those agents are of particular interest that exhibit a sensitive fluorescence and/or mass spectrometry (MS) signal.

1. Indirect methods of amino acid enantiomer separation

The use of CDAs to form diastereomeric derivatives of AAs that can be separated on achiral columns was introduced the early 1970s. Indirect separations require SA functional groups that are easily derivatizable; in the case of AAs, this is preferentially an amino group. The reaction with a CDA should be fast and nonstereoselective; a difference in the rates of formation of the diastereomers may otherwise cause a kinetic resolution which would eventually lead to false quantitative results. Consequently, it is essential that the chiral derivatization proceeds quantitatively for both enantiomers, and racemization does not occur on any of the chiral centers of the CDA, SA or the diastereomeric derivatives. Besides the low cost of the achiral stationary phase, the relatively facile method development, the favorable chromatographic properties [~~higher sensitivity~~ the lower limit of detection (LOD)] and the better resolution after the

derivatization of AAs are advantages of indirect methods. However, the ~~increased efforts to be taken in consideration for~~ method validation may be cumbersome. Since a fair number of CDAs are commercially available in both enantiomeric forms, the elution sequence can be changed, *i.e.* the peak of the enantiomeric impurity can be eluted before the main peak, with the result of easier quantification. The reactions of the amino group of the AAs are mainly involve the formation of amides, carbamates, ureas and thioureas. Information on this subject may be found in numerous reviews [4,6-10]. Recent relevant publications are detailed in Table 1.

Recent applications

Diastereomeric derivatives of amino alcohols and nonproteinogenic AAs with (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester [(*S*)-NIFE] have been analyzed by reversed-phase (RP)-HPLC [11]. Validation demonstrated that the method can be utilized for pharmaceutical applications. The derivatization conditions were optimized for the reaction between *o*-phthalaldehyde (OPA) and *N*-isobutyryl-L-cysteine (IBLC) reaction and for the analyses of ~~β -amino acids~~ AAs [12]. Test of the validated method in cell extract samples gave 25 pmol as the limit of quantification (LOQ). It can be pointed out that ultrahigh-performance liquid chromatography (UHPLC) can be performed more easily for the indirect than for the direct approach, which typically lacks the corresponding UHPLC column technology; however, this situation will probably change in the near future. An indirect RP-HPLC method developed and validated for the analysis of L-cycloserine in bulk D-cycloserine was based on derivatization with OPA and *N*-acetyl-L-cysteine (NAC) [13]. The optimized method proved sufficiently robust for application in routine quality control in pharmaceutical analyses. ~~*S*-Naproxen-based CDAs were applied for different model compounds by Bhushan *et. al.*~~ Phthalimidyl-(*S*)-naproxen has been used to separate the enantiomers of selenomethionine [14], and (*S*)-naproxen-benzotriazole (Nap-Btz) to separate the enantiomers of different proteinogenic AAs [15,16]. ~~Isothiocyanate-based~~

~~CDA~~s, (*R*)-Methyl benzyl isothiocyanate (MBIC) and (*S*)-1-(1-naphthyl)ethyl isothiocyanate (NEIC) were used by the same research group to separate the enantiomers of selenomethionine [17], while proteinogenic AAs were derivatized with cyanuric chloride-based reagents [18-20]. Higher stability of the diastereomers, enhanced resolution and a lower ~~limit of detection (LOD)~~ were generally achieved in comparison with earlier-described methods.

2. Direct methods of separation of structurally diverse amino acid enantiomers

Direct chromatographic methods of enantiomer separation on CSPs have undoubtedly taken over in recent years. Direct enantioseparation generally has the advantages that *i*) the chiral impurity in the SO molecules bound to the chromatographic support material is not critical, but its content should be controlled for the sake of reproducible stereoselectivity of the prepared CSPs, *ii*) partial racemization of the SO of the CSP is very unlikely as long as the chromatographic conditions are reasonable, *iii*) there is no reagent excess that could interfere with the detection or worsen ~~deteriorate~~ the chromatographic resolution in the analysis of free AAs (the issue of excess reagent peaks remains in the case of AA tagging), and *iv*) scaling-up for preparative purposes on chromatographic columns is readily achievable and can become relevant, *e.g.* in the fine chemical production of *N*-protected AA commodities with very high enantiomeric purity.

However, AA-type SAs are often derivatized (tagged) with nonchiral reagents in order to modify the analytes so that the enantiomers can be better separated on the CSPs and/or become more sensitive for detection. Naturally, partial racemization of the analyte must be avoided in such cases. ~~It should be emphasized that the concept of non-chiral derivatization preferentially of the amino group can often lead to a win-win situation as on the one side the enantioselective molecular recognition process with a given CSP can be supported and on the other side also the detection sensitivity and selectivity can be strongly enhanced.~~

These aspects are particularly emphasized throughout this paper. We ~~argument~~ focus especially more specifically on publications in which commercially available CSPs are used, making this review useful from practical aspects. Promising, though not yet commercialized CSPs are naturally also included.

This main section discusses the direct resolution of free (nontagged) AAs, the stereoselective resolution of tagged AAs and applications, particularly for complex sample matrices.

2.1. CSPs frequently applied for the enantioseparation of free amino acids

Comprehensive information on recent articles is presented in Table 2. This section relates particularly to the most promising and frequently employed CSPs.

2.1.1. Macrocyclic glycopeptide-based chiral stationary phases

Unlike other classes of SOs, macrocyclic antibiotics display a large variety of structural types. In general, these compounds have molecular masses in the interval 600-2200. There are acidic, basic and neutral types, and they often contain aromatic groups. These favorable characteristics of SOs allow them to interact with AAs of different types, taking advantage of the basket-like three-dimensional structures of the SOs in combination with diverse H-bonding and hydrophobic interaction sites derived from the intrinsic peptidic structure elements. The concept of using macrocyclic antibiotics as CSPs was introduced by Armstrong in 1994 [21] and their usefulness in AA resolutions has since been amply proven. The commercially available glycopeptide-based CSPs for HPLC are related to teicoplanin, teicoplanin aglycon, vancomycin, vancomycin aglycon and ristocetin A. However, Staroverov *et al.* used similar macrocyclic antibiotic-type SO (eremomycin) for AA analyses [22].

Recent applications

Teicoplanin and teicoplanin aglycon-containing CSPs have been successfully applied for the enantiomeric separation of synthetic monoterpene-based 2-aminocarboxylic acids [23]. Mechanistic aspects of the chiral recognition process have been discussed with respect to the structures of the analytes. Similarly, the effects of the separation conditions and temperature on the enantioselective resolution of isoxazoline-fused 2-aminocyclopentanecarboxylic acids [24] and bicyclo[2.2.2]octane-based 2-amino-3-carboxylic acids [25] have been studied on different macrocyclic glycopeptide-based (Chirobiotic) columns. Apparent thermodynamic parameters have been calculated, and entropically- and enthalpically-driven separations discussed as part of the molecular recognition process. The enantioresolutions of free unusual AAs and constraint β -AAs on teicoplanin- and polysaccharide-based columns have been compared [26,27]. The optimized methods proved applicable for enantiomeric purity (enantiomeric excess, ee) control. [27]. The unexpected and extraordinarily high selectivity and resolution obtained for these free constraint AAs on a polysaccharide-based column ensures suitability for preparative purposes. A selection of 19 pairs of free α -AAs and 6 pairs of free dipeptides were applied to test the efficiency of macrocyclic antibiotic-based SOs covalently attached to fully or superficially porous silica particles [28]. The results were compared with the enantioresolutions achieved on commercialized (Chirobiotic and cyclofructan-based Larihc) columns. With the same mobile phase, the superficially porous particle-based CSP provided the greatest efficiency and shortest analysis time. In some cases, ultrafast chiral separations could be realized in seconds. Examples of acidic or basic free AAs have not been reported. LC-MS applications are generally well feasible.

2.1.2. Crown ether-based chiral stationary phases

Chiral crown ethers with a cavity of specific size and with chiral structure elements of macrocyclic polyethers with repeating $-\text{O}-\text{C}_2\text{H}_4-$ units, where the oxygen atoms can serve as electron donors, serve as the SO moieties. Inorganic and alkylammonium cations can be incorporated into such a cavity. Unlike in cyclodextrins (CDs) where the host-guest complexation is based on hydrophobic SO-SA interactions, in crown ethers the inclusion complexation is based mainly on multiple H-bonding between the H atoms of the ammonium group of the SAs and the O atoms of the polyether of the SO. Enantioselective recognition is accomplished *via* additional intermolecular interactions of the substituents of the chiral SO and SA moieties. As a consequence, chiral crown ethers can be used to resolve enantiomers containing a protonated primary amine functional group which ~~acquires~~ requires a low pH of the mobile phase (typically pH around 2-3). The mobile phase conditions are in many cases unfavorable for MS detection. For AAs with a secondary amino function, enantioselective resolution usually fails.

Recent applications

A (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP was utilized for the enantioseparations of β^2 -amino acids [29] and isoxazoline-fused 2-aminocyclopentanecarboxylic acids [30]. The effects of the mobile phase composition, the pH, the concentration and the nature of the modifiers were investigated and discussed. CSPs based on (3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 were found to be useful for the resolution of the enantiomers of vigabatrin and its analog γ -AAs [31] and of valacyclovir and its analogs [32].

2.1.3. Ligand exchange-based chiral stationary phases

Introduced by Davankov and Rogozhin [33], chiral ligand-exchange chromatography (CLEC) for the direct separation of free α -AA enantiomers is based on the formation of diastereomeric mixed

chelate complexes through use of a chiral chelating agent, mainly derived from an α -AA together with a transition metal ion [most frequently copper(II), nickel(II), or zinc(II)] as SO. This can be carried out *via* a mobile phase additive of the SO or *via* an immobilized SO with chelating properties.

Numerous CLEC CSPs have been applied so far with different SO motifs, most often proline-type AA derivatives. The retention and separation are strongly affected by the concentrations and natures of the mobile phase components, together with other variables, such as pH and temperature. As the mobile phase must contain a certain amount of the metal ion in the form of a salt plus a buffer, this enantiomer separation methodology, although highly stereoselective, is not well applicable for LC-MS.

Recent applications

As an interesting variant *O*-benzyl-(*S*)-serine [(*S*)-OBS]] was applied as SO for the chiral discrimination of racemic AAs [34]. Computational modeling was utilized to shed light on the ~~network~~ multitude of interactions and perturbations that can play a relevant role in the enantioselective molecular recognition and retention process. Analogs of isoxazoline-fused aminocyclopentanecarboxylic acids were enantioseparated on a sodium *N*-((*R*)-2-hydroxy-1-phenylethyl)-*N*-undecylaminoacetate-based column [35]. The calculated thermodynamic parameters suggested that the *trans* analogs induce more efficient binding to the SO, as reflected by the larger negative free energies. In an application-oriented paper, the D-AA content of cheese samples was determined by CLEC by applying (*S*)-trityl-L-cysteine (L-STC) dynamically coated onto octadecylsilica [36]. To ensure food safety the combination of CLEC and conventional achiral chromatography was utilized to assess the impact of natural or artificial interventions on the product quality.

2.1.4. Zwitterionic ion-exchange-type chiral stationary phases

A novel class of zwitterionic (ampholytic) chiral ion-exchange-type CSPs as chiral SO motif was recently introduced. These are based on a weak cationic site derived from the *Cinchona* alkaloid moiety quinine (QN) or quinidine (QD) (a weak anion exchanger, WAX) and an anionic site derived from a chiral *trans*-aminocyclohexanesulfonic acid molecule moiety (a strong cation exchanger, SCX) [37].

Free AAs are ampholytic in character; the number of bonds between the AA group components and hence the distance between the chargeable sites within the molecule vary between the α -, β - and γ -AAs. As a consequence of the ampholytic properties, both the SO and SA ampholytes can undergo double ion pairing with each other due to the attractive electrostatic interactions of the charged and chargeable sites of the SO and SAs in a polar ionic (PI) or hydro-organic mobile phase environment.

Due to their nature, these ZWIX selectors allow the separation of the enantiomers of a remarkably broad spectrum of ionizable chiral analytes, ranging from acidic to basic and zwitterionic compounds as *N*-tagged, *O*-tagged or free AAs and small peptides. The occurrence of anionic, cationic and zwitterionic ion-exchange processes has been confirmed and these processes are predominantly involved in SO-SA interactions as part of the overall enantioselective retention and molecular recognition processes. Additional H-bonding and hydrophobic interactions are very likely to support the chiral recognition phenomena.

Recent applications

Free α -, β - and γ -AAs with different substitution patterns, including C-methylated α -AAs, were enantioresolved by *Cinchona* alkaloid-based CSPs [38]. Through the application of slightly acidic polar organic (PO) and ~~polar ionic (PI)~~ mobile phases, fairly good enantioseparations

could generally be achieved. The effects of structural variants of monoterpene-based β -AAs on the resolution were investigated [39]. The elution sequence was determined for all the investigated analytes, and was found to be opposite on the ~~quinine~~ QN-based ZWIX(+)TM and the ~~pseudoisomeric~~ pseudoenantiomeric ~~quinidine~~ QD-based ZWIX(-)TM columns. (The *Cinchona* alkaloids QN and QD are diastereomers, but the configurations of the QN (8*S*,9*R*) and QD (8*R*,9*S*) molecular moieties and the resulting steric environment largely determine the chiral recognition abilities of these SOs, and ZWIX(+)TM and ZWIX(-)TM are therefore frequently referred to as “pseudoenantiomers” because they behave as quasi-enantiomeric CSPs [38-40].) The same set of model compounds was applied to carry out a temperature dependence study [40]. Calculation of the apparent thermodynamic parameters revealed unusual temperature-induced retention behavior in some cases: the retention time increased with increasing temperature. HPLC methods were also developed for the enantioresolution of sixteen unusual β^2 -AAs [41]. The mobile phases applied were found to support ionic interactions between the zwitterionic SOs and free, ampholytic AAs. Similar behavior was found for the resolution of bicyclo[2.2.2]octane-based aminocarboxylic acids [42]. Various AAs and their analogs were utilized to develop schemes for efficient generic screening methods for ZWIXTM columns [43]. Another study was conducted for the enantioseparation of 27 unusual cyclic secondary α -AAs on QN- and QD-based CSPs [44]. For some applications, the QN- or the QD-based CSP appeared more suitable for the enantiomeric separations, which are SA-dependent, and relates to the fact that the SOs on ZWIX(+)TM and ZWIX(-)TM CSPs are only pseudo-enantiomeric to each other. The calculated thermodynamic parameters were found to depend on the structures of the SAs and the SOs employed. Structure-selectivity relationships were investigated through the enantiomeric separations of isoxazoline-fused aminocyclopentanecarboxylic acids [45]. SAs with methyl substituents in most cases interacted more strongly than ethyl-substituted ones with the SO,

which resulted in higher selectivity and resolution. This confirms the potentially strong effects of even small changes in the residues at a stereogenic center of the chiral SA. The performances of QN- and QD-based CSPs in separating β^2 - and β^3 -AAs as model compounds revealed, that the QD-based column mostly exhibited better selectivity for both β^2 - and β^3 -AAs [46]. The double ion pairing concept works more or less equally well for aminocarboxylic, -sulfonic and -phosphonic acids. Novel CSPs recently prepared through the coupling of a crown ether moiety to QN derivatives exhibited specific selectivity for chiral acidic SAs and for some free AAs [47]. From this example, it might be argued that the crown ether moiety acts as an interaction site for the protonated amino group of the AA, and the carboxylate group interacts with the charged quinuclidine moiety of QN.

Another interesting field of application of zwitterionic CSPs that may be expected to gain in importance in the near future is the stereoselective separation and analysis of small peptides [48-51].

2.2. Chiral stationary phases frequently applied for the enantioseparation of tagged amino acids

As indicated above, a specific tagging (derivatization) of AAs preferentially on the amino group can lead to molecular species with better chromatographic properties as the highly polar ampholytes become less polar and only acidic, and can promote higher detection sensitivity. Overall, such a strategy can result in higher enantioselectivity and/or lower LOQ. Information on recent relevant publications is provided in Table 3.

In this context, it should be remembered that the *N*-derivatization (tagging) of any AA results in acid-type SAs, offering new possibilities in the selection of suitable CSPs. The chemical

structures of frequently used *N*-protecting (tagging) nonchiral reagents are presented in Scheme 2.

2.2.1. Resolution on polysaccharide-type chiral stationary phases

To illustrate the long history of polysaccharide-based chiral separations, resolution of the enantiomers of some free AAs on cellulose as CSP in the form of paper chromatography was described in 1951 [52]. Polysaccharides, and especially amylose and cellulose, are naturally occurring polymers that can serve as the basis for the fabrication of more efficient and enantioselective CSPs by a dedicated derivatization of the free OH groups of the saccharide subunit. In this context, cellulose and amylose derivative-related CSPs based on coated silica were first reported by Okamoto in 1984 [53]. These phases have subsequently become highly popular CSPs suitable for the resolution of a broad spectrum of chiral compounds comprising neutral, acidic and basic SAs. The recent introduction of immobilized polysaccharide-type CSPs, has markedly improved their chemical stability and hence their more flexible usage [54], as widely proven, particularly in the industrial environment. These chiral columns are also highly feasible for SFC applications. However, these phases are usually not the primary choice for free AAs.

Recent applications focused on N-derivatized amino acids

Cellulose *tris*(3-chloro-4-methylphenylcarbamate) was recently applied as packing material for nano-LC and capillary electrochromatographic measurements [55]. The packed column was evaluated for the separation of Fmoc AA enantiomers, where 20 of 23 AA could be resolved. An HPLC method was also developed for the enantioresolution of α -AA esters [56]. Of the commercially available polysaccharide-based CSPs provided by several suppliers, the covalently bonded Chiralpak IA and IC columns (Daicel) proved to be the preferred choice for the

enantiodiscrimination of the investigated α -AA esters. Interestingly, the *N*-naphthoyl (*N*-NPTH), *N*-phthaloyl (*N*-PTH), *N*-tetrachlorophthaloyl (*N*-TCPHT) derivatives of AAs and their esters were well enantioresolved by polysaccharide-derived CSPs [57]. The interactions between the polymeric SO moieties and the SAs were described by computational modeling. Of numerous practical examples, the Boc derivatives of β -phenylproline stereoisomers were resolved on analytical and preparative scales on amylose- and cellulose-based CSPs [58]. These CSPs performed excellently for large-scale HPLC and SFC applications. All the applications described involved the use of mobile phases suitable for MS detection.

2.2.2. Cyclodextrin-based (brush type) chiral stationary phases

CDs are naturally occurring, nonreducing oligosaccharides. The most widely utilized α -, β - and γ -CD are composed of 6, 7 and 8 D-glucopyranose units bonded through α -1,4 glycosidic linkages forming a toroidal, hollow, truncated cone shape. The exterior of the toroid is relatively hydrophilic because of the presence of 18, 21 and 22 hydroxy groups, while the internal cavity is much more hydrophobic, which favors the enantioseparation of partially nonpolar compounds via selective inclusion. The hydroxy groups of the CDs can be derivatized, so that a wide variety of modified CDs can be prepared. Thanks to their relatively simple production, natural and modified CDs have found various applications, and CD-based CSPs are among the more popular CSPs used in chiral analysis. Because of their solvent compatibility, they have the ability to operate in different modes of HPLC: RP, normal-phase (NP), PO and PI modes, *i.e.* they may be regarded as multimodal-type CSPs. However, the spectrum of enantioselectivity is more restricted than for other SO types as the inclusion complexation is a rather specific and residue size-dependent property. As a consequence, these CD-based CSPs for HPLC have somewhat lost in popularity in recent years. However, CDs are still widely used for CE applications [1,2].

Recent applications

A novel bilayer CD-based CSP was fabricated by a two-step click approach, in which a native CD (down layer) and a perphenylcarbamoylated CD (top layer) were introduced onto a silica surface [59]. The enantioseparation abilities of this CSP were tested with dansyl (Dns) AAs among others, and were found to be tunable by endowing functionality to one of the CD layers. A covalently bonded cationic CD-based CSP was prepared by thiol-ene click chemistry and tested on a set of chiral compounds involving ~~dansyl~~ Dns AAs [60]. A surface-up click approach was applied for the fabrication of a triazole-bridged bilayer CD-based CSP [61]. The as-prepared bilayer CSP afforded enhanced binding ability and enantioselectivity toward ~~dansyl~~ Dns AAs. It should be mentioned that an aryl group can be particularly well inserted into the hydrophobic cavity of the CD-based CSPs as compared with aliphatic residues.

2.2.3. Pirkle-type (brush type) chiral stationary phases

These CSPs were introduced by Pirkle and Sikkenga [62], who immobilized (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol on a silica support. Dinitrobenzoyl (DNB) derivatives of AAs and other racemates could be separated well. These phases became the first commercially available CSPs in the early 1980s. Mechanistically, they preferentially function in NP mode and the enantioselectivity is based on multiple H-bonding, π - π interactions and dipole-dipole stacking between the SO and the SAs. As a follow-up, synthetic chiral SOs with low molecular masses and a π -acidic or π -basic and H-bonding character have been prepared and attached to solid supports in a brush-type format. In recognition of his pioneering work, they became known as Pirkle-type phases. As an option these CSPs are usually available in both enantiomeric forms, leading to the very important consequence that the elution sequence for two enantiomers can be

switched by exchanging an (*R*)-SO-based CSP with an (*S*)-SO-based CSP. This is not possible for SOs from the natural chiral pool, such as polysaccharides, proteins and macrocyclic antibiotics. Exceptions include AA-derived SOs and *Cinchona* alkaloid-based SOs, which exist as (pseudo)enantiomers as outlined above. The concept of enantiomeric “chiral columns” is of fundamental importance and is encountered for the pseudoenantiomeric ZWIX(+)TM and ZWIX(-)TM columns developed by Lindner et al. [37]. ~~For the cyclodextrin, polysaccharide and macrocyclic antibiotics based CSPs such possibilities do not apply.~~

3.3.3. Ion-exchange-based chiral stationary phases

Ion-exchange-based CSPs are frequently applied for the resolution of variety of acids, including derivatized AAs [63]. They exist as weak anion-exchangers based on *Cinchona* alkaloids (QN-AX, QD-AX columns) or as strong cation-exchangers based on *trans*-1,2-aminocyclohexanesulfonic acid derivatives [64]. The ampholytic ZWIX-type CSPs likewise function in these modes (see above).

Recent applications

In order to evaluate the impact of the bonding chemistry on the overall enantioselectivity of a given SO unit, anion-exchange-type thioether-linked and triazole-linked CSPs were synthesized by click chemistry and evaluated with structurally diverse racemic acids, including *N*-protected [dinitrobenzoyl (DNB), benzoyl (Bz), acetyl (Ac) or Z] AAs [65]. Dinitrophenyl (DNP) and DNB derivatives of AAs were used to test the enantiorecognition abilities of QN-based CSPs [66,67]; the elution sequence was reversed for the DNP- relative to the DNB-tagged AAs. This clearly indicates a substantial difference of the stereoselective molecular recognition principle and the need to prove the elution sequence of a pair of enantiomers. To improve the UV detectability and to enhance the enantioselectivity, the AAs were also modified with 2-

acyldimedone, and 12 structurally related compounds were investigated [68]. As a very favorable application, the QN- and QD-based CSPs were employed for the enantioresolution of fluorescent (6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate, AQC)-tagged AAs [69]. From systematic studies of the chiral and chemoselective recognition processes, it became evident that the urea-type tagging functionality supports the overall chiral recognition substantially and more universally. Essentially all AQC-tagged AAs could be resolved and also sensitively detected by MS/MS through use of a ZWIX-type column. Specifically an organic monolithic capillary column based on a QD derivative was prepared and applied for the enantioseparation of several *N*-derivatized [DNB, dichlorobenzoyl (DCIB), Fmoc, *etc.*] AAs [70]. 2,5-Dioxopyrrolidin-1-yl(2-(6-methoxy-4-oxoquinolin-1(4H)-yl)ethyl) carbonate was designed for the pre-column derivatization of proteinogenic AAs [71]. The derivatives were analyzed on Chiralpak™ QN-AX and ZWIX(+)TM with MS/MS detection. The simultaneous diastereo- and enantioseparation of *N*^α-Boc-*N*⁴-(hydroorotyl)-4-aminophenylalanine was investigated by applying ZWIXTM columns [72]. A molecular dynamics computational protocol was applied to gain a better understanding of the molecular basis of the retention mechanism. All the mobile phases used for the cation and anion-exchangers are well suited for MS detection.

4. Chiral analyses of amino acids in biological matrices

Determination of the enantiomeric distribution of various AAs in a range of biological matrices is a highly important task in life sciences. Information on recent publications is listed in Table 4.

4.1. Indirect methods

An LC-MS/MS method was developed and validated for the determination of D-serine in human plasma [73] based on pre-column derivatization with (*R*)-1-Boc-2-piperidine carbonyl chloride.

The optimized method was applied for the analyses of plasma samples from patients receiving a 5-day intravenous infusion of ketamine. In another example, MS-based methods were developed and compared for the determination of the ee of proteinogenic AAs [74]. The enantiomers of Trp and kynurenine were separated by pre-column fluorescence derivatization with *R*(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*(-)-DBD-PyNCS] in rat plasma samples [75,76]. The experimental results led to the conclusion that the administered D-Trp was partially converted to L-Trp *in vivo*. A ~~ultra-high-performance liquid chromatography~~ (UHPLC)-MS/MS method based on *R*(-)-DBD-PyNCS derivatization was developed for the simultaneous determination of partial AA racemization and isomerization of an *N*-terminal amyloid- β -peptide sequence [77]. The method was applied in the analysis of human brain tissues. In another relevant paper, the D-AA contents of human plasma, serum and urine samples were determined via OPA/IBLC derivatization and MS detection [78]. The method displayed excellent sensitivity (LOD at the pmol/L level) and it was claimed that no matrix effects or stability problems occurred. A distinction between D- and L-AAs from the broth of *Streptomyces* sp. was carried out by derivatization procedures using 2,3,4,6-tetra-*O*-acetyl- β -D-glycopyranosyl isothiocyanate (GITC) and 1-fluoro-2,4-dinitrophenyl-5-L-alanin amide (FDAA) prior to LC-MS assay [79]. Isotopic labeling combined with MS detection was applied for the quantification of DL-AAs from human serum samples [80]. The proposed method utilizing L-pyroglutamic acid succinimidyl esters (L-PGA-OSu) as derivatizing agents seemed to be applicable not only for biological samples, but also for food products. A method based on chiral derivatization was developed for the enantiodiscrimination of AAs from body fluids [81]. In a comparison 7 different CDRs, (*S*)-NIFE offered the best performance in terms of selectivity and sensitivity. This selection of examples reveals that the indirect methodology is not obsolete for the analysis of real-world samples.

4.2. Direct methods

Conceptually, these methods also most often integrate a derivatization strategy, but with the use of nonchiral reagents. In this context, an analytical procedure using model peptides was established to discriminate the innate D-Ala and D-Phe generated during the common acid-hydrolysis of peptides (proteins) through LC-MS/MS detection and a Chirobiotic TAGTM column [82]. The developed method was expected to be applicable for the determination of other AAs too.

Very significant contributions from the application of AA fluorescent tagging protocols emerged from the work of Hamase *et al.* who used 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as labeling agent and the sample analysis by a two-dimensional (2D) LC system [83-88]. NBD-F-AAs were separated on an RP column in the first dimension, and the collected fractions containing the peaks of the respective NBD-AAs were then introduced into an enantioselective column representing the second dimension, where the D and L enantiomers of the NBD-AAs could be baseline-resolved. The enantioselectivity was preferentially accomplished on Pirkle-type columns, but chiral ion-exchange systems were also suitable.

In the course of specific applications, the developed 2D-HPLC system based on NBD-F tagging was applied for the analysis of AAs of different biological origins. Similarly as in the above mentioned method, a 2D-HPLC system was used for the analysis of AAs from mice tissues with a ChiralpakTM QD-AX column [89]. Good peak shapes, good selectivities and high resolution power were achieved. A 1D chromatographic method was also described for the analysis of proteinogenic AAs on anion exchange-type chiral columns [90]. Similar results were attained with hydroxy succinimidyl ferrocenyl propionate (SFP) as tagging reagent. The enantioselectivity was sufficient for most of the proteinogenic AA derivatives. It was recently shown that *p*-*N,N,N*-

trimethylammonioanilyl *N*'-hydroxysuccinimidyl carbamate iodide (TAHS) or AQC (AccQ) (see Scheme 2) derivatized AAs could be separated well on ZWIX phases with excellent sensitivity in an LC-MS mode [91]. The method was successfully applied for the analyses of AAs in rat plasma and tissue samples.

Another group analyzed the level of D-serine in the mouse brain by using a chiral crown ether column and an HPLC-MS/MS system [92]. The proposed surrogate analyte method provided quantitative data with good accuracy and precision without pre-column derivatization.

For a dedicated enantioselective single AA analysis, 1D LC-MS/MS systems may be sufficient. However, it is very likely that the simultaneous and sensitive determination of several pairs of AA enantiomers in complex matrices can be solved only by a tagging strategy involving a 2D-LC system in combination with fluorescence and/or MS/MS detection.

5. Conclusions

The accurate and sensitive analysis of the individual enantiomers of natural (proteinogenic and nonproteinogenic) but also unusual AAs remains an important and challenging field in the life sciences, but also in pharmaceutical product analysis, *etc.* In this context, the following main subjects of enantioselective AA analysis have been addressed:

1. The chemical analysis of synthetic AA-type products and their characterization in terms of enantiomeric purity. This includes the individual *N*-protected AA building blocks for peptide synthesis, *etc.*
2. The analysis of biological samples (blood, serum, urine, tissue, *etc.*) for the levels of individual D-AAs besides the large excess of L-AAs in these matrices. Such data could be of diagnostic value, for instance, as early disease markers.

3. D-AAs may occur in proteins as “markers” of aging due to *in vivo* racemization. The hydrolyzates of proteins may therefore contain biologically relevant information based on the content of proteinogenic D-AAs ~~amino acids~~.

4. Proteinogenic and also many nonproteinogenic AAs found in D- and L-form in food and feed stuffs may have physiological effects, and investigations of the individual enantiomers will therefore certainly be of significance.

5. Analysis of the metabolomic profile regarding the diverse D- and L-AAs in urine will be of high interest. Parameters reflecting the metabolomic profiles of the gut microbiota, the source of food intake and the biological systems of living species in general, for example are not yet fully explored sources of information related to health and disease statuses.

A most challenging area is the sensitive enantioselective analysis of the variety of AAs in complex matrices, which can currently be approached best with 2D concepts of *N*-tagged AA derivatives together with fluorescence and/or MS/MS detection. For free AAs, 2D concepts have not yet been established, and in this case MS detection seems the only option to reach high sensitivity and specificity. From a chromatographic aspect the introduction of very efficient UHPLC-type chiral columns using CSPs based on fully porous particles of sub-2- μm size or on partially porous particles (core shell particles) is emerging [28,93], boosting the peak capacity of a column and reducing the overall analysis time. This is also of significance for the engineering of fully automated 2D-UPHPLC systems. 1D- and 2D-SFC systems for the analysis of biological samples have not yet been described and it remains to be seen how quickly the SFC technology will enter this field.

An upcoming challenge will be the resolution of peptide enantiomers and stereoisomers. The latter should in principle be accomplishable by RP chromatography, though complementary selective chromatographic systems will be needed to solve the problem comprehensively.

CSPs may meet such demands very well. 2D systems will have the greatest flexibility in this context, whereby the mobile phase compatibility of both dimensions is a crucial factor to be dealt with.

To solve these challenges efficiently in the near future, the combinations of RP and macrocyclic or ion-exchange-type CSP systems appear most promising.

In summary, the aim of our discussion of the recently published enantioselective methodologies to resolve free and tagged AAs was to provide an overview of the technical and practical solutions. Insight into molecular recognition has been integrated. There is clearly room for further advances of the applied methodologies to solve the current and future challenges based on “state-of-the-art” enantioseparations. The development of new and novel CSPs still makes sense.

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Scheme 1

Several structures of investigated amino acids

α-amino acids		β-amino acids			γ-amino acids
primary	secondary	β^2 -amino acids	β^3 -amino acids	cyclic β^3 -amino acids	
$\begin{array}{c} \text{COOH} \\ \\ \text{R}-\text{HC} \\ \\ \text{NH}_2 \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{HC} \\ \\ \text{R}-\text{NH} \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{R}-\text{CH} \\ \\ \text{H}_2\text{C}-\text{NH}_2 \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{H}_2\text{C} \\ \\ \text{R}-\text{CH}-\text{NH}_2 \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{HC} \\ \\ \text{HC} \\ \\ \text{NH}_2 \end{array}$ <p>$n = 3, 4$</p>	$\begin{array}{c} \text{COOH} \\ \\ \text{H}_2\text{C} \\ \\ (\text{CH}_2)_n \\ \\ \text{R}-\text{CH}-\text{NH}_2 \end{array}$ <p>$n = 1, 2$</p>

R₁: alkyl, cycloalkyl, aromatic, substituted aromatic, hetero aromatic

R₂: H, alkyl

Scheme 2 Structures of frequently used Tag elements and reagents

Name (abbreviation)	Structure	Name (abbreviation)	Structure
acetyl (Ac)		9-fluorenylmethyl-chloroformate (Fmoc-Cl)
benzoyl (Bz)
3,5-dinitrobenzoyl (DNB) 3,5-dichlorobenzoyl (DCB)		<i>t</i> -butyloxycarbonyl (Boc)
dansyl chloride		6-aminoquinoyl- <i>N</i> -hydroxy-succinimidyl carbamate (AQC)
phthaloyl (PTH)		<i>p</i> - <i>N,N,N</i> -trimethylammonioanilyl <i>N'</i> -hydroxysuccinimidyl carbamate iodide (TAHS)
naphthoyl (NPTH)		4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)
benzyloxycarbonyl (Cbz, Z)		1-fluoro-2,4-dinitrobenzene (DNP-F)

Table 1. Indirect methods: derivatization of amino group of amino acid enantiomers prior to separation by HPLC method

Analytes	Chiral derivatizing agents (CDAs)	Chromatographic conditions	Detection	References
Phg, Nva, 2-aminoadipic acid, 2-aminobutyric acid, pipecolic acid, 2-aminooctanoic acid	(S)-NIFE	Eurospher C18/ aq. TFA-MeOH	UV, 205 nm	[11]
β -Phe, β -Tyr	OPA/IBLC	Hyperclone ODS/ Na ₂ HPO ₄ -MeOH	Fl, Exc/Em 235 nm/ 442nm	[12]
D-cycloSer	OPA/NAC	Zorbax SB phenyl/ aq. Na ₂ HPO ₄ -MeCN	UV, 335 nm	[13]
D,L-selenoMet	phthalimidyI-(S)-naproxen	Lichrospher C18/ aq. TEAP/MeCN	UV, 231 nm	[14]
18 proteinogenic D,L-amino acids	Nap-Btz	Eurospher C18/ aq. TEAP/MeCN	UV, 231 nm	[15]
Cys, homoCys	Nap-Btz	Spherisorb ODS/ aq. TEAP/MeCN	UV, 231 nm	[16]
D,L-selenoMet	MBIC and NEIC	Spherisorb ODS-2/ aq. TEAP/MeCN	UV, 254 nm	[17]
proteinogenic D,L-amino acids	monochloro-s-triazine and dichloro-s-triazine derivatives	Eurospher C18 or Lichrospher C18/ aq. TFA/MeCN or aq. TFA-MeOH	UV, 230 nm	[18,19]
D,L-selenoMet, D,L-Met, D,L-Cys	monochloro-s-triazine and dichloro-s-triazine derivatives	Spherisorb ODS/ aq. TFA/MeCN	UV, 230 nm	[20]

Table 2. Direct enantioselective high-performance liquid chromatographic determination of amino acids

Analytes	Chiral stationary phase (CSP) or Selector	Experimental conditions/ Eluent	Detection	References
Macrocyclic glycopeptide-based CSPs				
monoterpene-based 2-aminocarboxylic acid	Chirobiotic T TM , T2 TM , TAG TM ,	aq. TEAA-MeOH; 100% MeOH; MeOH-AcOH-TEA	UV, 210 nm	[23]
isoxazoline fused 2-aminocyclopentanecarboxylic acid	Chirobiotic T TM , T2 TM , TAG TM , V TM and VAG TM	aq. TEAA-MeOH; 100% MeOH	UV, 210 nm	[24]
bicycle[2.2.2]octane based 2-amino-3-carboxylic acids	Chirobiotic T TM , T2 TM , TAG TM , R TM	aq. TEAA-MeOH; MeOH-AcOH-TEA; MeOH-MeCN-AcOH-TEA	UV, 215 nm	[25]
unusual amino acids	Chirobiotic T TM and T2 TM	aq. NH ₄ OAc-MeOH	UV, 254 nm	[26]
β -amino acids	Chirobiotic T TM	H ₂ O-MeOH; H ₂ O-EtOH	UV, 220 nm	[27]
proteinogenic and unusual amino acids	teicoplanin and teicoplanin aglycon on 2.7 μ m superficially porous silica	H ₂ O-MeOH	UV, DAD	[28]
Crown ether-based CSPs				
β^2 -amino acids	(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid	H ₂ O-MeOH-acid	UV, 205 nm	[29]
isoxazoline fused 2-aminocyclopentanecarboxylic acid	(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid	H ₂ O-MeOH-AcOH H ₂ O-alcohol-acid	UV, 205 nm	[30]
γ -amino acids, vigabatrin	(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6	H ₂ O-MeOH-H ₂ SO ₄ -NH ₄ OAc	UV, 251 nm	[31]

Table 2. (continued) Direct enantioselective high-performance liquid chromatographic determination of amino acids

Analytes	Chiral stationary phase (CSP) or Selector	Experimental conditions Eluent	Detection	References
Crown ether-based CSPs				
acyclovir, valacyclovir	(3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6	H ₂ O-MeOH-HCl H ₂ O-alcohol-ac:	UV, 254 nm	[32]
Chiral ligand-exchange chromatography				
protein and non-protein amino acids	(<i>S</i>)-OBS	H ₂ O-Cu(II)	UV, 254, 210 nm	[34]
isoxazoline fused 2-aminocyclopentanecarboxylic acid	sodium <i>N</i> -((<i>R</i>)-2-hydroxy-1-phenylethyl)- <i>N</i> -undecylamino acetate	H ₂ O-MeOH-Cu(I) H ₂ O-EtOH-Cu(I)	UV, 230 nm	[35]
D-Ala, D-Asp, D-Glu	L-STC	aq. HFBA-MeCN-Cu(II)	ELSD	[36]
Cinchona alkaloid-based CSPs				
proteinogenic and non-proteinogenic amino acids	zwitterionic QN- and QD derivatives	MeOH-MeCN-FA-DEA	UV, 254 nm; CAD	[38]
monoterpene-based cyclic β^3 -amino acids	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 215 nm; CAD	[39,40]
β^2 -amino acids	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 215 nm; CAD	[41]
Bicyclo[2.2.2]octane-based 3-amino-2-carboxylic acid	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 215 nm; CAD	[42]
27 unusual secondary amino acids	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 230, 254 nm; CAD	[44]

Table 2. (continued) Direct enantioselective high-performance liquid chromatographic determination of amino acids and small peptide epimers

Analytes	Chiral stationary phase (CSP) or Selector	Experimental conditions Eluent	Detection	References
isoxazoline-fused 2-aminocyclopentanecarboxylic acids	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 215, 230 nm; CAD	[45]
β^2 - and β^3 -amino acids	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-acid-amine	UV, 215, 230 nm; CAD	[46]
proteinogenic and non-proteinogenic amino acids	QN modified by <i>meta</i> - or <i>para</i> -substituted benzo-18-crown-6	MeOH-FA-DEA; MeOH-H ₂ O-FA-DEA; MeOH-FA-DEA-NH ₄ OAc; <i>etc.</i>		[47]
Separation of small peptide epimers				
all-L/all-D, Ala-n, Phe-n, Val-n	<i>cinchona</i> -sulfonate-based CSP	MeOH-FA-DEA aq. TFA-MeCN	CAD	[48]
D-Pro-D-Pro, D-Pro-L-Pro, L-Pro-D-Pro, L-Pro-L-Pro, Gly-D-Pro, D-Pro-Gly	<i>cinchona</i> -sulfonate-based CSP	MeOH-FA-DEA	CAD	[49]
D,L-Leu-D,L-Ala; D,L-Leu-D,L-Val Gly-D,L-Leu-D,L-Ala; D,L-Leu-D,L-Ala-Gly	ZWIX(+) TM and ZWIX(-) TM	MeOH-MeCN-H ₂ O MeOH-THF-H ₂ O MeOH-H ₂ O		[50]
Leu-Leu, Leu-Leu-Leu; Gly-Asn; Gly-Asp Gly-Ser	ZWIX(+) TM	MeOH-MeCN-FA-DEA MeOH-THF-FA-DEA	CAD	[51]

acid: AcOH, FA; amine: EA, DEA, TEA, PA, TPA, BA, TBA; alcohol: MeOH, EtOH, 2-propanol

Table 3. Direct methods: separation of tagged amino acid enantiomers by HPLC method on different types of CSPs

Analytes	Derivatizing agents (DA)	Chromatographic conditions column or selector/eluent	Detection	References
Polysaccharide-based CSPs				
proteinogenic and non proteinogenic amino acids	FMOC	Sepapak-2, Lux Cellulose-2/NH ₄ OAc-MeOH; NH ₄ OAc-MeCN	UV, 210 and 260 nm	[55]
Ala, Asp, Asn, Leu, Phe, Phg, Val	benzophenone imine	Chiralcel OD, Chiralpak AD, AS/2-propanol-hexane	UV, 254 nm	[56]
Ala, Val, Leu, Ile, Phg, Phe	<i>N</i> -PTH, <i>N</i> -TCPHT, <i>N</i> -NPHT	Chiralcel OC, OD, OF, OJ, AD, AS; Chiralpak AD, AS/2-propanol-hexane	UV, 230 nm	[57]
<i>cis/trans</i> - β -phenylPro	Boc	Chiralpak IA,IB,IC/hexane-2-PrOH-CHCl ₃ (acetone, <i>t</i> -BuOMe)	UV, 210 or 230 nm	[58]
β-cyclodextrin-based CSPs				
Nva, Val, Leu, Phe, Ser, Thr	Dns	perphenylcarbamoylated- β -CD/H ₂ O-MeOH, aq. TEAA-MeOH	UV, 220-230 nm	[59]
Thr, Nva, Val, Nle, Leu, Phe, Glu	Dns	imidazolium β -CD/ aq. TEAA-MeOH	UV, 220-230 nm	[60]
Nva, Val, Leu, Phe, Ser, Thr	Dns	triazole bridged bilayer β -CD/aq. TEAA-MeCN	UV, 220-230 nm	[61]

Table 3. (continued) Direct methods: separation of tagged amino acid enantiomers by HPLC method on different types of CSPs

Analytes	Derivatizing agents (DA)	Chromatographic conditions column or selector/eluent	Detection	References
Ion-exchange type CSPs				
Leu, Ala, Asp, Glu, β -Phe, <i>t</i> -Leu, Phe, Trp	DNB, Bz, Ac, Z	1,2,3-triazolo linked <i>Cinchona</i> <i>t</i> -butylcarbamate-type CSP/ aq. NH ₄ OAc-MeOH;	UV, 254 nm	[65]

MeCN-AcOH-TEA				
20 proteinogenic and non-proteinogenic amino acids	2,4-DNP	<i>Cinchonidine</i> - and QN-based CSP/ /aq. NH ₄ OAc-MeOH; NH ₄ OAc-MeCN	UV, 365 nm	[66,67]
proteinogenic amino acids, 2- and 3-aminobutyric acid	2-acyl-dimedone	MeOH-FA-DEA; MeOH-MeCN-FA-DEA; MeOH-H ₂ O-AcOH-NH ₄ OAc	UV, 254 nm	[68]
Ile, Thr, homoSer, 4-OH-Pro, β -amino acids, Phe derivatives, <i>etc.</i>	AQC	QN-AX, QD-AX/ MeOH-AcOH-NH ₄ OAc; aq. NH ₄ OAc-MeOH	Fl, Ex/Em 248/395 nm	[69]
Ala, Leu, Met, Thr, Phe, Val, Ser, Ile, Trp and Cys	3,5-DNB, 3,5-DCB, 3,5-DCIB, FMOC, <i>etc.</i>	MQD	UV, 254 nm	[70]
proteinogenic amino acids	2,5-dioxopyrrolidin-1-yl(2-(6-methoxy-4-oxoquinolin-1(4H)-yl)ethyl) carbonate	ZWIX(+) TM , Chiralpak QN-AX MeOH-MeCN-FA- HCOONH ₄	MS/MS, CAD	[71]
N ^{α} -Boc-N ⁴ -(hydroorotyl)-4-aminophenylalanine	Boc	ZWIX(+) TM , ZWIX(-) TM /MeOH-MeCN-FA-DEA	UV, 254 nm	[72]

Table 4. Determination of chiral amino acids in biological and natural samples by HPLC methods

Analytes	Sources/Matrixes	Chiral derivatizing agents (CDAs)	Experimental conditions Column/eluent	Detection	References
Indirect methods: application of CDA and non-chiral selector					
D,L-selenoMet, D,L-Met, D,L-Cys		(<i>R</i>)-1-Boc-2-piperidine carbonyl chloride	Zorbax Eclipse XDB C18/aq. TFA-MeOH	LC-MS/MS	[73]
19 chiral proteinogenic amino acids	Marfey's reagent	Marfey's reagent	Zorbax SB-C18/aq. FA-MeCN	HPLC-MS	[74]
Trp, kynurenine	rat plasma	<i>R</i> (-)-DBD-PyNCS	Inertsil ODS-3/aq. AcOH-MeCN, aq. FA-MeCN	Fl, Ex/em, 440 nm/565 nm	[75]
Trp	rat plasma	(-)-DBD-PyNCS	Inertsil ODS-3/aq. AcOH-MeCN	Fl, Ex/em, 440 nm/565 nm	[76]
L,D-Asp, L,D-isoAsp	human brain tissue	<i>R</i> (-)-DBD-PyNCS	BEH C18/aq. FA-MeOH	UPLC-MS/MS	[77]
proteinogenic D,L-amino acids	serum, plasma, urine, gut	OPA/IBLC	BEH C18/aq. NaOAc-MeCN-MeOH	LC-TOF-MS	[78]
Phe, Leu, Ala, Ile, Lys	Surugamides A-E, cyclic octapeptide	GITC, FDAA	Cosmosil C-18/aq. AcOH-MeCN	LC-MS/MS	[79]
18 proteolytic amino acids	human serum, food products	L-PGA-OSU	BEH C18/ aq. FA-MeCN	LC-MS/MS	[80]
proteinogenic D-amino acids	human plasma, cerebrospinal, urine	(<i>S</i>)-NIFE	BEH C18/aq. NH ₄ HCO ₃ -MeCN	UPLC-MS/MS	[81]

Table 4. (continued) Determination of chiral amino acids in biological and natural samples by HPLC methods

Analytes	Sources/Matrixes	Non-chiral derivatizing agents	Experimental conditions Column/eluent	Detection	References
Direct methods: application of non-chiral derivatizing agent and chiral selector					
Ala, Phe	D-Phe ^{2,6} -LHRH	NBD-F	Chirobiotic TAG/aq- TFA-MeOH	LC-MS/MS	[82]
His, Ser, Asn, Gln, Arg, Asp, <i>allo</i> -Thr, Glu, Thr	rat cerebrum, plasma, urine	NBD-F	2D-HPLC; 1D, monolithic ODS, aq. TFA-MeCN; 2D, Sumichiral OA/aq. citric acid-MeOH-MeCN	Fl, Ex/Em 470 nm/530 nm	[83]
<i>N</i> -Me-D-Asp <i>N</i> -Me-D-Glu	Wistar rats	NBD-F	2D-HPLC; 1D, monolithic ODS, aq. TFA-MeCN; 2D, Sumichiral OA/aq. citric acid-MeOH-MeCN	LC-MS/MS	[84]
D-Ser, D-Ala	mouse lumbar spinal cords	NBD-F	2D-HPLC; 1D, C18 2D, Sumichiral OA	Fl, Ex/Em 470/530 nm	[85]
D-Asp, D-Glu, D-Pro, D-Arg, D-Asn, D-Gln, D-Glu, D-His, D-Ser, D-Thr, D- <i>allo</i> -Thr	mammals tissue	NBD-F	2D-HPLC; 1D, monolithic ODS, aq. TFA-MeCN; 2D, Sumichiral OA or Chiralpak QN-AX	Fl,Ex/Em 470 nm/530 nm	[86]
Ala, Val, 2-aminobutyric acid, Nva, <i>N</i> -MeAla, isoVal, 3-aminoisobutyric acid	extraterrestrial amino acids in meteorite	NBD-F	2D-HPLC; 1D, monolithic ODS, aq. TFA-MeCN; 2D, Sumichiral OA, aq. FA-MeCN	Fl, Ex/Em 470 nm/530 nm	[87]
D-Ala	blood of Wistar rat	NBD-F	2D-HPLC; 1D, microbore ODS/ 2D, Sumichiral	Fl, Ex/Em 470 nm/530	[88]

OA/

nm

Table 4. (continued) Determination of chiral amino acids in biological and natural samples by HPLC methods

Analytes	Sources/Matrixes	Non-chiral derivatizing agents	Experimental conditions Column/eluent	Detection	References
Direct methods: application of non-chiral derivatizing agent and chiral selector					
D-Asp	thymus and spleen of mice	NBD-F	2D-HPLC; 1D, monolith ODS/aq, TFA-MeCN 2D, QD-AX/citric acid-MeOH-MeCN	Fl, Ex/Em 470 nm/530 nm	[89]
proteinogenic amino acids	human urine	SFP	QN-AX and QD-AX; aq. NH ₄ COOH-MeOH, FA-MeOH	LC-MS	[90]
D-Ala, D-Asp, D-Ser	rat plasma and tissues	AQC, TAHS	ZWIX(+)	LC-MS/MS	[91]
Direct methods: application of CSPs					
D-Ser	mouse brain	-	Crownpak CR(+)/aq. TFA-MeOH	LC-MS/MS	[92]