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REVIEW

Enantioresolution of Amino Acids: A Decade's Perspective, Prospects and Challenges

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Abstract Both from academic and industrial points of view enantioseparation of DL-amino acids continues to be a subject of immense importance and enjoys a great analytical significance in various fields, such as in the studies of fossils, origin of life, disease diagnosis, quality of food and beverages, etc. The present paper is a topical collection of recent advances along with a discussion on possible challenges in chiral amino acid analysis and is intended to present the existing state of knowledge on the topic as a particular facet of chromatography (and electrophoretic techniques). It presents a critical overview of the state-of-the-art of the topic, with critically selected examples to point the reader to trends and likely future developments and to give a selection of important references to the current literature.

Keywords Amino acids · Enantioseparation · HPLC · TLC · CSPs · Chiral derivatizing reagents

Abbreviations

AA	Amino acid
Aib	Aminoisobutyric acid
CC	Cyanuric chloride
CD	Cyclodextrin
CDR	Chiral derivatizing reagent
CD-CZE	Cyclodextrin-modified capillary zone electrophoresis
CDEKC	Cyclodextrin-modified electrokinetic chromatography
CD-MEKC	Cyclodextrin-modified micellar electrokinetic chromatography
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CILs	Chiral ionic liquids
CMC	Critical micelle concentration
CMCD	Carboxymethyl-β-cyclodextrin
CMPA	Chiral mobile phase additive
CSF	Cerebrospinal fluid
CSP	Chiral stationary phase
DFDNB	1,5-Difluoro-2,4-dinitrobenzene
HPLC	High performance liquid chromatography
2D-HPLC	Two-dimensional high performance liquid chromatography
DNP	Dinitrophenyl
FDAA	1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide
FDLA	1-Fluoro-2,4-dinitrophenyl-5-L-leucinamide
GC	Gas chromatography
GC–IRMS	Gas chromatography–isotope-ratio mass spectrometry
HPLC	High performance liquid chromatography
ILEC	Indirect ligand-exchange chromatography
LC	Liquid chromatography
MS	Mass spectrometry
TLC	Thin layer chromatography
LEC	Ligand-exchange chromatography
LE-CE	Ligand-exchange capillary electrophoresis
LE-MEKC	Ligand-exchange micellar electrokinetic chromatography
LIF	Laser-induced fluorescence
LOD	Limit of detection

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LOQ	Limit of quantification
MBIC	(*R*)-Methyl benzyl isothiocyanate
MNPs	Magnetic nanoparticles
MR	Marfey's reagent
NACE	Nonaqueous capillary electrophoresis
NBD-F	4-Fluoro-7-nitro-2,1,3-benzoxadiazole
NEIC	(*S*)-1-(1-Naphthyl) ethyl isothiocyanate
NMDA	*N*-Methyl D-aspartate
NP	Normal phase
OPA	*o*-Phthalaldehyde
PenA	Penicillamine
RP	Reversed phase
RTILs	Room temperature ionic liquids
SIM	Selected ion monitoring
SINP	*N*-Succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate
SRM	Selected reaction monitoring
STC	Spirothiazolidine
TFA	Trifluoroacetic acid
ToF	Time of flight

Amino Acids

Amino acids (AAs) are unique in terms of their structural features and multipurpose applications [1, 2]. With the ready availability of both enantiomers, AAs not only serve as a chiral pool for synthesis, but also provide an inexpensive pool for resolution studies [3]. AAs have been used either as the target racemic analyte for enantiomeric resolution studies or as the chiral auxiliary for resolution of racemic mixtures of several other compounds. Most of the basic methods and principles reported in literature for enantiomeric resolutions are based on experimental investigations carried out on amino acids or through their application as chiral selectors.

Demand for (single) enantiomerically pure AAs has continued to grow in such areas as pharmaceuticals [4], biochemistry [5], archeology [6], chiral synthesis [7], etc. The syntheses of optically active compounds (whether of pharmaceutical or synthetic importance, or as promising candidates as chiral ligands and auxiliaries in asymmetric syntheses) result in the formation of a mixture of products with one enantiomer predominating.

Proteinogenic Amino Acids

There are 20 AAs, which are genetically encoded and are the building blocks of peptides and proteins, which in turn are the most important constituents of all living systems. AAs also play important role in food industry on account of their contribution to the nutritive and biological value of food, affecting aspects of quality such as taste, aroma and color among others [8, 9]. D- and L-AA display different absorption kinetics and follow partly different metabolic pathways in humans. All natural α-AAs (except glycine) are chiral and occur predominantly in L-form. Proteins in the living systems consist exclusively of L-AAs; fossil samples contain D-enantiomers that are slowly converted from L-AAs, finally leading to equilibrium of L- and D-isomers. Prokaryotic and eukaryotic organisms showed existence of D-AAs [10]. D-AAs considered as biomarker of liver or kidney dysfunction because enzymes present in liver and kidney metabolize D-AAs [10, 11]. The generation of D-AAs in mammals has been investigated; submandibular gland and oral epithelial cells of mammals generate D-Ala and D-Asp [12] while brain produced D-Asp and D-Ser [13]. Since the discovery of glycine in cosmic dust, there has been a lot of interest in existence of AAs in extra-terrestrial space [14]. In 1995, scientists reported the presence of AAs more in L-form than D-form in Murchison meteorite [15].

Non-Proteinogenic Amino Acids

Non-proteinogenic AAs are those amino acids which are neither found in proteins nor generated by post-translational modifications. Non-proteinogenic AAs are useful building blocks for synthesis of analogs of biologically active peptides such as antibiotics, hormones, toxins and enzyme inhibitors [16]; in addition, they are also versatile chiral auxiliaries in many other organic syntheses [17]. Presence of many uncommon AAs has been reported in various living metabolites, such as antibiotics and some other microbiological products [18]. An important non-proteinogenic AA is penicillamine (PenA, 3,3-dimethylcysteine), which L-isomer occurs 'naturally' while D-isomer is pharmacologically active and widely used in the treatment of Wilson's disease, polyarthritis cystinuria, and heavy metal intoxication and rheumatoid arthritis. Therefore, it becomes very important to control the enantiomeric purity of PenA and to develop rapid and sensitive methods for its detection and enantiomeric separation. The pure D (or *S*) form of drug is used because L (or *R*) form and DL (*RS*) racemate are very toxic and have mutagenicity [19]. The chemical processes underlying the pharmacological action of this drug are still unknown. Thus, if D-PenA (eutomer) is employed for therapeutic purpose, even with a caution and under careful medical supervision, the high toxicity of L-PenA (distomer) and of the racemate mixtures restricts its use. Copper chelation of D-PenA has been shown to generate reactive oxygen species that are cytotoxic to human leukemia and breast cancer cells [20]. A detailed review on direct and indirect methods of enantioseparation of PenA using liquid chromatography, along with a brief comparison of the two approaches, has been published by Bhushan and Kumar [21]; it suggests that there is a scope for development of

methods for determination of enantiomers of PenA in biological specimens.

Stereospecificity of Enantiomers

Enantiomers of AAs are known to have different physiological and biological activities; these arise due to high stereospecificity causing differences in metabolic rates, mechanism and their binding to different receptor types. The AAs are also known to undergo racemisation reaction that has found application in determining the age of living mammals, and in estimating the temperature histories of various environments and thus serve both as a geochronological and biochronological tool [22]. Measurement of racemisation of AAs in dental enamel, for forensic sample, has been applied to estimate the age at death [23]. Changes in D-AA concentrations indicated changes in the microbial colonization of the gut as a result of chronic inflammatory bowel diseases or diet [24].

Chiral Recognition

Much of the explanations on chiral recognition have been provided for direct approach of resolution, particularly using CSPs; chiral recognition in practice is a molecular recognition phenomena. In a chromatographic system, it means preferential interaction of one enantiomer (from the racemic/non-racemic mixture of the analyte), with another chiral species in the chromatographic system working as chiral selector (e.g., a different enantiomerically pure molecule or the chirality of the stationary phase). When the enantiomers pass through the CSP, each must necessarily form a transient diastereomeric complex, on the surface of the column packing, of different free energies; but this is an insufficient condition for the ultimate separation of the enantiomers to occur. Therefore, for chiral recognition to take place, the enantiomeric molecules must have functional groups which provide interactions with the CSP (or any other chiral selector), otherwise introduction of appropriate substituents (having appropriate electronic and stereochemical structure) is required (via derivatization of the analyte). The enantiomer which forms the most stable diastereomer will be most retained, whereas the opposite enantiomer will form a less stable diastereomer and will elute first. Different aspects of chiral recognition models have been discussed in certain reviews. However, detailed investigations are required to understand the mechanism of retention of enantiomers, via intermolecular interactions, leading to separation of enantiomeric pair in chiral systems [25, 26].

Present Report

Enantioseparation of DL-AAs continues to be a subject of immense importance. There has been an increasing need to develop rapid, robust, highly sensitive methods for separation and quantification of both the configurations of AAs in different situations, particularly, in asymmetric synthesis (of pharmaceutical or synthetic importance) and clinical diagnostics. Despite the progress made, enantioselective separation and quantification (of AAs, as focused in this report) remain an analytical challenge owing to frequently incomplete resolution of enantiomers and insufficient sensitivity for determination of trace amounts of D-AAs typically found in biological fluids and tissues.

Literature search reveals that a large number of papers were published on 'enantioseparation of amino acids' from 2004 to 2014; however, the number of citations increased up to 4500 in 2014 which clearly depicts that enantiomeric separation of AAs is gaining more attention now (Fig. 1).

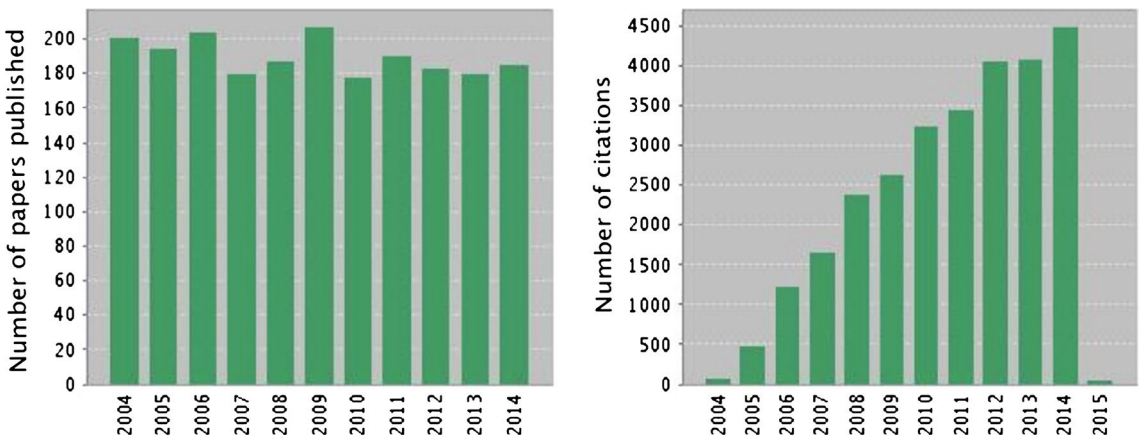


Fig. 1 Number of papers published and citations on 'separation of chiral amino acids' from 2004 to 2014 (data obtained from web of science [v5.16])

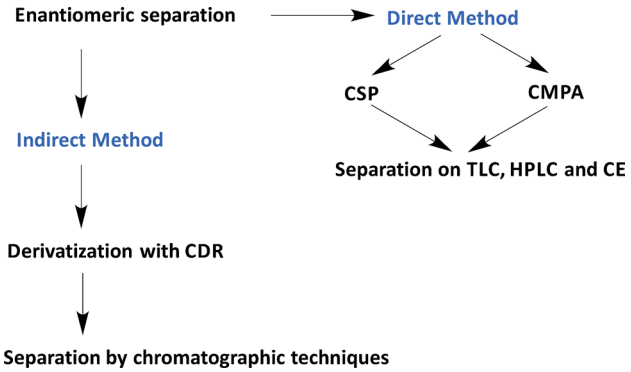


Fig. 2 Approaches to enantiomeric separation

Therefore, the present review is aimed to highlight the methods for enantiomeric separation of AAs reported in the literature from 2004 to 2014 with an objective to focus on recent advances in the area and to identify the challenges along with a discussion on possibilities to address them.

Approaches to Enantioresolution

There are two approaches for enantiomeric resolution as shown in Fig. 2. One is direct approach and the other is indirect approach as discussed in the following sections. To make this review reader friendly, a summary of the literature survey on enantiomeric resolution of amino acids by direct and indirect approach is presented in Tables 1 and 2, respectively.

Direct Approach

Resolution through reversible short-lived diastereomeric association between the chromatographic chiral environment and the solute enantiomers is termed as direct approach. This environment may be due to structural feature of the chiral stationary phase (CSP), or a chiral selector added to the mobile phase (CMPA) or by immobilization of chiral selector (by different methods, especially in TLC) on the stationary phase.

Possible interactions in enantioselective sorption process include co-ordination to transition metal ions, charge transfer interaction, ion exchange, and inclusion phenomena (host–guest complex). There is no chemical derivatization prior to separation process. CMPA and CSPs are adopted both in TLC and HPLC.

Methods for Direct Enantioseparation

Various chromatographic techniques like TLC, HPLC, and GC are frequently used for chiral separation and analysis of AAs [27–31]. Schurig [28] reviewed historical development of enantioseparation of derivatized α-AAs by high-resolution capillary gas chromatography on chiral stationary phases derived from α-AA derivatives and modified cyclodextrins along with the pioneering work emerging from Emanuel Gil-Av and his associates at the Weizmann Institute of Science, Israel; nevertheless, GC has limitations, well known now.

Literature shows certain reviews [29, 32] and monographs [33–35] on separation of AAs including enantioresolution. Several chromatographic [36–38] and electrophoretic methods [39, 40] are based on using chiral stationary phases or chiral selectors as CMPA for separation of AA enantiomers.

Enzymatic reactions are highly stereoselective [40, 41]. Oxidation and decarboxylation constitute the basis of enzymatic catalysis. Though enzyme catalysis of AA transformations is particularly useful for exact determination of high enantiomeric purity, it results in the loss of one of the enantiomers. It is the oldest method for chiral analysis of AAs [42].

Impregnation and TLC

Impregnation is caused either by mixing a suitable chiral selector with the adsorbent prior to plate-making or by immersion of the untreated plate in the solution of chiral impregnating reagent prior to development. A stereoselective separation obtained in a system containing chiral impregnating reagent or the chiral additive in the mobile phase can be due to one or a combination of the following 'mechanisms': (a) stereoselective complexation in mobile phase; (b) adsorption of the chiral selector to the solid phase; (c) formation of the labile diastereomeric complexes with different distribution properties between the mobile and stationary phase.

The role of impregnation in resolving enantiomers or in improving the separation of mixtures of AAs or their derivatives in terms of ion pairing, complex formation, ligand exchange or other steric interactions has been reviewed by Bhushan and Martens [43]. Nevertheless, the chiral selector must be a pure enantiomer and should provide interactions such as π–π, n–π, hydrogen bonding, ion–ion and ion–dipole interactions with at least at three points of one enantiomer (of the analyte) and at two points of the other enantiomer of the chiral molecule (the analyte). Generally, two enantiomers have different interactions with the chiral selector; however, the presence of two or more functionalities with similar nature in the chiral selector may lead to competitive binding of analog sites. This effect decreases the chiral recognition. Bhushan and Agarwal [44] reported a method for direct TLC resolution and isolation of enantiomers of DL-PenA using L-tartaric acid and (*R*)-mandelic

Table 1 Direct enantioseparation of amino acids

Method	Amino acid	Direct method	Detection	References
TLC	DL-Selenomethionine	Reversed phase TLC using (−)quinine chiral selector	Ninhydrin and iodine vapors	[45]
TLC	DL-Penicillamine	(*R*)-Mandelic acid and L-tartaric acid as chiral impregnating reagents and as chiral mobile phase additive	Iodine vapor	[44]
HPLC	Free amino acids	CHIRALPAK ZWIX(+) and ZWIX(−) cinchona alkaloid-derived zwitterionic CSP	DAD	[47]
HPLC	Eleven unnatural β2-amino acids	CSP using 11-methylene-unit spacer of aminoundecyl silica gel for the bonding of (+)-(18-crown-6)-2,3,11,12- tetracarboxylic acid as selector	UV-205	[108]
HPLC	Proteinogenic amino acids	6-Methoxyquinoline-4-carboxylic acid-succinimide ester (MQ-Osu) as CDR on anion exchange CSP	MS	[50]
LE-HPLC	DL-Methionine, DL-leucine, DL-valine and DL-tyrosine	Copper (II) complex of *N*,*N*-dimethyl-L-phenylalanine	UV-PDA	[78]
HPLC	β^2-Amino acids	Chiral stationary phases containing quinine- or quinidine-based zwitterionic selectors	UV	[191]
HPLC	DL-Penicillamine and DL-cysteine	α-Acid glycoprotein and β-cyclodextrin columns	UV-231 nm	[77]
HPLC	γ-Amino acids	CSP based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid	UV-210 nm	[107]
HPLC	*N*-Fluorenylmethoxycarbonyl α-amino acids	Polysaccharide-derived chiral stationary phases	DAD	[109]
HPLC	β^3-Homo-amino acid	(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP	UV-210 nm	[104]
HPLC	Dansyl amino acids	Cyclic hexapeptide as chiral selector		[192]
HPLC	β-Amino acids	(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP		[105]
HPLC	*N*-(3,5-Dinitrobenzoyl)-α-amino acids	(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP		[100]
HPLC	Amino acids	NBD-Cl and FDAA derivatives on Pirkle-type CSP	UV-470 nm	[98]
Micro-HPLC	Natural and unnatural amino acids	Ristocetin A-based CSP		[95]
HPLC	Dansyl amino acids	Norvancomycin-bonded CSP		[96]
GC–MS	Serine enantiomers and glycine in cerebrospinal fluid	Chiralsil-L-Val	MS	[118]
GC	D-Amino acid analysis	FITC derivatives on capillary column packed with vancomycin-modified silica–diol particles	UV-205 nm	[149]
CLEC	α-Amino acids	Cinchona alkaloids as chiral selectors along with Cu(II) ions	DAD	[46]
CE	Amino acids	Fluorescein isothiocyanate (FITC)	LIF	[125]
CLE-CE	Dansyl amino acids	γ-Cyclodextrin (γ-CD)	UV	[84]
CLE-CE	Amino acids and glycyl dipeptides	Chiral selectors Cu(II), Co(II), Ni(II) and Zn(II) complexes of three different sugar acids	DAD	[83]
LE-CE	Dansyl amino acids	Cu(II) complexes with L-prolinamides as chiral selector		[80]
CE–MS^2	Free amino acids	Cyclodextrin as chiral selector	MS	[130]
LE-MEKC	Free amino acids	Cu(II)-L-proline as chiral selector		[81]
CE–MS/MS	Amino acids	7-Fluoro-4-nitrobenzoxadiazole (NBD-F) and 1-fluoro-2,4-dinitrobenzene (DNB-F) on teicoplanin CSP	MS	[97]
EKC	Dns-amino acids	γ-Cyclodextrin as chiral selector		[91]
Column chromatography	α-Amino acids	Dynamic CSP prepared by grafting of methylmethacrylate onto xanthan gum (XG)	Polarimeter	[193]

acid as chiral impregnating reagent as well as CMPA. The detection limits were found to be 0.12 μg for each enantiomer of PenA with L-tartaric acid, under both the conditions, and 0.11 μg with (*R*)-mandelic acid.

Application of Alkaloids as Chiral Selectors

In this section, application of cinchona alkaloids has been discussed for enantioseparation of AAs. Taking into account the importance of faster analysis and low detection limit, enantiomers of DL-selenomethionine (DL-SeMet) were isolated in their native form by impregnated TLC using (−)-quinine (a cinchona alkaloid) as chiral impregnating reagent and CMPA [45]. The results were compared for (i) CMPA approach, (ii) impregnation of plain plates carried out by mixing the chiral selector with slurry, and (iii) by ascending development of plain plates in a solution of chiral selector; the method (ii) provided the highest resolution. Chromatographic analysis took only a few minutes so the method could be useful to determine enantiomeric purity of the samples obtained from different sources even without much sample preparation requirements, and detection was possible at a concentration lower than the limit of 1 % prescribed for pharmaceutical industry. Enantioseparation of several racemic α-AAs (native and derivatized) was obtained using cinchona alkaloids as chiral selectors in mobile phase along with Cu(II) ions in chiral ligand-exchange chromatography by HPLC [46].

Cinchona alkaloid-derived zwitterionic chiral stationary phases (CSPs) containing a chiral sulfonic acid motif [namely, CHIRALPAK ZWIX(+) and ZWIX(−)] which serve as negatively charged interaction site for direct enantioseparation of AAs [47, 48] have been developed. In zwitterionic CSPs, the chiral selector contains a positive and a negative charge in suitable mobile phase. Similarly, the ampholytic analyte may also bear a positive and negative charge in the same mobile phase. It is assumed that the analyte cation interacts with the anionic site of the chiral selector and the analyte anion interacts with the cationic site of the chiral selector through strong coulombic attraction which led synergetic double ion-pairing between the chiral selector and the analyte. The zwitterionic character of these CSPs as well as appropriate mobile phase offered the selective features of a double ion-pairing resulting in improved retention and resolution. Similarly, two other CSPs containing quinine-, or quinidine-based zwitterionic ion-exchanger as chiral selector were investigated for the enantioseparation of 27 unusual cyclic secondary α-AAs [48]. They have also studied the effect of the parameters such as nature and concentration of the bulk solvent, acid and base additives, the structures of the analytes and temperature on enantioseparation.

To enhance MS/MS detection sensitivity on cinchona alkaloid-based CSPs, Reischl et al. investigated the potential of an *N*-acyl-bound metalloferrocene tag to promote chemoselective and enantioselective separation of proteinogenic AAs [49]. Later, 6-methoxyquinoline-4-carboxylic acid-succinimide ester (MQ-OSu) was used as a tag to yield the corresponding stable *N*-acyl amino acid (MQ-AA) that allowed to combine the simplicity of one-dimensional chromatography on anion exchange CSP with the sensitivity of fluorescence and the specificity of MS/MS detection [50] as shown in Fig. 3. Thus, the labeling concept for all individual chiral proteinogenic AAs brought improved separation and sensitive quantification. The LOD and LOQ with MS detection were found to be 100 times lower than those obtained using fluorescence detector; it thus established suitability of this method for analysis of MQ-D-AAs in biological fluids.

Chiral Ligand-Exchange Chromatography (CLEC)

It can be regarded to have begun when Davankov et al. [51, 52] modified commercial HPLC columns by grafting alkyl derivatives of α-AAs such as *n*-decyl-L-histidine or *n*-hexa decyl-L-proline, onto the resin for enantiomeric resolution. It was suggested by Davankov and Rogozhin [53] that CLEC is a typical case of complexation chromatography. It has been shown to be a powerful tool for chiral separation of AAs and other chelate complex forming compounds [53, 54].

Copper (II) Amino Acid Complexes as Reagents in LEC

While discussing application of TLC in enantiomeric resolution it becomes necessary to mention the ligand-exchange TLC plates. TLC plates developed at Degussa AG [55–58] during 1984–1986 were made commercially available by Macherey–Nagel under the trade name of Chiralplate®). The plates contain (2*S*, 4*R*, 2′*RS*)-*N*-(2′-hydroxy dodecyl)-4-hydroxy proline [59] as the chiral selector. Resolution of several proteinogenic and non-proteinogenic AAs, their derivatives and certain other compounds with more than 100 racemates has been accomplished [55–58, 60–66] using this ligand-exchange TLC.

The method was capable of resolving enantiomers in trace amounts, for example, the lowest level of detection of D-dopa in L-dopa samples was >0.25 % [57], and that for D-penicillamine in a DL-mixture was >0.5 % [58].

Davankov observed that the stability of the diastereomeric complexes formed in CLEC is higher than the stability of the diastereomeric adducts formed by other chiral selectors [67]. This technique is usually employed in three separate forms: (i) in the "chiral-bonded ligand exchanging stationary phases" the chiral selector is covalently attached to the stationary phase [68]; (ii) ligand-exchange columns are prepared by dynamically coating the commercially

Table 2 Indirect enantioseparation of amino acids

Method	Amino acid	Indirect method	Detection	References
HPLC–UV	DL-Selenomethionine	CDRs based on difluorodinitrobenzene	UV-340 nm	[45]
2-D HPLC	Amino acids	Derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole	UV	[194]
HPLC	Amino acids	NBD-Cl and FDAA derivatives on Pirkle-type CSP	UV-470 nm	[98]
HPLC	Selenomethionine	CDR phthalimidyl-(*S*)-naproxen	UV-231 nm	[181]
HPLC	Aromatic amino acids	Adsorption onto modified magnetic nanoparticles and separation on Column-Chirex Phenomenx	UV-258	[110]
HPLC	Proteinogenic amino acids	(*S*)-Naproxen-benzotriazole	UV-231	[180]
HPLC	15 Proteinogenic amino acids and DL-selenomethionine	*R*-(+)-Naphthylethyl amine and (*S*)-(+)-1-benzyl-3-aminopyrrolidine as chiral auxiliaries in cyanuric chloride or its 6-butoxy derivative	DAD	[163] [164]
HPLC	Selenomethionine, methionine and cysteine	12 CDRs were synthesized (based on DFDNB and cyanuric chloride moieties)	UV	[166]
HPLC	Selenomethionine	(*R*)-Methyl benzyl isothiocyanate (MBIC) and (*S*)-1-(1-naphthyl) ethyl isothiocyanate (NEIC)	UV	[165]
HPLC	13-Proteinogenic amino acids	6 Cyanuric chloride-based CDRs	UV	[162]
HPLC	D-Amino acids in milk and oyster	OPA-*N*-acetyl-L-cysteine		[177]
HPLC	Amino acid in food analysis	4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)	UV and CD	[145]
HPLC	Penicillamine, cysteine and homocysteine	(*S*)-Naproxen-benzotriazole as CDR	UV	[179]
HPLC	Protein and non-protein amino acids	*N*-(4-Chloro-6-piperidinyl-[1, 3, 5]-triazine-2-yl)-L-leucine amide and *N*-(4-chloro-6-piperidinyl-[1,3,5]-triazine-2-yl)-L-leucine triazine-based CDRs	UV	[161]
HPLC	Non-protein amino acids, and PenA	(*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester	UV-205	[182]
HPLC	Proteinogenic and non-proteinogenic amino acids	Four CDRs FDNP-L-Ala, FDNP-L-Val, FDNP-L-Phe, and FDNP-L-Leu,	UV-340 nm	[153]
HPLC	Serine enantiomers and glycine in cerebrospinal fluid	Marfey's reagent	UV	[118]
HPLC	Penicillamine	*N*-Succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate	UV-231 nm	[178]
HPLC	α-Amino acids	Marfey's reagent	UV-340 nm	[158]
HPLC	α-Amino acids	CDRs based on s-triazine chloride	UV-230 nm	[160]
HPLC	α-Amino acids	CDR N-(*R*)-mandelyl-L-cysteine (*R*-NMC)	UV-340 nm	[176]
GC–MS	Amino acids	Non-chiral derivatization on a chiral column and chiral derivatization on non-chiral column	MS	[112]
GC–MS	Amino acids	Heptafluorobutyl chloroformate in situ derivatization on Chirasil-Val	MS	[111]
TLC	Amino acids	Marfey's reagent	UV-340 nm	[155]
TLC	Amino acids	Marfey's reagent and its chiral variants as CDR	UV-340 nm	[156]
TLC and HPLC	Penicillamine	Marfey's reagent	UV-340 nm	[157]
TLC and HPLC	Non-protein α-amino acids	Marfey's reagent and its four variants	UV-340 nm	[154]

available reversed-phase columns with a hydrophobic chiral selector [69, 70] and (iii) the chiral selector predominantly resides in the mobile phase, in this case, the selector complex is continuously introduced into the column. Analytes to be resolved form the mixed-ligand complexes with the selector, which are diastereomeric in their structure.

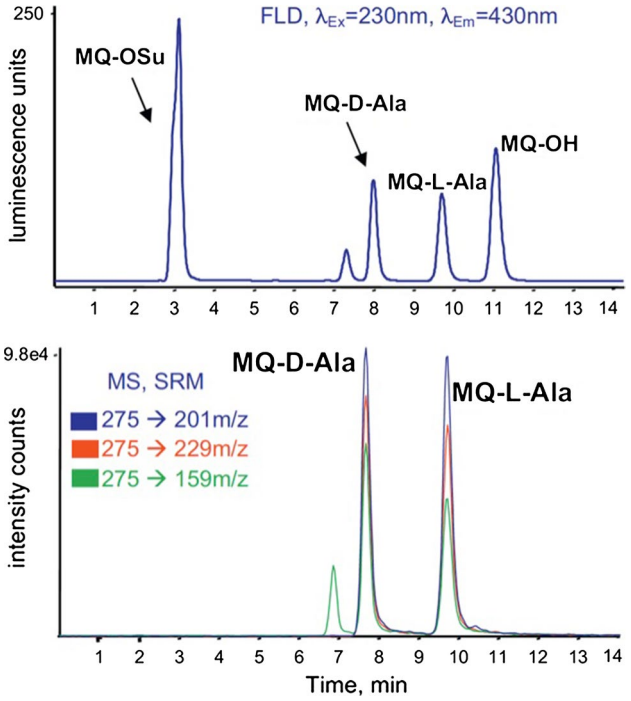


Fig. 3 Comparison of measurements of the same sample, above the LC–FLD signal is shown, below LC–MS/MS in SRM mode. Because of the high fluorescence activity of MQ tag, measurements were carried out to investigate the applicability of fluorescence detection compared to MS. For this purpose, identical samples were evaluated with LC–MS as well as LC–FLD. The representative measurement of racemic alanine is shown. Figure taken from [50] with permission

Such diastereomers interact differently with the column packing and hence get separated [71–74]. Schmid et al. [75] highlighted their own research developments on chiral separation using the principle of ligand-exchange.

CLEC model suggests that the complex undergoes specific interaction with the enantiomer (i.e., with the ligand donating a lone pair of electrons); the metal ion simultaneously coordinates the chiral selector and the enantiomer to be separated. The metal ion used in CLEC especially the Cu(II) causes large background noise and decreases the sensitivity of UV–visible detector [38]. At pH higher than 5 the precipitation of Cu is also reported and such a precipitation results in choking of chromatographic column. Application of metal ions other than Cu(II) has not been found successful. Higher concentration of Cu in the mobile phase has deteriorating effect on column efficiency and lifetime. The CLEC in its present form has been found to be unsuitable for chiral trace analysis especially in complex biological matrices as plasma.

Taking into account the atypical behavior of PenA in its reaction with ninhydrin, Sotgia et al. [76] reported an HPLC method for separation and quantification of stereoisomers of PenA after their spirocyclization with ninhydrin. Cu(II)-L-proline complex was used as a chiral selector in the mobile phase which provided resolution via ligand exchange, but due to copper in the mobile phase there was a very high background noise that limited the sensitivity of the detection system particularly with fluorescence detector. The method was able to detect traces of L-PenA in samples of D-PenA below 0.1 % in fairly short times. By tagging ninhydrin with cysteine (Cys) and PenA, Bhushan and Kumar [77] developed an optimized HPLC method for enantioseparation (Rs > 2) and semi-preparative separation in the form of their spirothiazolidine (STC) derivatives (i.e., DL-1,3-dione-4′-carboxy-5′,5′-dimethyl-spiro [indane-2,2′-thiazolidine] using α-acid glycoprotein and β-CD columns. The enantiomers, so separated as STC derivatives, were detagged using Zn dust and 10 % aq TFA and the native enantiomers were obtained. This method was found to be superior in comparison to the literature report [76] as there was no background interference (due to absence of copper in mobile phase), and the LOD was 0.01 % for the recovered native enantiomers.

Selectivity and Retention in LEC

Dimitrova and Bart [78] investigated the influence of non-ionic surfactants on the selectivity and retention in LEC for the enantioselective separation of racemic mixtures of methionine, leucine, valine and tyrosine using copper(II) complex of *N*,*N*-dimethyl-L-phenylalanine as the chiral additive. They studied the Armstrong model [79] by varying the surfactant concentration; the partition coefficients of the AA complexes between the aqueous phase and the micelles and between the micelles and the stationary phase were calculated for Brij 35. The selectivity for all the AAs changed with increase in the concentration of different surfactants. Sodium dodecyl sulfate was found to improve the selectivity between analytes for enantioseparations of dansyl AAs by ligand-exchange capillary electrophoresis [80] and of three underivatized AAs by ligand-exchange micellar electrokinetic chromatography (LE-MEKC) [81].

Dimitrova and Bart [82] reported separation of DL-methionine, leucine, and tyrosine using copper (II) complex of *N*,*N*-dimethyl-L-phenylalanine as the chiral selector. Two LEC processes were investigated. These were (i) chiral selector in the mobile phase and non-chiral column, and (ii) a chiral stationary phase with the chiral selector in mobile phase as the combination of two chiral selectors (dual system) in one chromatographic separation system. The adsorption behavior of the three amino acids was predicted using dispersive equilibrium model. The dual system could be used for preparative separations, whereas the single one was appropriate for fast analytics.

Chiral Ligand-Exchange Capillary Electrophoresis (CLE-CE)

Giuffrida et al. [29] compiled literature on ligand-exchange capillary electrophoresis (LE-CE) and capillary electrophoresis (CE) coupled on line with mass spectrometry highlighting the progresses in chiral recognition and separation of AA enantiomers by capillary electromigration techniques, using different chiral selectors, especially cyclodextrins, covering the literature published from January 2010 to March 2014.

Using the principle of ligand-exchange, Höld et al. [83] made a comparative study on the application of Cu(II), Co(II), Ni(II) and Zn(II) complexes of D-gluconic acid, D-saccharic acid and L-threonic acid as chiral selectors for enantioseparation of aromatic AAs by CLE-CE. In terms of high resolution, the most suitable complexing agents were Cu(II) with saccharic acid and threonic acid, Co(II) with gluconic acid and Ni(II) with gluconic acid, while Zn(II) did not show any positive results with all three sugar acids. Studies demonstrated that resolution strongly depended on concentration and pH of the selector.

Chiral recognition is largely influenced by change in pH; it was investigated for enantioseparation of dansyl AAs by CLE-CE using Cu(II) complexes of L-prolinamides [80] where the change of species distribution of complexes with pH values was observed by visible spectrophotometry. CLE-CE attained increased attention in enantiomeric separation and accurate determination of enantiomeric purity of dansyl derivatives of AAs as well as dipeptides [84]. They compared the efficiency of three types of systems, viz., Zn(II)-L-4-hydroxyproline complex, and γ-CD as chiral selectors, and CLE-CE system with Zn(II)-L-4-hydroxyproline complex coordinated with γ-CD (as chiral selector). It was found that successful separation was achieved using Zn(II) as the central ion and L-4-hydroxyproline as the chiral ligand coordinated with γ-CD. This is because γ-CD participated in chiral recognition process and played synergistic effect in enantioseparation of AAs and dipeptides; the studies can be considered to have opened new opportunities for enantioseparation based on the synergistic effect of two chiral selectors. Integration of LE approach in CE led to the development of microchip CE for enantiomeric separation of labeled amino acids using LIF detection [85] and chemiluminescence detection [86].

Another important application of ligand exchange principle has been seen in the clinical auxiliary diagnosis of diabetes by LE-MEKC. Cu(II) complex of L-proline as chiral additive provided good selectivity and high resolutions for enantioseparation and determination of three underivatized AAs in human urine, AAs injection, and AA oral liquid [81].

Chiral Stationary Phase (CSP)

Chiral HPLC columns are made by immobilizing the chiral selector onto the stationary phase or packing the column with chiral material. Commercially available CSP columns are classified into following categories.

Cavity-Phase (Cyclodextrin α, β, γ)

The most commonly used CSP in liquid chromatography for pharmaceutical analysis and enantioseparations is the cyclodextrin-bonded silica material where immobilization of cyclodextrin (CD) provides enantioselectivity as well as the stability to the chiral stationary phase. The very first use of CDs for chiral separations as a selective precipitation/crystallization agent for enantiomers was published in 1959 [87]. There after, a large number of articles were published on the use of CD-CSPs with countless analytical methods, grown in academia and industry [88, 89]. CDs have also been used as CMPA in liquid chromatography.

An interesting study for enantiomeric separation of AAs has been reported by Giuffrida et al. [90] with the use of modified CDs (mCDs), namely 6-deoxy-6-[1-(2-amino)ethylamino]-*β*-CD, 6-deoxy-6-[*N*-(2-methylamino) pyridine)]-*β*-CD and 3-monodeoxy-3-monoamino-*β*-CD, which were used as chiral selectors for CE-LIF and CE–TOF-MS. The potential applicability of these three mCDs as chiral selectors in CE was also compared with the unmodified β-CD and γ-CD; and it was found that LOD values obtained by chiral CE–TOF-MS were in the nM range comparable or only slightly worse to those obtained by CE-LIF. Furthermore, with the advantages of less analysis time, high resolution and efficiency values, and the chiral CE–TOF-MS method was successfully applied for the detection of main D-, and L-AAs in different real samples including transgenic versus wild soy and vinegar. The chiral-CE–MS profile was found very similar for wild and transgenic soya. Arg, Lys, Gly, Ala, Glu and Asp were detected as main AAs in both soy varieties. However, some interesting differences were highlighted using the chiral-CE–MS approach; namely D-Arg was found only in the transgenic variety as shown in Fig. 4. Later on, they used 3-amino derivative of γ-CD (3-deoxy-3-amino-2(*S*),3(*R*)-γ-cyclodextrin/GCD3AM) for enantioseparation 10 dansyl derivatives of α-AAs in electrokinetic chromatography [91]. GCD3AM showed very good chiral recognition, high selectivity and resolution towards several pairs of AAs.

Protein and Peptide-Based CSPs

Armstrong et al. introduced macrocyclic antibiotics (of the glycopeptides nature) as chiral selectors in HPLC and

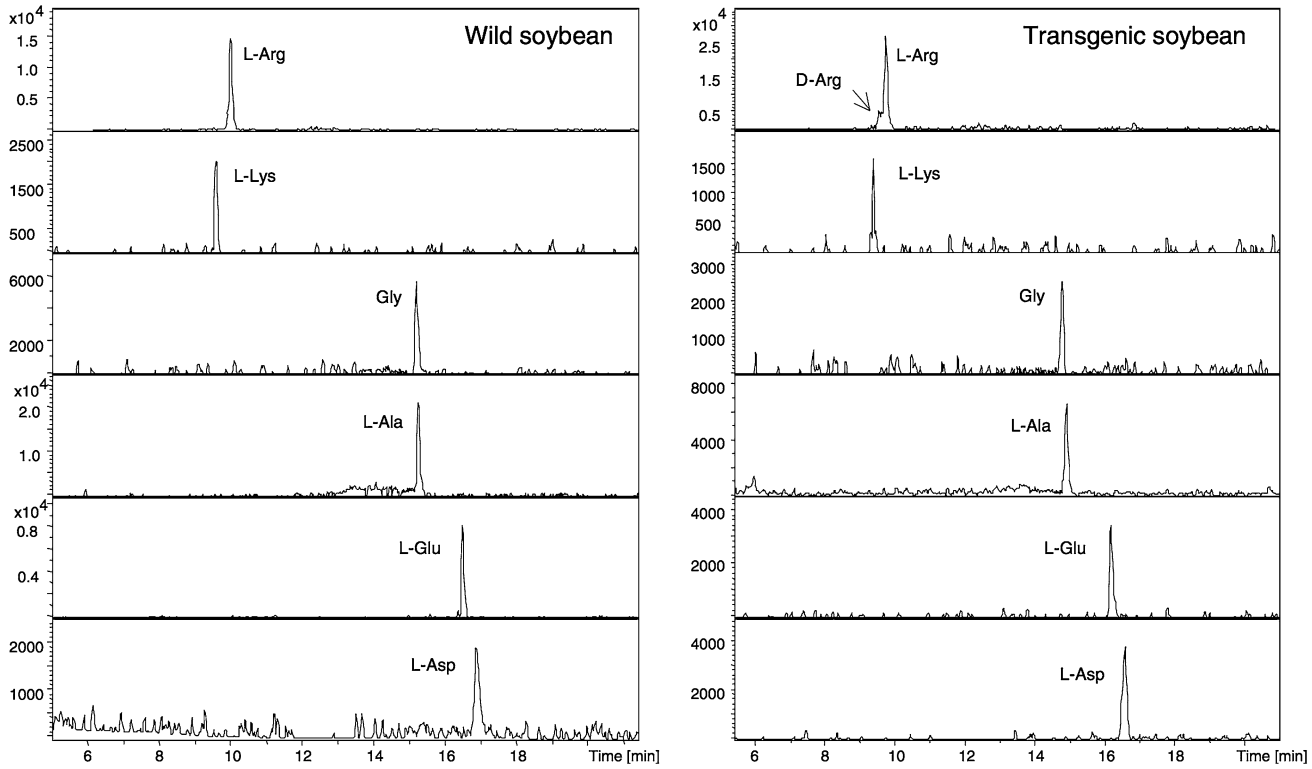


Fig. 4 CE–ESI-TOF-MS extracted ion electropherograms from a wild and transgenic soybean. Figure taken from [90] with permission

CE [92–94]. The aglycone portion with four joined macrocyclic rings and five sugar chains along with various functional groups in Ristocetin A are considered to be responsible for chiral recognition and enantioselectivity in protected AAs [92]. The interactions involved in chiral recognition are hydrophobic interactions, hydrogen bonding, dipole–dipole interactions, electrostatic interactions, as well as π–π interactions. Piccinni et al. [95] performed chiral separation of Fmoc- and Z-derivatives of natural and sulfur-containing unnatural AAs by micro-HPLC using microbore columns packed with the macrocyclic antibiotic Ristocetin-A, chemically bonded to 3.5 μm silica gel. Ding et al. [96] synthesized norvancomycin-bonded CSP for separation of enantiomers of several dansyl AAs; chromatographic results showed that both ionic and hydrophobic interactions were engaged between the analyte and macrocycle. Derivatization of AAs with 7-fluoro-4-nitrobenzoxadiazole (NBD-F) and 1-fluoro-2,4-dinitrobenzene (FDNB) followed by their separation on teicoplanin CSP capillary HPLC/tandem mass spectrometry has been reported by Song et al. [97]. NBD-AA enantiomers (12 pairs) were baseline resolved while the efforts to separate FDNB tagged AA enantiomers on this CSP were not successful as shown in Fig. 5 for DL-Phe.

An important application of the two columns (β-CD and α-acid glycoprotein) was seen in the separation of Cys and PenA. Tagging of Cys and PenA with ninhydrin yields their enantiomeric spirothiazolidine (STC) derivatives (i.e., DL-1,3-dione-4′-carboxy-5′,5′-dimethyl-spiro[indane-2,2′-thiazolidine], which were separated (Rs > 2) by HPLC with UV detection using α-acid glycoprotein and β-CD columns with the advantage of semi-preparative separation [77]. The enantiomers, so separated as STC derivatives of DL-PenA were subjected to detagging, using Zn dust and 10 % aq TFA and the native enantiomers were obtained. For analytical purposes dinitrophenyl (DNP) derivatives of DL-PenA were also prepared and resolved on both the columns with LODs 0.01 and 0.004 % for STC and DNP derivatives, respectively. The optimized analytical method was further scaled up for preparative separation of enantiomers. This method was found best compared to the reported one [76] in terms of low LODs, lack of background interferences and recovery of the native enantiomer.

Charge Transfer CSPs

The CSPs contain π-acidic or π-basic aromatic groups and capable of donor–acceptor interaction (charge transfer complexation). Cui et al. used two types of derivatization reagents, i.e., achiral derivatization reagent (NBD-Cl) and chiral derivatization reagent (FDAA) for AA analysis; the derivatives were separated on chiral column (Pirkle type, consisting of dinitrobenzoyl phenylglycine ionically bound to aminopropylsilica) and achiral column,

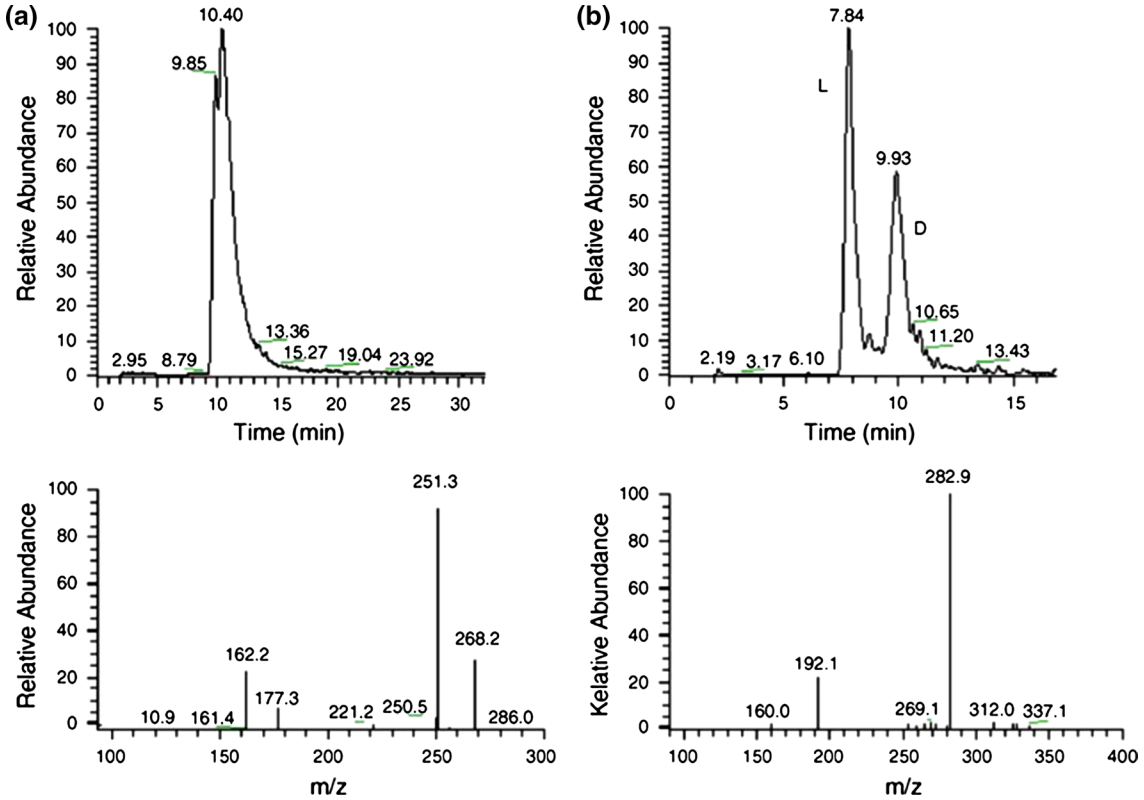


Fig. 5 A comparative study on separating DNB-Phe and NBD-Phe enantiomers on teicoplanin CSP: **a** extracted mass chromatogram of *m/z* 330 → 251 from separating DNB-DL-Phe and the full scan MS/MS spectrum for peak identification; **b** extracted mass chromatogram of *m/z* 327 → 282 from separating NBD-DL-Phe and the full scan MS/MS spectrum. Figure taken from [97] with permission

respectively [98]. The comparison study in terms of sensitivity and selectivity of the two reagents was carried out for three pairs of AA enantiomers (Ser, Val and Phe). FDAA derivatives showed reversed elution order with more interference in the chromatogram. On the other hand, the NBD-derivatives were efficiently analyzed and seemed to be more appropriate for determination of enantiomeric purity of a series of (L)-AAs.

Crown Ether-Based CSPs

Since the introduction of chiral crown ethers such as CSPs [99] a CSP based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid has been successfully applied for enantioseparation of *N*-(3,5-dinitrobenzoyl)-α-AAs [100] and β^3-AAs [37, 68, 101–105]. The chromatographic retention and resolution behavior were found to depend on the structure of the substituents in β-position and ionic strength of the mobile phase [104]. Péter and co-workers [106] separated β^2-AAs bearing aliphatic and aromatic side-chains on a CSP of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid attached to silica with a short, 3-methylene-unit spacer: all aromatic β^2-AAs enantiomers were baseline-resolved, while AAs with alkyl side-chains showed partial separation. Lee et al. [107] used two different CSPs based on 3 methylene-unit or 11 methylene-unit spacer group in (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid and the two were compared for the enantiomeric separation of γ-AAs; longer spacer unit provided higher resolution. The improved lipophilicity of the long-tethered CSP might be responsible for its higher chiral recognition ability which was further investigated by Ilisz et al. for enantiomeric separation of 11 unnatural β^2-AAs on CSP using the 11-methylene-unit spacer of aminoundecylsilica gel for the bonding of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid as selector [108]. Moreover, the nature and position of the substituents on the aromatic ring substantially influence the retention and enantioseparation [105] in CSP based on crown ether.

CSPs Based on Polysaccharides and MNP

Lee et al. resolved enantiomers of *N*-protected fluorenylmethoxycarbonyl (*N*-FMOC) α-AAs on three polysaccharide-derived chiral stationary phases, such as cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD), amylose tris(3,5-dimethyl-phenylcarbamate) (Chiralpak AD) and cellulose tris(4-methylbenzoate) (Chiralcel OJ) using

reverse mode chromatography [109]; and found that hydrophobicity played an important role on the retention of the *N*-FMOC α-AAs in the reverse mode.

Magnetic nanoparticles (MNPs) have been suitable candidates for enantioselective separation of chiral biomolecules due to their unique size, large surface area, biocompatibility, low toxicity, superparamagnetic properties and well understood surface chemistry. Ghosh et al. investigated resolution of underivatized aromatic AAs (DL-Trp, DL-Phe and DL-Tyr) utilizing superparamagnetic Fe_3O_4 nanoparticles functionalized with silica and carboxymethyl-*β*-cyclodextrin (CMCD) [110]. Resolution of enantiomers from racemic mixture was quantified in terms of enantiomeric excess using HPLC. The MNPs selectively adsorbed L-enantiomers of DL-Trp, DL-Phe, and DL-Tyr from racemic mixture and enantiomeric excess (ee) for each was found to be 94, 73 and 58 %, respectively. The enantioselectivity was confirmed first by FTIR studies demonstrated that hydrophobic portion of enantiomer penetrated into hydrophobic cavity of cyclodextrin molecules to form inclusion complex. Secondly, XPS study revealed that amino group at chiral center of the AA molecule formed hydrogen bond with secondary hydroxyl group of CMCD molecule and favorability of hydrogen bond formation resulted in selective adsorption of L-enantiomer.

CSPs in GC

Gas chromatography (GC) is a commonly used technique in AA analysis; nevertheless, the main requirements of GC application for AA analysis are that the polar analyte has to be converted into their volatile, thermally stable derivatives. Earlier, alkyl chloroformates became attractive derivatization reagents in GC as they react immediately with most AA functional groups in aqueous matrices. In situ derivatization of AAs with heptafluorobutyl chloroformate followed by subsequent chiral as well as non-chiral GC/mass spectrometric analysis on a respective nonpolar fused silica and Chirasil-Val capillary column has been reported by Simek et al. [111].

GC and Chloroformate Derivatives

Bertrand et al. reported two GC–MS methods for enantioselective separation of the 20 proteinogenic AAs [112] using selected ion monitoring (SIM) detection. In the first method, ethyl chloroformate and 2-chloropropanol were used to derivatize enantiomers of AAs. The resulting *N*(*O*,*S*)-ethoxycarbonyl-2-chloropropyl ester derivatives were separated on an achiral column by capillary GC–MS. In the second method, ethanol and ethylchloroformate were used for derivatization and the *N*(*O*,*S*)-ethoxycarbonyl ethyl ester derivatives were separated on Chirasil-L-Val column. The separation performances of the two methods were compared; second method was found to be better resulting in the separation of 14 out of 19 enantiomeric pairs while the first method could resolve only 10 enantiomeric pairs. One of the main advantages of these methods was the use of SIM detection mode, which could identify the co-eluting peaks and allowed detection at very low concentration. The simplicity of these methods makes them readily applicable to many fields, such as foods.

GC and D-Amino Acids

A review on gas chromatographic enantioseparation of derivatized α-AAs on chiral stationary phases has been summarized by Schurig [28]. Advances in chromatographic techniques improved sensitivity in AA analysis which further revealed the presence of D-AAs in food, plants and higher animals [113]. Higher concentration of D-serine has been identified in mammalian central nervous system [114]. D-Serine acts as a neuromodulator where it binds to *N*-methyl D-aspartate (NMDA) [114] which is excitatory AA receptor [115] involved in central nervous system development, brain plasticity, memory, and learning. NMDA dysfunction has been associated in various pathological conditions, including stroke, schizophrenia, epilepsy, and neurodegenerative conditions [116]. Further studies showed endogenous D-serine metabolism and synthesis from L-serine [117]. These reports highlighted the need to precisely detect D-AA so that clinical studies can further be improved. In view of the above studies, Fuchs et al. [118] separated and quantified D-serine (0.14 μM) and L-serine (0.44 μM) in cerebrospinal fluid (CSF) on CSP (chirasil-L-val column) by GC–MS system and determined age-dependent human concentration ranges. In their previous study [13], it was suggested that D-serine plays a pivotal role in normal and aberrant human brain development because D-serine deficiency was found in patients with 3-phosphoglycerate dehydrogenase deficiency.

Capillary Electrophoresis (CE)

CE has become a useful analytical technique for chiral analysis because of its high efficiency and flexibility of incorporating various chiral selectors of only a small amount [119]. However, the main drawback associated with CE is that photometric detection is less sensitive in CE and this aspect was overlooked while using various schemes of detection [120–122]; therefore, different on-line (after injection) or offline (before injection) preconcentration techniques were employed [123–125]. Furthermore, use of laser-induced fluorescence (LIF) detection together with derivatizing reagents can be the best choice to increase the sensitivity of chiral CE of AAs and reaching low limits

of detections (LOD) in the range of 10^{-8}–10^{-10} M [122, 125–127].

Several papers as well as review articles have been published on enantioseparation of AAs by capillary electrophoresis. In the CE analysis of AAs, the selection of separation mode is one of the most important issues to obtain good resolution of target enantiomers. Kitagawa et al. [128] presented a comprehensive review on the progresses in CE analysis of AA enantiomers in the last decade. They discussed various chiral selectors including CDs, bile salts, crown ethers, cinchona alkaloids, metal–chiral AA complexes, macrocyclic antibiotics and proteins that have been employed to separate AA enantiomers. Moreover, several separation modes, CD-modified capillary zone electrophoresis (CD-CZE), CD electrokinetic chromatography (CDEKC), micellar EKC (MEKC), CD-modified micellar electrokinetic chromatography (CD-MEKC), capillary electrochromatography (CEC), ligand-exchange CE (LE-CE), and nonaqueous CE (NACE) have also been used for analysis of chiral AAs. Coupling of mass spectrometry (MS) with CE has become a powerful technique for rapid, efficient, selective, and sensitive analysis as well as chiral analysis [129]. The coupling significantly increases signal-to-noise ratio, and the ability to simultaneously measure and identify several compounds in the same sample can be obtained using MS^2 experiments [130]. Use of CE–MS in chiral AA analysis has gained attracted attention during the last decade [90, 130–135]. Two new electrophoretic techniques have been developed by Liang et al. One is microchip capillary electrophoresis based on bovine serum albumin-conjugated Fe_3O_4@Au nanoparticles as stationary phase [136] and other is open-tubular capillary electrochromatography using bovine serum albumin-conjugated polydopamine–graphene oxide (PDA/GO) nanocomposites (PDA/GO/BSA) as stationary phase [137]. Both of the stationary phases have been tested for enantiomeric separation of tryptophan and threonine, and a successful separation of the enantiomers was achieved. The materials showed good stability, reusability and reproducibility for enantioseparation and hence can be considered as a promising candidate for high-throughput screening of chiral compounds.

2-Dimensional HPLC

In comparison to simple HPLC, two-dimensional HPLC (2D-HPLC) methods combining reversed-phase separation (as the first dimension) and chiral separation (as the second dimension) are useful techniques for trace analysis of L-AAs and, therefore, suitable for chiral AA metabolome analysis in physiological fluids and tissues [138–143], as well as in the fermented foods and beverages. In 2014, Miyoishi et al. [128] used 2D-HPLC after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) for AA analysis, by fluorescence detection, in *Kurozu* vinegars. They found that *Kurozu* vinegars made by traditional earthenware jar fermentation contain large amounts of L-AAs. Miyoshi et al. also described 1D-HPLC and 2D-HPLC analysis of naturally occurring free L-AAs in mammals [144]. A new D- and L-AA analysis method was developed using a CD detector. A comprehensive analysis of D- and L-AAs carried out by ultra-HPLC circular dichroism (CD) detector after pre-column derivatization with NBD-F and its application to food samples was explored [145].

Amino Acids in Environment and Origin of Life

While discussing enantioresolution of AAs it becomes relevant and important to address the question why D-AAs were eliminated and organisms have evolved only L-AAs. Today, the occurrence of D-AAs is not generalized in higher organisms, whereas many more D-AAs have been detected in primitive organisms such as bacteria. The homochirality of AAs in organisms, the so-called L-AAworld, is likely to be established being accompanied by evolution of life on earth.

It is interesting to note that detection of trace amounts of aminoisobutyric acid (Aib) in ice samples of Antarctica indicated the presence of Aib-producing microorganisms. Aib, a non-proteinogenic AA, functions as a highly specific marker. In 2014, Magi et al. [146] published a review article stating the presence of certain AAs in Antarctic environment. Presence of AAs Aib (α-aminoisobutyric acid, 2-methylalanine) and DL-Iva (isovaline, 2-ethylalanine) has been seen in organic extracts of mycelia after total acidic hydrolysis and derivatization by GC/SIM-MS on Chirasil-L-Val column [147].

Carbonaceous chondrites comprise many local organic compounds and can be an essential source of prebiotic compounds required to study the origin of life on earth and other planets. Burton et al. studied enantiomeric and isotopic compositions of simple primary AAs by LC–TOF-MS and GC–IRMS in six metal-rich CH and CB carbonaceous chondrites [148]. Extracts of CH chondrites were found to be predominantly rich in AAs (1316 mg L^{-1}) while CB chondrite extracts had much lower abundances (0.22 mg L^{-1}).

Indirect Approach: Use of Chiral Derivatizing Reagents (CDRs)

Indirect method involves a formation of chemical bond between analyte and chiral derivatizing reagent (CDR) to convert a pair of enantiomers into diastereomers.

Diastereomers have different physicochemical properties and can be separated in achiral environment. These methods are suitable for trace analysis of enantiomers in biological samples, such as blood and urine, because of option of coupling the enantiomeric mixture with highly sensitive reagents with high molar absorptivity (ε) or high fluorescence quantum yield (φ). Separation of diastereomeric pair via indirect technique is sometimes simpler to perform and often has better resolution than with a direct method because chromatographic conditions are much easily optimized.

CDRs were investigated to attain high sensitivity and selectivity which gained high popularity because the conventional reversed-phase columns (achiral columns) were able to separate the diastereomers. Further development of hyphenated techniques (liquid chromatography coupled to mass spectrometer, LC–MS) replaced optical detectors because of superior sensitivity and selectivity of MS. An extensive use of coupling technique can be seen in food chemistry where nano-liquid chromatography (nano-LC) coupled with mass spectrometry used for enantiomeric separation and determination of D- and L-AAs in orange juices [149].

Chiral Derivatizing Reagents (CDRs)

The choice of the derivatizing reagent has significant effect on success of separation, and detection of resulting diastereomer and accuracy of method. Various CDRs are reported in the literature for enantiomeric separation of AAs; in addition their applications have gained attention to determine the enantiomeric purity of various other chiral compounds. Illisz et al. presented two comprehensive reviews on application of CDRs in HPLC separation of AA enantiomers [150, 151] which highlighted a comparison study of different derivatizing agents for resolution of complex mixtures of proteinogenic DL-AAs, non-proteinogenic AAs and peptides/AAs from peptide syntheses or microorganisms.

CDRs Based on Difluorodinitrobenzene, Cyanuric Chloride and Isothiocyanate

Marfey [152] introduced 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA), synthesized by the reaction of 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and L-Ala-NH2; so named as Marfey's Reagent (MR). Amide was chosen because it is quite stable, neutral and does not undergo racemization. The reaction involves substitution of one of the two fluorine atoms in 1,5-difluoro-2,4-dinitrobenzene by L-alanine amide to yield MR which reacts stoichiometrically, without racemization, within 1 h under alkaline conditions at 40 °C with the α-amino group of L- and D-AAs yielding diastereomers as depicted in Fig. 6. The diastereomers can be separated and quantified (in nanomole range) by HPLC due to large difference in their relative retention.

Derivatization of free AAs with other reagents suffers inherent problems, e.g., inability to react with all proteinogenic AAs, unstable derivatives, poor detectability of certain AA derivatives, or lack of quantitative yield of the reaction. Marfey's reagent overcame these problems and proved to be a highly successful reagent for structural characterization of peptides, confirmation of racemization in peptide synthesis, and detection of small quantities of D-AAs. Application of MR and its structural variants for chiral analysis of AAs has been summarized in a review by Bhushan et al. [31]. The structures of MR and its chiral variants are shown in Fig. 6. Diastereomers of 18 proteinogenic and 08 non-proteinogenic AAs prepared with FDAA and four of its structural variants (FDNP-L-Phe-NH_2, FDNP-L-Val-NH_2, FDNP-L-Leu-NH_2 and FDNP-L-Pro-NH_2) were separated on RP-HPLC [153, 154]. Separation of a complex mixture of 18 racemic proteinogenic AAs was achieved in a single chromatographic run of 65 min, as depicted in Fig. 7; the method was found successful to determine D-AA in excess of L-isomer with LOD of 0.001 % for D-enantiomer. Separation and determination of D-serine and L-serine, in cerebrospinal fluid (CSF) has been carried out by derivatization with Marfey's reagent and LC–MS analysis [118].

Bhushan et al., for the first time, reported TLC resolution of AA diastereomers prepared from chiral variants of MR; the diastereomers were resolved using mobile phase phenol–water 3: 1 (v/v) and acetonitrile in triethylamine–phosphate buffer (50 mM, pH 5.5) in NP-TLC and RP-TLC, respectively [154, 155]. Later on, they used 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (FDLA) to prepare the diastereomers of proteinogenic and non-proteinogenic AAs which were separated on NP and RP-TLC [156]. The resolutions were compared with FDAA; FDLA provided better separation in terms of resolution; this was because the leucine-amide side chain moiety in FDLA is more hydrophobic and so exhibits high affinity with column compared to alanine side chain in FDAA.

In spite of the presence of thiol group, and like other AAs without aromatic chains, PenA is relatively a poor absorber in the UV/vis region; therefore, functionalization of stereoisomers was necessary for enantioseparation. Derivatization of DL-PenA with MR and its structural variants (FDNP-Phe-NH_2 and FDNP-Val-NH_2) provided greater sensitivity and easier location on TLC plates due to their self visibility, in comparison to the methods reported prior to 2004. The diastereomeric pairs prepared from the three CDRs were successfully separated on RP and normal phase

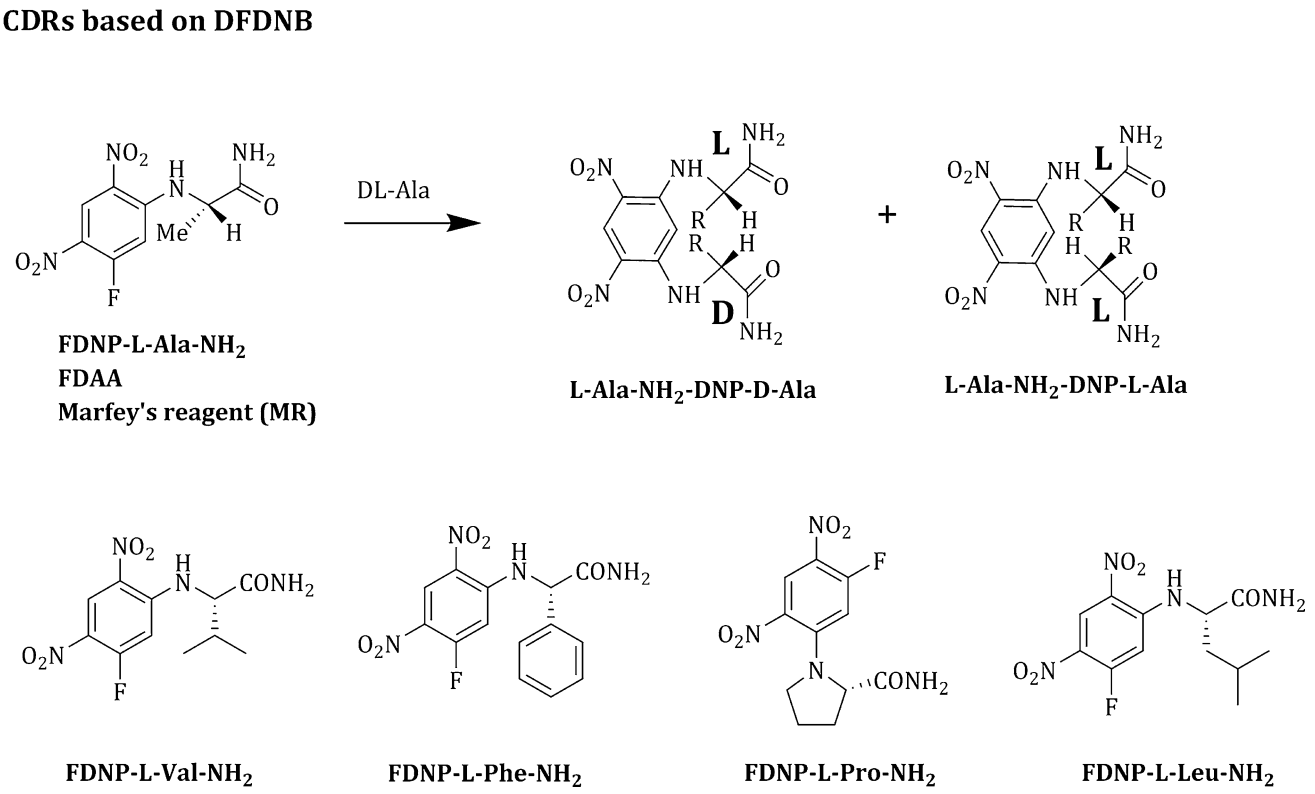


Fig. 6 Structures of CDRs based on DFDNB

TLC plates by using TEAP buffer (50 mM, pH 5.5)–MeCN (50:50, v/v), and phenol–water (3:1, v/v), at 25 °C, respectively, as the solvent systems [157]. The TLC method was successful for detection of L-PenA in D-PenA up to 0.05 %; elution order of diastereomers on RP-plates was found to be reverse of normal phase. Enantioseparation of the same set of diastereomeric pairs by RP-HPLC using acetonitrile–0.01 M TFA as mobile phase under a linear gradient (acetonitrile, 25–65 %, 45 min) at a flow rate of 1 mL/min with UV detection at 340 nm detected L-PenA in D-PenA up to 0.005 %.

Literature reveals that CDRs based on DFDNB [31, 152, 153, 158, 159] and cyanuric chloride (CC) moieties having AAs or AA amides [160–162] as chiral auxiliaries (Fig. 8a, b) have been applied for enantioseparation of AAs (including compounds containing amino group and pharmaceuticals). Two enantiomerically pure amines, viz., (*R*)-(+)-naphthylethyl amine and (*S*)-(+)-1-benzyl-3-aminopyrrolidine, were used as chiral auxiliaries for nucleophilic substitution of chlorine atoms in cyanuric chloride or its 6-butoxy derivative by Bhushan et al. Diastereomers of 15 DL-proteinogenic AAs [163] and Se-Met [164] were synthesized under microwave irradiation using these reagents and separated on RP-HPLC. Microwave irradiation required shorter reaction time (120 s or less) as compared to the time required by conventional derivatization (nearly 40–45 min). DFDNB-based CDRs containing chiral auxiliaries L-methionine, and *S*-benzyl-L-cysteine were also synthesized and used to prepare diastereomers of DL-SeMet which were separated by RP-TLC and RP-HPLC [45].

Indirect enantioseparation of Cys, methionine and SeMet have been investigated by Bhushan et al. using isothiocyanate-based CDRs [(*R*)-methyl benzyl isothiocyanate (MBIC) and (*S*)-1-(1-naphthyl) ethyl isothiocyanate (NEIC)] [165], and 12 CDRs based on DFDNB and CC [166] moieties, with UV detection.

CDRs Based on OPA and Chiral Thiol

Precolumn derivatization with *o*-phthalaldehyde (OPA) and a chiral thiol is one of the well-known methods in HPLC for efficient chiral analysis of α-AAs; the reaction gives diastereomeric isoindoles. *N*-Acetyl-L-cysteine (NAC) and *tert*-butyloxycarbonyl-L-cysteine with OPA were used for the first time [167, 168]. Afterwards many other chiral thiols were used for derivatization with OPA, such as thiosugars [169] and enantiomeric *N*-acyl-L-cysteines [170, 171], *N*-acetyl-D-penicillamine (NAP) [170], D-3-mercapto-2-methylpropionic acid [172]. The conclusion of these studies revealed that the structure of SH reagent determined

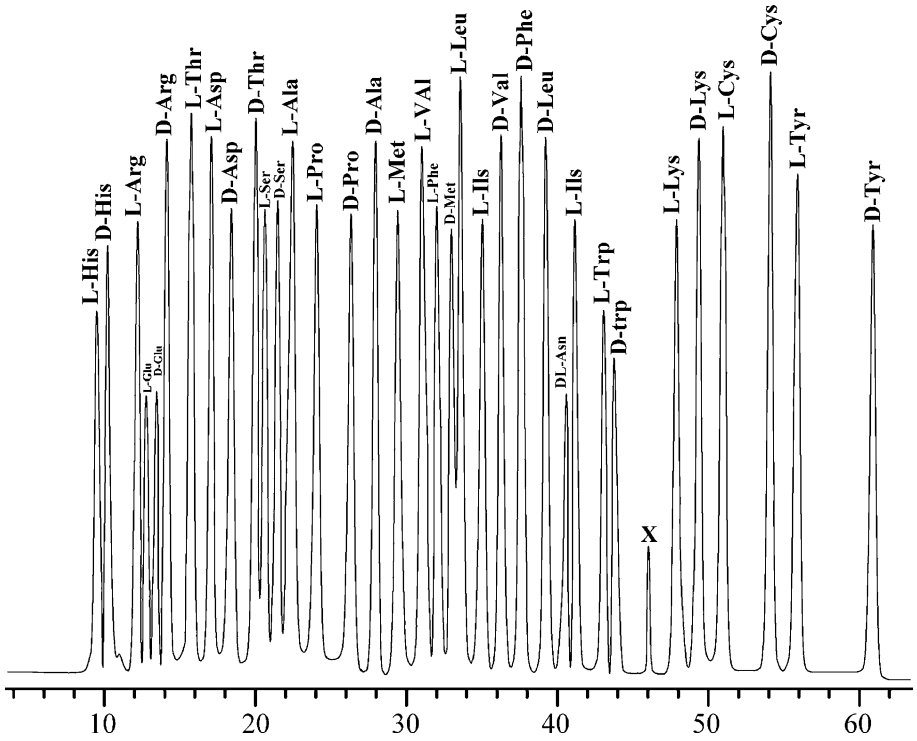


Fig. 7 The chromatogram showing resolution of mixture of 18 diastereomeric pairs of amino acids prepared with FDNP-L-Val-NH_2 (X unknown peak). Figure taken from [153] with permission

resolution and presumably the stability of isoindoles so formed. Application of different thiols (Fig. 9) allowed enantiomeric analysis of individual α-AAs [173–176]. An important application of chiral *N*-acetyl-L-cysteine with OPA has been investigated to determine D-AAs in milk and oyster with the advantage of simple sample preparation and improved separation [177]. In comparison to traditional enantiomeric thiols *N*-(*R*)-mandelyl-L-cysteine [(*R*)-NMC)] provides higher resolution for enantiomers [176] of α-AA.

Other CDRs

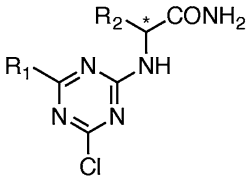
The structures of CDRs discussed in this section are presented in Fig. 10. *N*-Succinimidyl-(*S*)-2-(6-methoxynaphth-2-yl) propionate (SINP, Fig. 10) introduced by Bhushan and Tanwar showed successful separation of diastereomers of DL-PenA on RP-HPLC with the advantage of one step room temperature derivatization and low detection limits (0.5–2.5 pmol range) [178]. (*S*)-Naproxen-benzotriazole as a new CDR was introduced by Bhushan et al. to prepare diastereomers of PenA, Cys and homocysteine under microwave irradiation [179], and 18 proteinogenic amino acids by microwave irradiation and vortexing [180] followed by their separation on RP-HPLC with UV detection at 231 nm. In conclusion, they highlighted that the large, highly conjugated naphthyl ring of CDR attached to the chiral center offers high UV absorbance and gives detection at very low concentration (LOD 0.1–1.2 pmol range), and the hydrophobic property of the naphthyl ring facilitates enantioresolution. Recently, a new CDR phthalimidyl-(*S*)-naproxen ester was prepared and used for synthesis of diastereomers of SeMet via microwave irradiation or vortexing, by Bhushan and Nagar [181]. The diastereomeric pairs were successfully separated by RP-HPLC using binary mixtures of aqueous triethylammonium phosphate and acetonitrile with low limit of detection 0.11 and 0.10 pmol/mL for D- and L-SeMet diastereomers, respectively. Bhushan et al. also reported use of (*S*)-*N*-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(*S*)-NIFE] as a reagent for indirect enantioresolution of non-proteinogenic AAs by RP-HPLC [182].

Advances, Challenges and Possibilities to Address

Chiral Ionic Liquid

It can be easily synthesized and may be used as a chiral selector in solution. It seems that chiral ionic liquids (CILs) are good chiral selectors as they are capable of giving most types of interactions, especially ionic interactions, which are important in chiral recognition protocols [183, 184]. CILs derived from AAs are also known [185]. One recent application of CILs in enatioseparation was studied by Qing et al. [186]. They synthesized four AA ionic liquids as cations [L-Pro][CF_3COO], [L-Pro][NO_3], [L-Pro]$_2$[SO_4],

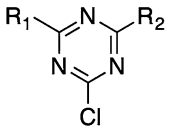
(a)



*Chiral center

CDR	R^1	R^2
1	-Cl	$-CH_3$
2	-Cl	$-CH(CH_3)_2$
3	-Cl	$-C_6H_5$
4	-Cl	$-CH_2CH(CH_3)_2$
5	$-OCH_3$	$-CH(CH_3)_2$
6	$-OCH_3$	$-CH_2CH(CH_3)_2$
7	$-OCH_3$	$-CH_2CH_2SCH_3$
8	$-OCH_3$	$-C_6H_5$
9	$-OCH_3$	$-CH_3$
10	$-NHCH(CH_2C_6H_5)CONH_2$	$-CH(CH_3)_2$

(b)



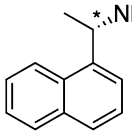
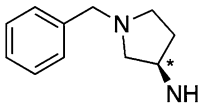
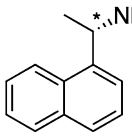
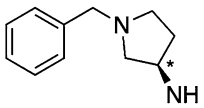
CDR	R^1	R^2
1	$-O(CH_2)_3CH_3$	
2	$-O(CH_2)_3CH_3$	
3	-Cl	
4	-Cl	

Fig. 8 Structures of CDRs based on cyanuric chloride

and [L-Phe][CF_3COO] and applied them for enantiomeric separation of tryptophan using LEC; good enantioseparation was achieved with resolution of 1.89. Furthermore, the room temperature ionic liquids (RTILs) have unique chemical and physical properties such as air and moisture stabilities, high solubility in water, wide electrochemical windows, high conductivities, high heat capacities and virtually no vapor pressures. Because of these properties, RTILs have shown tremendous potential in analytical applications [187] and enantiomeric separation of pharmaceuticals [188].

Chiral Ligand Exchange

Considering the fact that more stable ligand-exchange complexes are formed in solution compared to stationary phases, chiral ligand-exchange diastereomeric complexes can be formed off-column with respect to formation of ligand-exchange complex resulting in reduction in concentration and amount of chiral selector to overcome problems related to detection and sensitivity, and precipitation on column. However, it is required to be assessed for enantioseparation of different racemates including DL-AAs.

Resolution of Non-Racemic Mixtures Using Achiral Phase Chromatography

Since the physical properties, e.g., density, boiling point, vapor pressure, refractive index, IR and NMR spectra of the pure enantiomers, [(*R*) or (*S*)], are generally indistinguishable from their mixture in the vapor and liquid state (melt or solution), resolution of enantiomers (from racemic or non-racemic mixtures) in an achiral environment

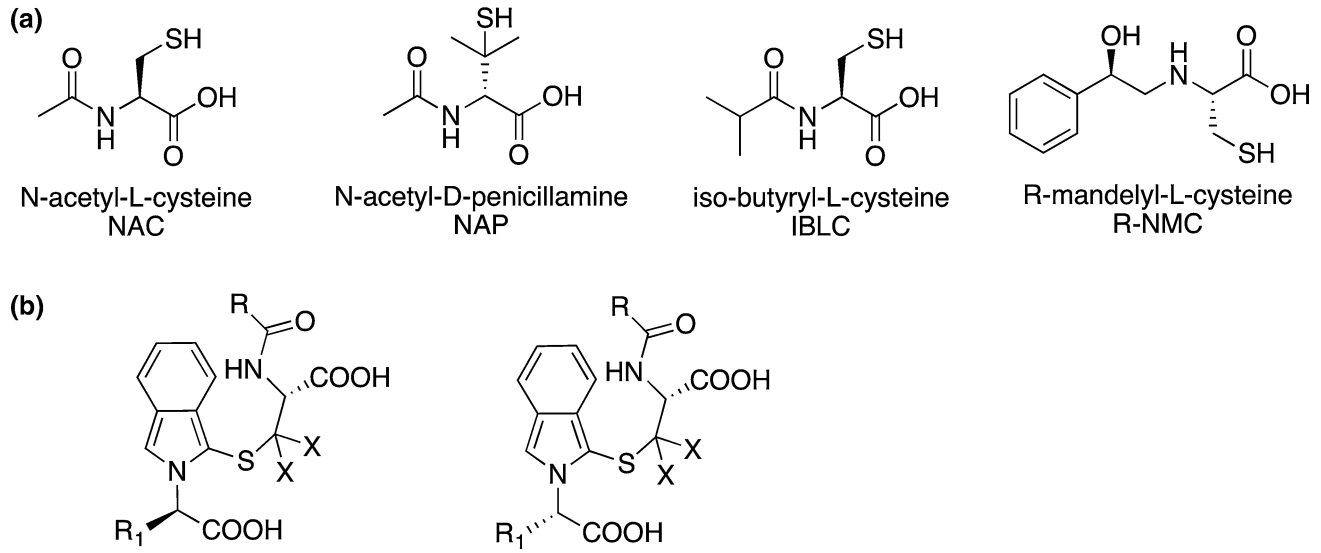


Fig. 9 **a** Structure of chiral thiols: traditionally used enantiomeric NAC, NAP, IBLC, and novel diastereomeric *R*-NMC. **b** Structure of the stereoisomeric isoindoles formed after derivatization of α-amino acids with OPA and a chiral thiol. Substituent X is CH_3 for NAP adducts, and H for the rest

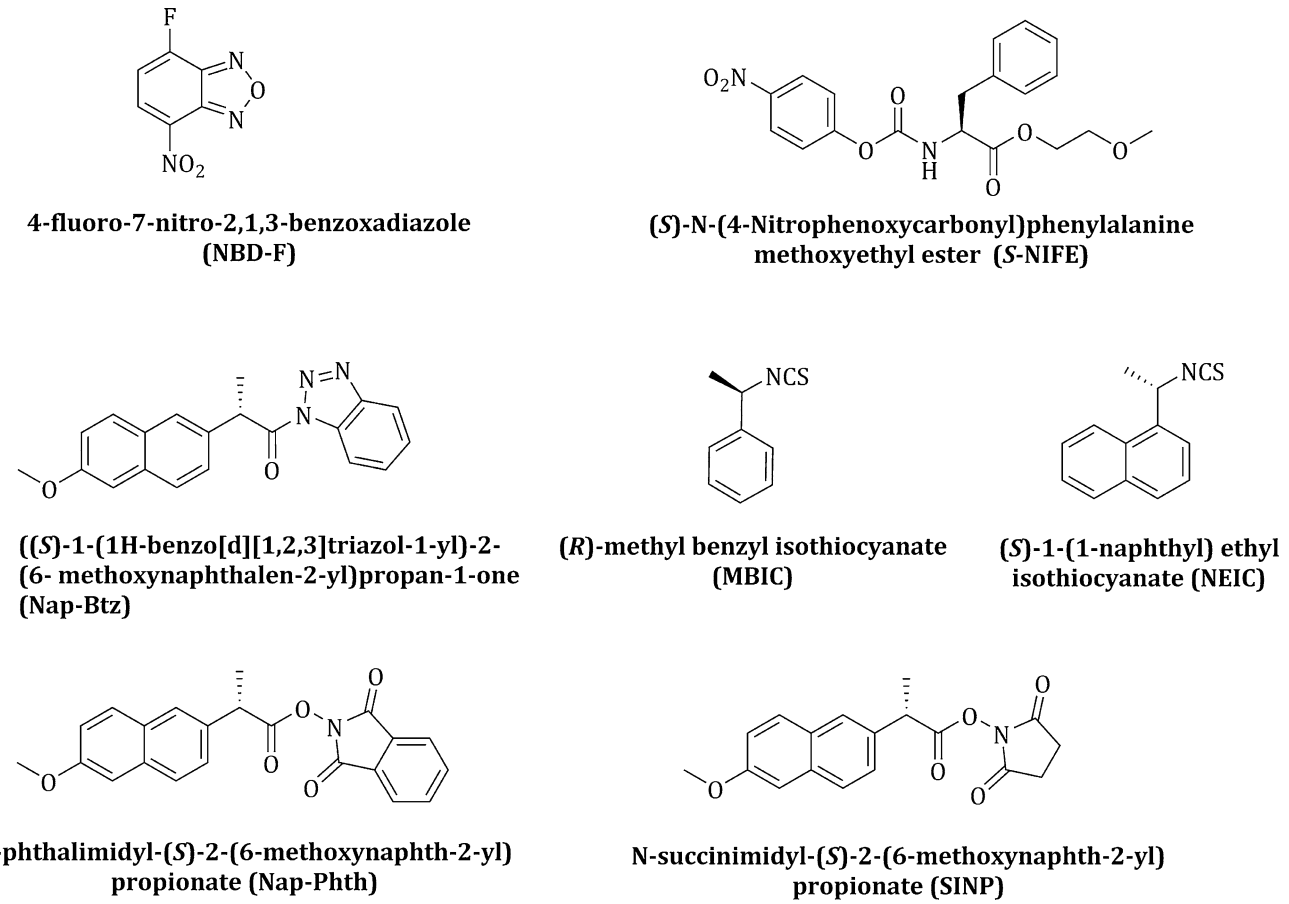


Fig. 10 Structures of other CDRs discussed under this review

is not brought into consideration in view of the prevalent concepts of enantiomers, diastereomers, racemic and non-racemic samples. Nevertheless, there are sporadic reports on enantioseparation of certain non-racemic mixtures in an achiral environment particularly from the people working in analytical chemistry; the sporadic reports till 1992 on such liquid chromatographic separations were presented as a short review [189]. Since then there appeared a few more reports on enantioresolution of certain non-racemic mixtures in achiral environment. The basic question and the concept 'why separation of one particular enantiomer (i.e., enantiomeric enrichment) should take place under achiral phase chromatography from the non-racemic mixture' has been discussed briefly with the possible mechanism of separation for enantioselective effects along with the technical terms used in literature and application of scientific terminology in the bounds of IUPAC [190]. The achiral phase liquid chromatography can be used as a general method for enantiomeric purification of non-racemic mixtures of liquids as well, since it provides high yields of enantiomerically pure compound (at the laboratory scale, currently).

Conclusion

AA analysis is required not only for determination of AA composition of protein/peptide hydrolysate, but it is much more important for simultaneous analysis and enantioresolution of AAs and determination of D-amino acid in excess of L-isomer. Most of the AAs lack a chromophore; therefore, derivatization methods are particularly required for their detection. Different methods and reagents were introduced for AA analyses, many of these (e.g., (+)-1-(9-fluorenyl) ethyl chloroformate, and OPA) are very good and are still successfully used in GC-, LC- and CE- applications. It is interesting to note from the literature that enantiomerically pure L-amino acids have been used as chiral auxiliaries in CC and DFDNB and the chiral derivatizing reagents (CDRs) so obtained were successful in resolving enantiomers from DL-mixtures of amino acids. In some cases, simultaneous analysis and enantioresolution of AAs has resulted in LOD and LOQ values, respectively, in the range of 50 and 150 pmol. Most of these reagents have been prepared easily in micromolar quantities, in laboratory. DFDNB-based CDRs are light sensitive and the whole analysis is protected from light. HPLC resolution of a mixture of 36 diastereomers (corresponding to 18 DL-AAs) in a single chromatographic run of 65 min has been successful. The work has proved to be useful to determine the content of D amino acids (up to 0.001 %) in excess of L-amino acids and to determine D-amino acids in absence of pure D-AA enantiomers.

Structural features of different AAs used as chiral auxiliaries (in CDRs) provided a flexibility to change the hydrophobicity of the reagent and enhanced possibility of resolution of a variety of racemates in different situations. Moreover, CDRs containing opposite enantiomers (e.g., FDNP-L-Ala and FDNP-D-Ala) have also been easily prepared and used to compare the effect of D-, and L-configurational moieties on enantiomeric resolution. Recent reports on syntheses of CC-based new CDRs, together with the data and features of reagents, are expected to stimulate further development and exploration to meet the quest for new suitable chiral selectors for resolution of various enantiomeric/racemic mixtures. These would increase sensitivity and selectivity of detection by incorporating groups with high molar extinction coefficients, luminophores, near-infrared chromophores, or laser-excitable fluorescent groups yielding high quantum yields.

TLC separation of enantiomers of 17 DL-amino acids on reversed-phase and homemade normal phase TLC plates has been successful as their derivatives prepared with FDNP-L-Phe-NH_2 and FDNP-L-Val-NH_2. TLC could be simpler (economical, fast, reliable, and semi-quantitative) for use in analytical research and in routine QC of pharmaceuticals and for direct resolution and analytical control of enantiomeric purity. It can be safely contented that there is still no match of Chiralplate® for direct enantioresolution of a variety of racemates. Yet the chiral TLC is currently underestimated as a tool for enantiomer analysis and separations. The commercial precoated TLC plates are considered to have better standardized layer of adsorbent. But they are expensive. The use of homemade plates is quite common for routine works. Comparison and publication of results of various enantiomeric resolutions with both types of plates from this laboratory has not shown any difference in terms of chromatographic considerations.

Direct enantioseparation by HPLC or CE has largely been dependent upon use of CSPs based on CDs, macrocyclic antibiotics, glycoproteins, crown ethers, cellulose and amylose and cinchona alkaloids while in TLC these chiral selectors have been used either as impregnating reagents or as mobile phase additives. There have been different explanations and theoretical models for enantioselectivity. These have not been included in this paper mainly because the focus of this report is AAs and none of these models is specifically discussed or established for enantioseparation of AAs.

Chiral columns are expensive and usually high amounts of HPLC grade solvents are required for elution of the desired compound from such columns. By no means is a competition advocated between HPLC and TLC. Both techniques are complementary rather than competing. Further investigations for the development of more facile and

simple separation techniques for enantiomeric resolution are still required. Development of novel techniques that do not require any outside chiral selector (or auxiliary) for separation of non-racemic mixtures should become more relevant and important both in academia and pharmaceutical industry. The homochirality of AAs in organisms, the so-called L-amino acid world, is likely to be established being accompanied by evolution of life on earth.

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