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Recent developments in separation methods for enantiomeric ratio determination of amino acids specifically involved in cataract and Alzheimer's disease.

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Abstract

The racemization of amino acids provides another example of how the analytical sciences sit at the confluence of several disciplines. Over the past 60 years a quantity of articles has been published on amino acid racemization in various fields such as anthropology, geology, paleontology, archaeology, history, forensic science, medicine, pharmaceutical analysis and food science. The degree of *postmortem* racemization of amino acids from their L- to their D-configuration, for example, has long been used in the relative and quantitative dating of long-lived proteins which link archaeology and bioanalysis. Since the 1970s, the presence of D-amino acids in these long-lived proteins has also been reported to be involved in aging-related diseases. Since then, a large number of articles have published with a constant improvement of the analytical methods culminating in their chiral separation and this review will focus on analysis of amino acid racemization in Alzheimer's disease and cataracts published between 2015 and 2020. The reader will herein find a review, including articles older than 2015 when of utmost importance, of analytical approaches toward explaining the involvement of amino acid racemization in Alzheimer's disease and cataract. A second section is devoted to chiral separation methods: "indirect" involving formation of covalent diastereoisomers, through the use of a chiral derivatizing agent (CDA) and "direct" involving transient formation of diastereoisomers. Methods employing a chiral stationary phase (CSP) are presented and these CSPs extensively described. Capillary electrophoretic methods and multidimensional liquid and supercritical fluid chromatography are also reviewed. A third section is devoted to the recent application of these methods for determination of the D/L ratio, including studies where the localization of racemization in specific peptides has been studied: various biological samples will be reviewed, with sections on sample preparation and application of these methods to Alzheimer's disease and to studies of cataracts. A brief conclusion will summarize the main points and future trends of this topic.

1. Introduction

Human lifespan is increasing, and there is a concurrent increase in the incidence of aging-related diseases such as senile cataract, dementia, Parkinson's and Alzheimer's diseases. While surgical intervention to remove cataracts is well established, no really effective treatment is currently available for neurodegenerative diseases and a better understanding of their physiopathology is constantly being sought. One emerging research topic concerns amino acids, in particular their isomeric forms, and it is a theme that has been gaining momentum since publications in the 1970s reported that, as well as the L-amino acids thought to be essential for life, D-amino acids also exist.

There are 20 so-called proteinogenic amino acids which make up proteins (Figure 1), nine of which are labelled as "essential" for humans as they cannot be synthesized by the body. Many amino acids, both proteinogenic and non-proteinogenic, have key biological functions and some may be further considered essential in certain pathologies or at different ages. They all include a carboxyl group and an amino group, both bonded to the α -carbon atom neighboring the carboxyl group, while this α -carbon is also linked to a hydrogen atom and to a side group making the generic amino acid structure chiral (excepted glycine where the side group is also a hydrogen atom). The different side groups for each amino acid lead to differing characteristics including acidity, basicity, hydrophobicity or hydrophilicity. The amino acids that constitute proteins may undergo several post-translational modifications, among which the conversion from their (left-handed) L- configuration to their (right-handed) D- form has been reported.

Over the last decade or so, it has become increasingly accepted that D-amino acids may be implicated in age-related diseases, and evaluation of this D/L enantiomeric ratio and localization of this stereo-inversion in the protein have become more important. In this review, covering the past five years, we focus upon Alzheimer's disease, in which D-aspartic acid and D-serine amino

acids play a pivotal role, and senile cataract. A **brief** description of the two pathologies will be presented, then the current analytical methods available for the chiral separation of amino acids will be presented. A third section will be devoted to recent applications of those analytical methods for enantioseparation of various amino acids in biological matrices in direct relation to cataract and Alzheimer's disease. The intention is not to be exhaustive, but rather to present the key milestones in each topic as a primer for those interested in this rapidly growing field.

2. Biological relevance of D-amino acids in ageing-related diseases

2.1 Alzheimer's physiopathology

Alzheimer disease (AD), which represents the majority of dementia cases, is a neurodegenerative brain disorder resulting in multiple cognitive impairments, and its incidence and prevalence increase significantly with age [1]. Extracellular accumulation and deposition of β -amyloid peptides ($A\beta$ – a product of a type 1 transmembrane protein (APP) processing), as well as the abnormal aggregation and phosphorylation of Tau proteins (known to promote the polymerization and stability of microtubules), are currently considered to be the main neuronal dysfunction and degeneration processes which contribute to this neuropathology [2].

The native $A\beta$ has three L-configuration aspartic acids (L-Asp) residues at positions 1, 7 and 23 and the D form of these 3 residues has been detected in plaques from the brains of senile patients with AD [3]. Recently, Lambeth *et al* [4], suggested that epimerization or isomerization of $A\beta$ -Asp residues prevents proteolysis at the site of modification, leading to accumulation of failed lysosomal bodies within cells and neurodegeneration. Several studies have also reported the presence of D-Ser at position 26 in $A\beta$ in the senile plaques from AD brains, and observed that this isomerization renders the protein more soluble and susceptible to proteolysis releasing toxic D-Ser₂₆ $A\beta$ (25-35) fragments both *in vitro* and *in vivo* [5].

In Tau peptide isoforms, studies of D-isomerization at various Asp or Ser positions have revealed that the impact of isomerization of certain amino acids on the transition (or not) from

random coils to β -sheets and the formation of fibrils by Tau isoforms has been suggested to play a crucial role in the onset of AD [6].

Early-stage Alzheimer's disease is characterized by a reduction in energy metabolism, and a concomitant reduction in the production of L-serine, an amino acid produced largely by astrocytes in the brain, and whose biosynthesis pathway is altered in AD. L-serine is the precursor of D-serine, known to stimulate *N*-methyl-d-aspartate receptor (NMDAR), which is essential for proper brain function and memory. Activation of NMDAR requires the concomitant binding of two ligands, glutamate at the glutamate binding site and a co-agonist, glycine or D-serine, at the glycine binding site. Reduced production of L-serine by astrocytes causes a decrease in the activation of NMDAR, which in turn leads to an alteration in neuronal plasticity and associated memory capacities [7]. However, as levels of soluble A β (amyloid- β oligomers) increase, the activity of astrocytes increases, leading to increased expression of serine racemase and greater release of D-ser: NMDAR activation by D-ser and related excito-toxic events leading to synaptic dysfunction have also been associated with AD pathology [8, 9].

2.2 Cataract physiopathology

Cataract is caused by the opacification of the lens and can be considered as an age-related disease.

The mechanism involved in the development of cataracts is not well characterized. Many authors have hypothesized and demonstrated that the water-soluble structural proteins present in the lens and cornea of the eye (α -, β - and γ -crystallins), whose main function is to focus light on the retina using the high refractive index of the cells in the lens fibers, aggregate abnormally [10].

The constant evolution of analytical methods has made it possible, particularly in recent years, to better characterize the racemized amino acids in crystallins, to quantify each enantiomer and isomer, and to better understand the impact of post-translational modifications on the structural modifications of these proteins and their role in the etiology of cataracts. Today, it is well

established that the amino acids most susceptible to spontaneous isomerization are aspartic acid and serine, and that deamidation of asparagine residues, by generating aspartic acid, can also be accompanied by isomerization [11]. The main sites of specific isomerisations are reported to be Asp 58, 76, 84 and 151 in α A-crystallin [12, 13], Asp36, 62, 96, 109 in α B-crystallin [13, 14], Asp37 and 4 in β A3- and β B2 crystallin, respectively, and Ser59, 62 and 162 in α A-crystallin [14, 15]. Surface-exposed Asn76 and 143 are also reported to be heavily modified by deamidation in γ S-Crystallin [16]. These modifications are likely to lead to alterations in the structure of the crystallin subunits, leading to their aggregation [17, 18].

3. Current analytical methods for the separation of L- and D-amino acids

Among the different methods that have been developed to resolve racemic mixtures, chromatography is the most widely used technology. Broadly speaking, two approaches can be pursued, hereafter called “indirect” and “direct”. The first “indirect” approach forms true diastereoisomers through the derivatization of the compound of interest **with a chiral derivatizing agent (CDA)** and involves subsequent separation of the diastereoisomers in an achiral environment. The second “direct” approach forms transient diastereoisomers between the solute and the chiral stationary phase (CSP) or employs a chiral additive to the mobile phase (CAMP) in order to resolve the enantiomers in order to identify and, ideally, quantify them. The indirect approach involves, on the one hand a chiral agent, which must be of high enantiopurity, and on the other hand time spent in removing the derivatization agent so as to recover the original enantiomers. Despite being more time-consuming than the direct approach, the indirect one is the most widely used in recently published chiral separation of amino acids in biological samples (see section 4). **The methods presented in this section are summarized in Table 1.**

3.1 Indirect method in liquid chromatography for amino acids separation

For this method, involving sample derivatization by a chiral agent prior to an injection on an achiral column, several *pros* and *cons*, must be considered and they are summarized in Table 1.

The main derivatization reactions involving amino acids are the formation of carbamates, amides, ureas and thioureas moieties. Frequently used derivatizing agents **include**: activated carboxylic acids and their ester-type derivatives, acid chlorides, chloroformates, *N*-succinimidyl esters, chiral isocyanates and mainly isothiocyanates, *O*-phtalaldehyde (OPA) or analogs with chiral thiol and lastly *N*-haloarylamino acid derivatives (Marfey's reagent, which is widely used, belongs to this last class). For more insight into those derivatizing approaches, the reader is invited to consult a very comprehensive review by Ilisz *et al.* summarizing more than three hundred articles [19].

3.2 Direct methods for amino acids separation

3.2.1 Separation of transient diastereoisomers on CSPs in liquid and supercritical chromatography

As attested by several recently published reviews, interest in the enantioresolution of amino acids continues to grow [20-22]. The direct methods of separation employing CSPs can be divided into two broad categories: one implements the resolution of underivatized/free amino acids on a suitable chiral column, whereas the second consists of a derivatization with an achiral reagent before chiral separation. For a long time, SFC has remained in the shadow of HPLC, but it has benefited from the development of CSPs as most of them are used in both LC and in SFC (except ligand exchange-based CSPs) and, aided by recent technological improvements, SFC has begun to carve itself a niche in the field of chirality [23].

3.2.1.1 CSPs usually used for enantioresolution of underivatized amino acids

Macrocyclic glycopeptide-based CSPs were introduced by Armstrong and colleagues in 1994. Also called macrocyclic glycopeptide antibiotics, they display a wide variety of structural types (vancomycin, ristocetin A, teicoplanin) characterized by an interconnected, amino acid-based macrocycle containing aromatic rings. The recognition process is driven by different interactions such as ionic, dipole-dipole and π - π interactions, while hydrogen bonds and steric hindrance also

play a role [24]. Initially developed on 5 μm fully porous particles, from around 2015, these stationary phases have been commercialized on sub-2 μm particles and can nowadays be found (from Azyp) as 2.7 μm , superficially porous particles (NicoShell, VancoShell, TeicoShell and TagShell), purported to enable higher efficiency. **All the mobile phases used for this CSP are well suited for MS detection.** Sanchez-Hernandez *et al.* reported the enantiomeric recognition capability of a teicoplanin column for 3 proteinogenic aromatic amino acids *i.e.* phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr). This was achieved by SFC in 7 minutes. Thanks to the intrinsic properties of the mobile phase in SFC, the possibility of simultaneously separating the six enantiomers was investigated by coupling two columns in series. The first of these was the chiral teicoplanin and the second was an achiral diol column and UV detection at 200 and 220 nm [25]. In an HPLC approach already implemented for the analysis of free amino acids, a very recent paper by Mazzocanti *et al.* reports the enantioseparation of 31 N α -Fmoc amino acids of varying physico-chemical properties on a zwitterionic-teicoplanin CSP. Three different particle sizes were examined (1.9 μm fully porous, or 2 and 2.7 μm superficially porous) and UV detection at 214 nm was performed [26].

Crown ether-based CSPs are composed of a cavity of a specific size, with chiral structure elements of macrocyclic polyethers with multiple -O-C₂H₄- units. Enantiorecognition is achieved by the formation of a complex between this cavity and an ammonium ion derived from the primary amino moiety, functional group of the amino acid under acidic mobile phase conditions. This complex is based mainly on hydrogen bonds between the protons of the ammonium ion and the electron donor oxygen atom of the crown ether [27]. This coated CSP was developed in 1987 by Shinbo and co-workers, and was commercialized by the Daicel Corporation as Crownpak® CR (+) and Crownpak® CR (-). However, under more than 15% MeOH or when using different solvents, significant column bleeding was observed. **Thus, in 2014** the Daicel Corporation developed an immobilized version, marketed as Crownpak® CR-I (+) and Crownpak® CR-I (-).

Very recently, Miller and Yue published an original work using SFC to resolve 17 free amino acids on a Crownpak CR-I (+) column [28]. **Mass spectrometry detection was enabled by the use of acidic additives such as trifluoroacetic- (TFA) or formic acid (FA).** The benefits of these CSPs have also been reported in HPLC coupled to time-of-flight mass spectrometers [29].

Ligand exchange-based CSPs are the oldest models introduced by Davankov and Rhogozhin in 1971, but were not transposed to SFC. This chromatography is based on the reversible chelation of the enantiomers into the sphere of the central transition metal ion complexed with a chiral, chelating ligand. Resolution results from the difference of each diastereoisomeric chelate's thermodynamic stability. The central metal ion is usually a divalent transition metal ion (Cu^{2+} ; Zn^{2+} ; Ni^{2+} or Mn^{2+}) and chiral ligands include amino acid derivatives. In chromatography, the latter can be either fixed to a solid support or added to the mobile phase [30]. More recently, *Cinchona* alkaloid derivatives were used as chelating ligand. In a paper by Echevarria *et al.*, quinidine (also used in ion exchange-based CSPs) was added to the mobile phase in the presence of Co^{2+} and Cu^{2+} for the enantioseparation of amino acids [31]. **In this case, the mobile phase contained a certain amount of the metal ion in the form of a salt plus a buffer, making it unsuitable for LC-MS.**

Ion exchange chiral chromatography relies for its resolving power upon a complex formed *via* ionic interactions and additional hydrogen bonds or π - π interactions. Anion-exchange selectors are quinidine (QD) or quinine (QN) derived from *Cinchona* alkaloids. Cation-exchange selectors usually contain carboxylic or sulfonic acid moieties. Zwitterionic ion exchange-based CSPs combine both **cationic and anionic** selectors, and were pioneered by Lindner and colleagues in 2008. Again commercialized by the Daicel Corporation, where quinine is combined with (S,S)-*trans*-2-aminocyclohexanesulfonic acid for CHIRALPAK® QN-ZWIX(+) and quinidine combined with (R,R)-*trans*-2-aminocyclohexanesulfonic acid for CHIRALPAK® QD-ZWIX(-), with chiral selectors being immobilized on 3 μm silica-gel. These stationary phases are especially

appropriate for the enantioseparation of underivatized amino acids, and have been described in SFC with mass spectrometric detection by West *et al.* [32], but have been also described for N α -protected proteinogenic amino acids using SFC-UV detection [33], as well as in HPLC methods [34]. An interesting work comparing separation capabilities of CHIRALPAK[®] QN-ZWIX(+) and CHIRALPAK[®] QD-ZWIX(-) was recently performed on free- and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatized uncommon amino acids and their isobaric analogs, such as allo-threonine, homoserine, allo-isoleucine and homocysteine, using HPLC-ESI-QTOF MS [35].

In 2018, Lipka *et al.* reported the successful enantioseparation of 10 free amino acids on a Lux i-Cellulose-5 column based on cellulose (3,5-dichlorophenylcarbamate) CSP with evaporative light scattering detection [36] – these CSPs are not usually the primary choice for free amino acids enantioseparation.

3.2.1.2 CSPs used for enantioresolution of derivatized amino acids

It should be **born** in mind that a derivatization reaction occurring preferentially on the amino group of amino acid decreases the polarity of highly polar ampholytes, which become merely acidic species. This strategy can improve sensitivity of detection and increase the choice of usable CSPs.

Polysaccharide-based CSPs, made from naturally occurring polymers, have a long history, having been reported for the first time in 1939 when Henderson and Rule used disaccharide lactose as a chiral adsorbent in column chromatography. The enantiorecognition process between the stationary phase and the amino acid enantiomers is mediated by hydrogen bonds involving the NH- or CO- moieties of the carbamate linker, and additional π - π and Van der Waals interactions with the phenyl rings, and involves inclusion of the enantiomers into the helicoidal three-dimensional structure of the stationary phase [37]. Polysaccharides, in particular amylose and cellulose coated onto silica gel, were introduced by Okamoto and coworkers in 1984 and

were commercialized by Daicel as Chiralcel® and Chiralpak®. Two main technological improvements have in recent years must be noted: the immobilization of the polysaccharide on the silica gel through covalent bonds, and the reduction of the particle size (to 2.5 μm in Trefoil AY1, CEL1, CEL2 phases commercialized by Waters in 2014, and more recently to 1.6 μm in the Chiralpak IA-u, IB-u, IC-u, ID-u, IG-u and IH-u phases from Daicel). **The mobile phases compatible with polysaccharide-based phases are well-suited for MS detection.** The separation of derivatized amino acids through polysaccharide-based CSP was reported by Dousa in 2019 [38]: twelve OPA-derivatized N-acetyl-DL-cysteine were completely enantioseparated on Chiralcel OZ-3R column, based on cellulose tris(3-chloro-4-methylphenylcarbamate), by HPLC with fluorescence detection.

Pirkle (or brush)-based CSPs belong to the π -electron acceptor/ π -electron donor category of chiral selectors. These phase were pioneered by Pirkle in 1975, who immobilized a (R)-2, 2, 2-trifluoro-1-(9-anthryl)ethanol on a silica support. These phases were the first CSPs to become commercially available in the early 1980s. The chiral selectors involved are small, chiral molecules enabling donor-acceptor interactions including hydrogen bonds, face-to-face or face-to-edge π - π interactions, or dipole stacking. Steric hindrance also plays a role in chiral recognition. It must be highlighted that these phases exist in both enantiomeric forms and the most popular Pirkle phases are (S, S)-Whelk-O1 and (R, R)-Whelk-O1, both based on 1-(3,5-dinitrobenzamido)-1,2,3,4,-tetrahydrophenanthrene [39]. The most recent technological improvement is the reduction of the fully porous particle size to 1.8 μm , commercially available from Regis® Technologies. **As for the previous CSPs, the mobile phases most-often used with Pirkle-based phases are compatible with MS detection.** The (R, R)-Whelk-O1 column was recently used for the enantioseparation of derivatized cysteine by HPLC with UV/vis detection [40].

Cyclofructan-based CSPs are cyclic oligosaccharides consisting of six, seven or eight β -2,1 linked d-fructofuranose units, hence they are named CF6, CF7 and CF8, respectively. Alkylation or aryl derivatization of the three -OH groups relieve strain inside the native cyclofructan units and makes the internal, crown ether-like structure accessible for the separation of primary amines, while native cyclofructan-based CSP has rather limited enantioselectivity because of these stable hydrogen bonds between the three -OH moieties which render the crown ether core largely inaccessible. These phases were invented by Armstrong and colleagues in 2009 and were made commercially available as LarihcShell-P from Azyp, who recently marketed a 2.7 μm superficially porous version of this CSP. This phase was reported by Lim *et al.* for chiral resolution of primary amines [41]. **It should be noted that these phases have also been successfully used in gas chromatography and with mass spectrometric detectors.**

Cyclodextrin-based CSPs are cyclic oligosaccharides composed of α -1,4-linked D-glucopyranose units (usually 6, 7 or 8 but larger rings containing 10 to 12 units have been also reported). The toroidal structure has a hydrophilic surface resulting from the hydroxyl groups in the 2, 3 and 6 positions, making them water soluble. The cavity is composed of the glucoside oxygens and methylene hydrogens, giving it an apolar character. As a consequence, cyclodextrins can include the apolar portion of a molecule of appropriate dimensions and bind them through dipole-dipole interactions, hydrogen bonding, or London dispersion forces. The -OH groups of the glucose units can be functionalized with various groups and numerous derivatized cyclodextrins are today used as chiral selectors. Developed in conjunction with Armstrong and introduced in 1983, this patented line of chiral stationary phases has the ability to form inclusion complexes through the interactions cited above, enabling numerous chiral separations by selectively preferentially including into the cyclodextrin cavity one of the two enantiomers. **The mobile phases used with cyclodextrin-based phases are well suited for MS detection.** A CYCLOBOND™ I 2000 HP-RSP column was tested to separate the enantiomers of

free tryptophan and its structurally related derivatives by HPLC with UV detection [42]. However, in this work several CSP types were compared: two polysaccharides (CHIRALPAK AD-RH and CHIRALPAK IB), two cyclodextran (Larihc CF7-DMP and Larihc CF6-RN) and two macrocyclic glycopeptide (CHIROBIOTIC™ T and CHIROBIOTIC™ T2), and this last was the most efficient towards these compounds. It can be seen that the spectrum of enantioselectivity is quite restricted for these CSP types, and as a consequence CD-based CSPs have recently diminished in popularity for liquid chromatography applications, despite remaining popular for capillary electrophoresis [43].

3.2.2 Separation of diastereoisomeric complexes by capillary electrophoresis

If, at the analytical level, liquid chromatography and, more recently, supercritical fluid chromatography have become very popular technologies, capillary methods are also potentially powerful. Indeed, Bernardo-Bermejo *et al.* published a review in 2020, dealing with the multiple advantages of those approaches, as attested by the distribution of these various analytical approaches among papers published between 2015 and 2019 for chiral separations: 63.2% in LC, 16.7% in CE; 12.7% in GC and lastly 7.4% in SFC [44].

As reported by recent **and** comprehensive reviews, capillary electrophoretic methods [45, 46] and capillary electromigration methods [47, 48] are particularly suitable for chiral separation of amino acids (usually derivatized), mainly because they are highly hydrophilic. Neutral cyclodextrins in CE were used in the chiral separation of LL- and DD-enantiomers of several small dipeptides, and among the three native CD and methyl- and hydroxypropyl-derivatives tested, the β -CD performed best [49]. A short word should be said, in the context of this review dedicated to the determination of D/L amino acid ratios, about the use of oligonucleotide sequences as chiral selectors in partial-filling CE for the enantioseparation of phenylalanine, tryptophan and tyrosine- and tryptophan-methylesters [50]. The reader is also directed to a very interesting review devoted to the contribution of capillary electrophoresis to neuroscience

(dealing with sample preparation and detection of neuroactive chiral compounds) by Sanchez-Lopez *et al.* in 2015 [51].

Capillary electrophoresis is a mature technique particularly suitable for amino acids analysis of limited sample sizes. Its main drawback, sensitivity, may now be overcome through the development of its hyphenation with mass spectrometry [52, 53]: this detection method will surely permit further development of capillary electrophoresis in the bioanalysis of complex matrices.

3.3 Multidimensional liquid and supercritical fluid chromatography for the separation of amino acids

In the context of quantification using mass spectrometry to enhance a technique's resolving power, a chiral separation step is still needed together with a chemical separation of each amino acid and 2D systems offer great flexibility. Firstly, a recent tutorial dealing with the principles of multidimensional chromatography is advisable for those readers unfamiliar with the technique [54]. Briefly, the four main different physical ways to implement 2D-LC are the following:

- i) heart-cutting 2D-LC (LC-LC) consists of a single fraction being injected onto the second dimensional column;
- ii) multiple heart-cutting (mLC-LC) where more than one fraction of the first dimension's column effluent is injected onto the second column;
- iii) comprehensive 2D-LC (LC×LC) is when the entire effluent of the first dimensional column is sequentially injected on to the second;
- iv) selective comprehensive 2D-LC (sLC×LC) consists of selected fractions from the first dimensional column being comprehensively injected onto the second.

Ali *et al.* [55] recently summarized the advances in chiral multidimensional liquid, gas and supercritical fluid chromatography in which several articles are devoted to separation of derivatized amino acids. Two dimensional-SFC reported in this review combined reversed-phase

LC (achiral C18 column) and chiral SFC in the first and second dimensions, respectively. Going further in multidimensional chromatography, a 3D-LC method was recently reported by Furusho *et al.* for determination of Asn, Ser, Ala and Pro racemization in biological matrices [56].

A two-dimensional approach for the identification of peptide isomers is detailed in section 4.2. Briefly, collision-induced dissociation is generally used as a first dimension to fragment ions, followed by a separation of the fragments using ion mobility spectrometry [57]. Ion mobility spectrometry coupled with mass spectrometry has recently gained in popularity for peptide analysis, and the reader is invited to consult a recent review for insight on the topic [58].

4. Recent applications of the determination of enantiomeric ratios

In this section the applications found in the review of the literature between 2015 and 2020 are presented and summarized in Table 2.

4.1 Biological matrices related to Alzheimer's diseases

Though the composition of amyloid- β (A β) plaques in human brains have been studied since the 1970s, and the amino acids in A β were sequenced over thirty years ago, several recent publications have investigated the enantiomeric ratios of D- and L-amino acids in relation to Alzheimer's disease. Using relatively conventional derivatization and reversed-phase chromatography with fluorometric or mass spectrometric detection, or else exploiting ion mobility technologies to resolve these epimers, the following publications all sought to improve our understanding of AD and its clinical management. Lin and co-workers investigated the roles of D-glutamate, D-serine and D-alanine in cognitive decline among over 140 Alzheimer's disease patients or patients with mild cognitive impairment [59]. They used HPLC with fluorescence detection to examine these three D-substituted amino acids in serum, extracting the serum with methanol before derivatization with a N-isobutyl-L-cysteine (IBC) and *O*-phthalaldehyde (OPA) mixture. While the relatively long run time (*ca.* 1h) may preclude its widespread adoption, their approach nevertheless achieved excellent separation of the D- and L-

substituted epimers of the three amino acids. They reported significant associations between low serum D-glutamate levels and impairment in naming objects, following commands and comprehension; conversely, higher levels of both D-serine and D-alanine were respectively associated with worse cognitive function and more behavioral symptoms.

In a comprehensive report, Inoue and colleagues [60] built on work from the 1990s suggesting that AD (and indeed Down's syndrome) was characterized by post-translational racemization (AAR) and isomerization (AAI) of amino acids in brain tissue. They developed a UPLC-MS/MS method to examine AAR and AAI in the N-terminal A β (1-5) peptide sequence from frontal lobe tissue in AD and control subjects, measuring D- and L-aspartic and iso-aspartic acids. Their approach employed tryptic digestion to isolate the N-terminal A β (1-5) sequence (Figure 2) and a covalent chiral derivatization (CCD) upstream of the LC analysis. The CCD procedure was performed with (R)-(-)-4-(N, N-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(R)-DBD-Py-NCS]. Subsequent chromatographic separation was performed on a conventional reversed-phase column (Waters Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 μ m) with tandem mass spectrometric detection. They reported excellent recoveries (>99 %) and found significantly higher concentrations of all four molecules in AD patients, as well as compared with controls.

Xing and colleagues [61] compared 18 D-amino acids in plasma from an AD rat model and age-matched controls. Derivatization of plasma proteins with (S)-N-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(S)-NIFE] preceded reversed-phase chromatographic separation on an Acquity UPLC BEH C18 column and detection with tandem mass spectrometry. This rigorously developed UPLC-MS/MS method had limits of quantification in the range 0.05 – 40 ng/mL, excellent precision and recoveries, and showed significantly lower concentrations of D-serine, D-aspartate, D-alanine, D-leucine and D-proline in AD rats' than controls' plasma. In further methodical work from this group, Li and colleagues report the development of UPLC-

MS/MS method for the simultaneous quantification of 11 D-amino acids in different regions of rat brains [62]. After protein precipitation, (S)-NIFE derivatization was again employed and the same reversed phase LC approach used to obtain baseline separation of the 11 epimer pairs, with lower limits of quantification in the range 0.06 – 10 ng/mL. Compared with controls, the concentrations of D-serine, D-alanine, D-leucine and D-proline were significantly lower in the hippocampus and cerebral cortex of a rat model of Alzheimer's disease, while no difference was observed in the concentrations of the 11 D-substituted amino acids in the cerebellum or olfactory bulb.

While there is an apparent contradiction between these reports from the same laboratory with respect to D-serine, it should be noted that the Xing *et al.* study was concerned with absolute concentrations in rodents' plasma while Lin *et al.* compared serum concentrations among humans with different degrees of cognitive impairment. The method showed good precision and recoveries, and was sufficiently sensitive to be successfully applied to plasma and tissue samples from rats.

Significant work in this field derives from studies which sought to enhance minute differences in ion mobility between D- and L- amino acids in order to create extra dimensions for their separation. While standard proteomics approaches cannot distinguish between D- or L- substituted amino acids, since their elemental composition and masses are identical, elegant exploitation of different charge states and the use of metalation have proved fruitful in resolving D- and L-amino acid peptides.

In a methodological study on the same A β amino acid epimers as Inoue *et al.* (2014) above, but this time in the A β (6-16) peptide sequence, Zheng *et al.* developed an IMS-MS method for their analysis [63]. They sought to improve the resolution of their IMS through the use of structures for lossless ion manipulations technology (SLIM), increasing the IMS path length, ion selection efficiency and trapping and accumulation capacity to enhance both separation and sensitivity.

Nagy and co-workers [64] furthered this SLIM-IMS-MS method, and they also employed metalation in a complimentary approach to that of Fouque and colleagues' (below), generating sodium adduct ions of the A β epimers to achieve baseline resolution of four sets of A β tryptic peptides from the A β (17-28) peptide sequence. The SLIM-IMS-MS method was augmented by a serpentine ultralong path with extended routing (SUPER) to achieve enhanced separation and ca.4-fold greater sensitivity, the very long IMS path length (99m) being key in being able to separate these epimeric mixtures.

Fouque and colleagues [65] used trapped ion mobility spectrometry (TIMS) and metalation to enhance tiny differences in the ion mobilities of D- and L-amino acids for their separation and quantification by time of flight mass spectrometry. They confirmed the utility of their approach for the analysis of ten epimer pairs across different IMS platforms, comparing different ion sources and investigating the use of travelling wave IMS, and underlining the advantage of online mobility separated collision-induced dissociation (CID) in conjunction with high resolution mass spectrometry (TIMS-CID-MS). They point out that real samples present real analytical challenges, with sometimes grossly unequal epimer mixtures compared to the 1:1 mixtures used in methodological studies. They comment that different metal ions may improve resolution and predict that the integration of electron transfer dissociation to the TIMS-TOF platform may further enhance epimer discrimination.

4.2 Biological matrices related to cataract physiopathology

In the case of cataracts, research is understandably focused on lens proteins, and in particular on the isomerization of L-Asp since, as described above, it is thought to be responsible for aggregation of proteins and subsequent loss of transparency of the cornea. Two main goals have been widely pursued: the quantification of the four Asp isomers L- α , L- β , D- α and D- β , and their localization in the protein sequence through the study of its constitutive peptides. **The**

methods developed until 2015 were time-consuming and involved multiple steps, but the process has been streamlined and is now broadly as follows:

- i) human lenses are solubilized in 2-mercaptoethanol/Tris HCl buffer (pH 7.4) solution then sonicated and homogenized before being centrifuged;
- ii) the supernatant is subjected to size exclusion chromatography to separate α , β and γ -crystallin proteins and the desired protein collected;
- iii) the sub-units of the protein (for instance α A and α B) are separated by reversed-phase chromatography on a short-chain apolar column (C4 for example) and collected;
- iv) the chosen protein's sub-unit of interest (for instance α A, α B or β B2 – the most-studied to date) are digested using a trypsin enzyme (known to hydrolyze peptide bonds at the carboxylic side of basic amino acids such as Lys or Arg);
- v) the resulting tryptic peptides are injected on a C18 column with tandem mass spectrometric detection, where peptide mass ion intensity is measured through an ion trap system, leading to a “forest” of peaks eluting while 120 minutes.

Mass spectrometric analysis is conducted by alternating full MS and MS/MS scans (done in collision-induced dissociation), hence digested peptides can be separated and identified. The peptide of interest (containing one Asp residue at a known site) is then chosen. For aged-human crystallin, considering the peptide of interest, four peptides of same molecular weight containing L- α , L- β , D- α and D- β Asp isomers are expected. Each of these peptides would be expected to elute as one peak on a C18 column and to have the same mass. However, due to differences in the hydrophobicity of each peptide (L- α -Asp is the most hydrophobic, whereas D- α -Asp, L- β -Asp and D- β -Asp are more hydrophilic) this peptide elutes as four different peaks, once extracted from the tryptic peptides by mass range. Exact retention times of the four Asp-containing peptides were attributed by comparison with corresponding synthetic peptides, and the D/L ratio determined on the basis of the respective peak area (D- α + D- β *versus* L- α + L- β).

It must be noted that while this approach is quantitatively accurate, synthesizing the four standards peptides is time consuming.

This method was reported by Fuji *et al.* in 2015 for the quantitative analysis of isomeric aspartic residues in α A 55-65 TVLD⁵⁸SGISEVR peptide [66], and then in α A 146-157 IQTGLD¹⁵¹ATHAER peptide in 2016, but this paper is not covered herein. In 2018, the same team studied the whole β B2-crystallin sub-unit composed of 204 amino acids, including 11 Asp, in the lenses of 11-, 42-, 63- and 83-year-old individuals. After trypsin digestion, 24 peptides were obtained among which 6 contained one Asp residue. Two others peptides were found to contain 2 or 3 Asp residues and were therefore not pursued. The method was the same as previously described and each of the 6 peptides, in which L- α -Asp was replaced by D- α -Asp and L- β -Asp by D- β -Asp, were chemically synthesized and used as references to assign Asp isomers. However, separation of the four L- α -Asp, D- α -Asp, L- β -Asp and D- β -Asp containing peptides was not achieved on a C18 column for most of these peptides, making it impossible to determine their enantiomeric ratios. The tryptic peptides of β B2-crystallin from human lenses were thus subjected to preparative, reversed-phase chromatography with tandem mass spectrometry being used to identify the fractions of each target peptide: these were then pooled, concentrated and collected for derivatization into diastereoisomers with BOC-L-Cysteine-OPA. The D/L ratio of each Asp residue was subsequently calculated from the area of the diastereoisomer peaks determined by fluorescence detection. This method successfully determined the D/L ratio of the Asp residue in 6 peptides: MASD⁴HQTQAGKPQSLNPK; WD⁸³SWTSSR; RTD⁹²SLSSLRPIK; TD⁹²SLSSLRPIK; IRD¹⁹²MQWHQR and D¹⁹²MQWHQR. The racemization of Asp4 was found to increase in an age-dependent manner, which was not the case for the Asp83, Asp92 and Asp192 residues [67].

In 2020, Mizuno *et al.* reported the study of Asp isomerization in α -crystallin from bovine lenses. Six Asp-containing peptides from both sub-units (α -A and α -B) were again targeted. The 24

synthetic peptides were synthesized and used for method development and optimization of derivatization and mass spectrometry parameters. Instead of the previously described, reversed-phase chromatography for separation of the two sub-units, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used and the two resultant bands were excised and digested by trypsin. Each of the tryptic peptides was subsequently derivatized by three different derivatizing agents, and each peptide separated on a C18 column by UHPLC. As previously reported, the D/L ratio of each Asp-containing peptide was calculated both in unheated peptides and after 2, 7 and 14 days of heating them at 60°C. In α A-crystallin, four peptides were targeted: TVLD⁵⁸SGISEVR; FVIFLD⁷⁶VK; HFSPED⁸⁴LTVK; IPSGVD¹⁵¹AGHSER. Two further peptides were examined in α B-crystallin: APSWID⁶²TGLSEMR and VLGD⁹⁶VIEVHGK. The most racemized were Asp58 and Asp151 in the α A-crystallin. The originality of this work is in the use of three derivatization reagents tested, namely (R)-(-)-4-(N,N-dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-APy), (R)-(-)-4-(N,N-Dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-Py-NCS), and 2,5-Dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazine-2-yl)pyrrolidine-2-carboxylate (DMT-(R)-Pro-OSu). The first of these was used to derivatize carboxyl groups in the tryptic peptides (after protection of the amino groups), and the last two were used to derivatize the amino groups of the tryptic peptides. Derivatization generally has two goals where enantioseparation is concerned: to improve separation and sensitivity. From a separation point of view, the results obtained for the 72 derivatized peptides indicate that their separation using derivatization at the amino group is more complete than for peptides derivatized at the carboxylic group. Concerning the derivatization group itself, isomerized peptides were better separated by C18 column with a lower molecular weight, less hydrophobic reagent (better separation with (DMT-(R)-Pro-OSu) than (DBD-Py-NCS)). From a detection point of view, derivatized peptides exhibited better sensitivity than non-derivatized; in addition, the LOD and

LOQ were lower when using (DMT-(R)-Pro-OSu), indicating that the ionization efficiency of the derivatized peptide in ESI was increased by the high proton affinity of the triazine structure of (DMT-(R)-Pro-OSu) [68].

Also in 2020, Wu and Julian reported a two-dimensional approach for identification and localization of isomers of serine, aspartic acid and glutamic acid in human lenses. The first dimension was collision induced dissociation of the fragments, followed by ion mobility spectrometry coupled to mass spectrometry [57]. Three peptide sequences were studied: $^{57}\text{APSWFDTGLSEMR}^{69}$ from human αB -crystallin, $^{202}\text{GYQYLLEPGDFR}^{213}$ from βB1 -crystallin and $^{79}\text{HFSPEDLTVK}^{88}$ from human αA -crystallin. By comparing differences in either fragment-ion intensities or arrival-time distribution for each isomer, it was possible to identify isomeric forms and the specific site of modification of the peptides. Even if both dimensions are individually capable of identifying isomers, ion mobility remains key for determining the modification site.

5. Conclusion and future trends

Aging-related diseases represent a major public health challenge and, while by no means offering a panacea, accurate and efficient analysis of the racemization of L-amino acids to their D-enantiomers may at least enhance our **knowledge** of the physiopathology of some of these diseases. The specific functions of this isomerization remain to be fully elucidated, in spite of our deepening understanding provided by extensive studies focusing D-Ser and D-Asp, but it is recognized that they play a mechanistic role in age-related pathologies. D-Ser is a pivotal neuromodulator involved in neurodegenerative disorders such as Alzheimer's disease. The presence of D-Asp may change the highly-ordered structure of a protein, which in turn may play a role in the age-dependent aggregation of crystallins in cataracts. The isomerization of amino acids does not occur uniformly but happens at specific, susceptible residues defined by the peptide sequence or structural neighborhood. Thus, two main goals are being actively pursued to

improve our knowledge of these pathologies: i) racemization quantitation, and; ii) racemization localization in peptides. While a plethora of analytical methods for separating L- from D-amino acids in simple aqueous matrices exists, for separating them in biological samples the possibilities are more restricted and involve 1D- or 2D- reverse-phase LC with tandem mass spectrometry and, less often, capillary electrophoretic methods. Both use aqueous media (mobile phase or background electrolyte) and are thus well-suited for amino acid separation and quantitation, with or without a derivatization step. In the more complex case where the studied racemization occurs in a peptide sequence, LC with tandem mass spectrometry is usually employed after a derivatization step. **In addition, ion mobility spectrometry coupled with mass spectrometry has also gained in popularity and holds significant promise in advancing our understanding.**

Besides helping to unravel complex physiopathologies, the identification of biomarkers can also be considered as an area where analysis of amino acid racemization has a role in monitoring molecular mechanisms associated with disease etiology. In this context, metabolomics has great potential, concerned as it is with the non-targeted characterization of the entire set of metabolites from a biological sample, though it does require powerful analytical tools [69]. A recently published (2019) facile method for the preparation of chiral amino acid standards for enantioselective metabolomics is of interest in this context. It goes a considerable way to solving the problem of matrix effects which affect enantiomers unequally, and enables different racemization levels of several amino acids to be investigated with accuracy by mass spectrometry [70].

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Figure captions

Figure 1 : The 20 proteinogenic amino acids.

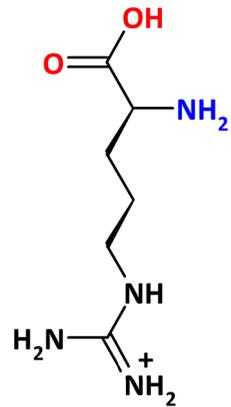
Figure 2 : Tryptic Digestion and Experimental Approach for the Post-Translational AAR and AAI Formation of N-Terminal A β Sequences.

Reprinted with permission from Simultaneous Determination of Post-Translational Racemization and Isomerization of N-Terminal Amyloid- β in Alzheimer's Brain Tissues by Covalent Chiral Derivatized Ultraperformance Liquid Chromatography Tandem Mass Spectrometry, Koichi Inoue, Daiju Hosaka, Nana Mochizuki, *et al* in *Analytical Chemistry* 86 (2014) 797-804
Copyright (2020) American Chemical Society.

A- Amino acids with electrically charged side chains (at physiological pH of 7.4)

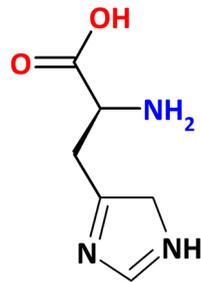
Arginine
(Arg)

R



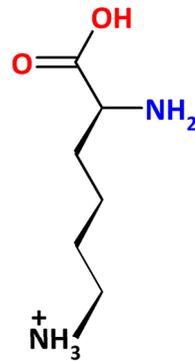
Histidine
(Hist)

H



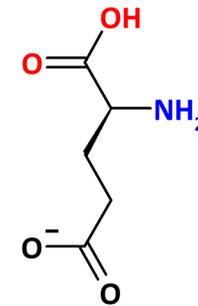
Lysine
(Lys)

K



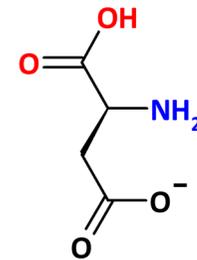
Aspartic acid
(Asp)

D



Glutamic acid
(Glu)

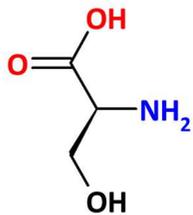
E



B- Amino acids with polar uncharged side chains

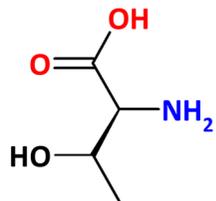
Serine
(Ser)

S



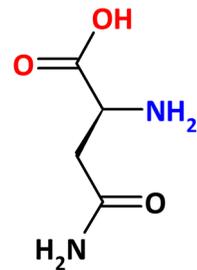
Threonine
(Thr)

T



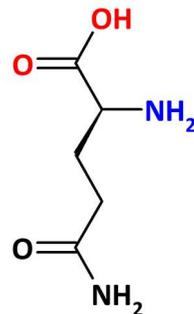
Asparagine
(Asn)

N



Glutamine
(Gln)

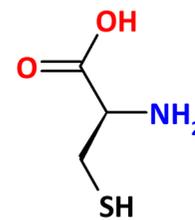
Q



C- Special cases

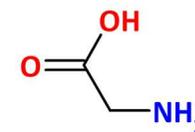
Cysteine
(Cys)

C



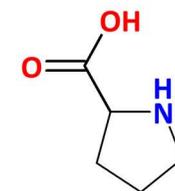
Glycine
(Gly)

G



Proline
(Pro)

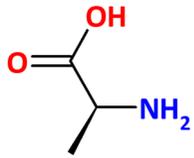
P



D- Amino acids with hydrophobic side chain

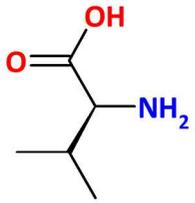
Alanine
(Ala)

A



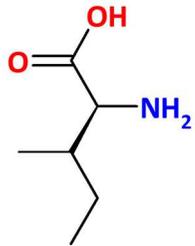
Valine
(Val)

V



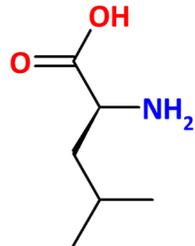
Isoleucine
(Ile)

I



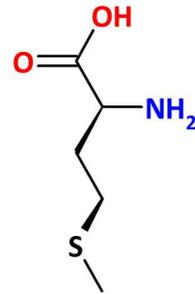
Leucine
(Leu)

L



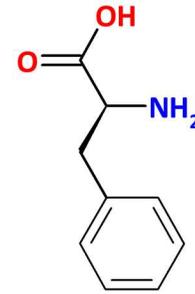
Methionine
(Met)

M



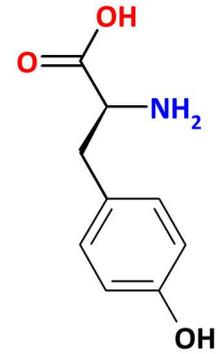
Phenylalanine
(Phe)

F



Tyrosine
(Tyr)

Y



Tryptophan
(Trp)

W

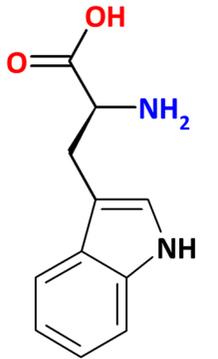


Figure 1

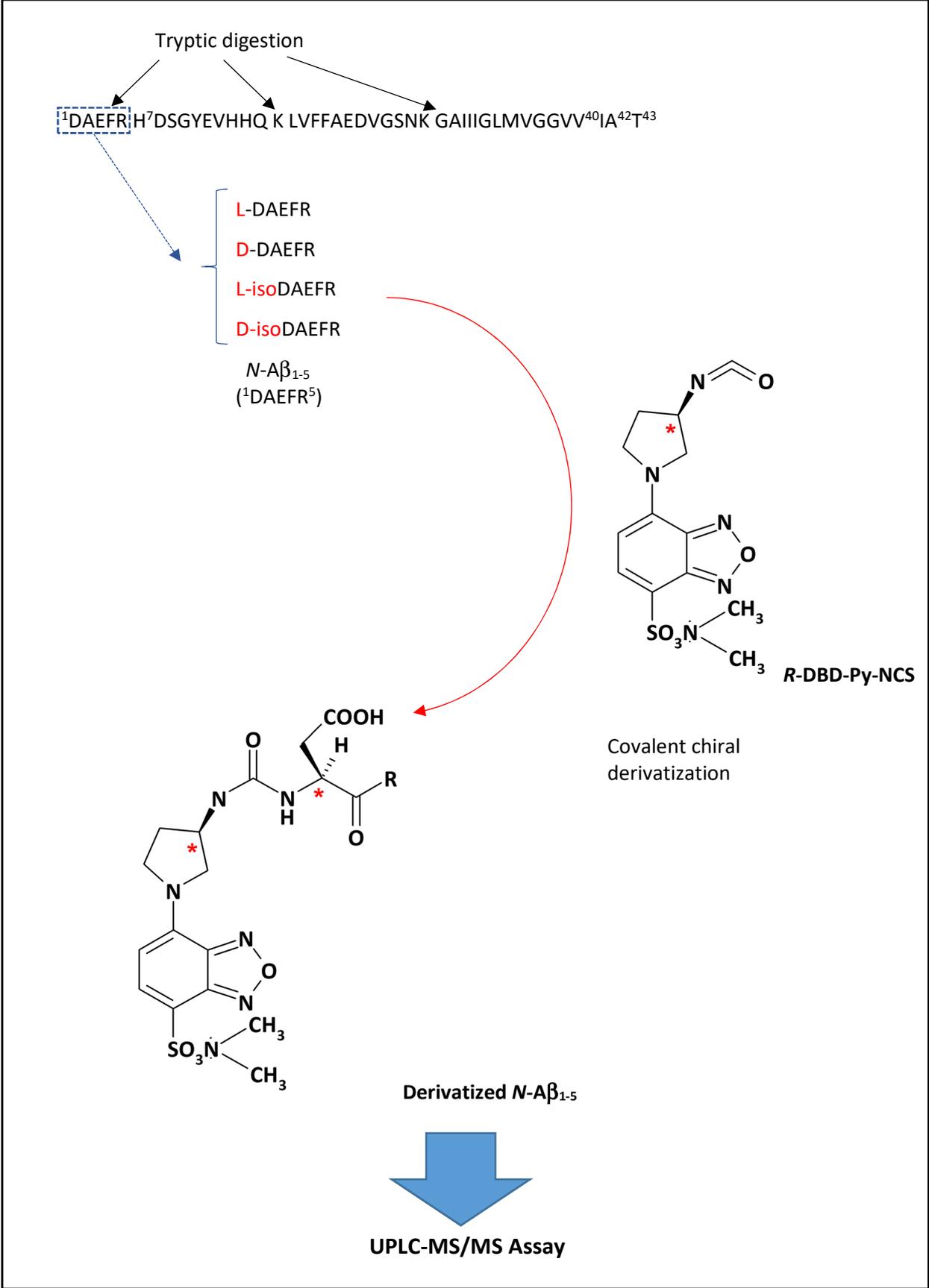


Figure 2

Table 1. Key features of the methods employed for chiral separation of amino acids

Indirect method – separation of true diastereoisomers on an achiral stationary phase			
Pros		Cons	
<ul style="list-style-type: none"> i) large number of chiral derivatizing agents (CDA) commercially available ii) CDAs sometimes available in both R and S configurations, enabling the analyst to reverse the elution to elute the minor enantiomeric impurity before the main peak in enantiomeric excess determination iii) CDAs are highly sensitive UV-visible or fluorescent tags suitable for trace analysis of amino acids in complex biological matrices 		<ul style="list-style-type: none"> i) both enantiomer and the CDA must possess derivatizable functional group ii) chiral derivatization reaction should proceed quantitatively for both enantiomers to avoid an analytical bias iii) no racemization should occur or overestimation of the minor enantiomer could occur (hydrolysis during sample treatment should be checked) iv) enantiomeric purity of the CDA must be higher than 99.9% to avoid supplementary enantiomeric excess leading to formation of additional stereoisomers which may co-elute with target peaks 	
<i>Guideline</i> : most widely used method, in particular for biological samples.			
Direct method – separation of transient diastereoisomers on a chiral stationary phase (CSPs)			
<i>For underivatized/free amino acids</i>			
Macrocyclic glycopeptide (<i>MS-compatible</i>)	Crown ether (<i>MS-compatible</i>)	Ligand exchange (<i>Poorly MS-compatible</i>)	Ion exchange (<i>MS-compatible</i>)
Pros		Cons	
<ul style="list-style-type: none"> i) partial racemization of CSP' chiral selector unlikely as long as chromatographic conditions not extreme ii) no reagent excess could interfere with the detection or worsen the chromatographic resolution in the analysis iii) scale-up for preparative applications possible (depending on the CSP) 		<ul style="list-style-type: none"> i) lower stability of the diastereomers ii) lower resolution iii) higher LOD in comparison with indirect method or direct method for tagged amino acids (below) 	
<i>Guideline</i> : method recommended for non-biological samples			
Direct method – separation of transient diastereoisomers on a CSP			
<i>For derivatized/tagged amino acids</i>			
Polysaccharide (<i>MS-compatible</i>)	Pirkle (<i>MS-compatible</i>)	Cyclofructan (<i>MS-compatible</i>)	Cyclodextrin (<i>MS-compatible</i>)
Pros		Cons	
<ul style="list-style-type: none"> i) highly available, free primary or secondary amino and carboxylic groups of amino acids can be targeted for labeling reactions ii) fluorenylmethylchloroformate (FMOC), t-butylloxycarbonyl (BOC), 9- and benzyloxycarbonyl (CBZ) are common derivatization agents iii) these groups permit induction of a fluorescence or a mass spectrometry signal iv) when subsequently cleaved-off under specific conditions, the peptide structure remains unaffected 		<ul style="list-style-type: none"> i) reagent excess could interfere with the detection or worsen the chromatographic resolution ii) partial racemization of the analyte could occur during derivatization reaction 	
<i>Guideline</i> : method recommended for biological samples thanks to mass spectrometry detection			
Direct method – separation of diastereoisomeric complexes			
<i>Guideline</i> : used for biological samples, ranked just after the Indirect method			

Table 2. Characteristics of methodologies developed for enantiomeric ratio determination in biological matrices

Biological matrices related to Alzheimer's disease					
<i>Amino acids</i>	<i>Matrix</i>	<i>Derivatizing agent</i>	<i>Method</i>	<i>Column</i>	<i>Reference</i>
D and L glutamate D and L serine D and L alanine	Serum	N-isobutyl-L-cysteine (IBC) and O-phthalaldehyde (OPA) mixture	HPLC/Fluo	Grom-Sil OPA-2 (4.0 x 250 mm ; 5µm)	[59]
D- and L-aspartic and iso-aspartic acids	Aβ (1-5) peptide in human brain tissue	(R)-(-)-4-(N, N-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole [(R)-DBD-Py-NCS]	UPLC-MS/MS	BEH C18 (2.1 x 100 mm, 1.7 µm)	[60]
18 D and L amino acids	Rat plasma	(S)-N-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(S)-NIFE]	UPLC-MS/MS	BEH C18 (2.1 x 50 mm, 1.7 µm)	[61]
11 D and L amino acids	Rat brain tissue	(S)-N-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(S)-NIFE]	UPLC-MS/MS	BEH C18 (2.1 x 50 mm, 1.7 µm)	[62]
<i>Ion mobility spectrometry</i>					
D- and L-aspartic and iso-aspartic acids	Aβ (6-16) peptide in human brain tissue		SLIM-IMS-MS		[63]
D- and L-aspartic and iso-aspartic acids	Aβ (17-28) peptide in human brain tissue		SLIM-IMS-MS		[64]
	<i>Various peptides :</i>		TIMS-CID-MS		[65]
D and L phenylalanine	acathin-I				
D and L arginine	dermorphin 1-4				
D and L alanine	deltorphan I				
D and L methionine	WKYMVM				
D and L serine and tyrosine	LHRH				
D and L tryptophan	γ-MSH				
D and L tryptophan	somatostatin-14				
D and L tyrosine	Tyr11-neurotensin				
D and L tryptophan	Trp11-neurotensin				
D and L alanine	GRF				

Biological matrices related to senile cataract

<i>Amino acids</i>	<i>Matrix</i>	<i>Derivatizing agent</i>	<i>Method</i>	<i>Column</i>	<i>Reference</i>
D- and L-aspartic and iso-aspartic acids	In human α A-crystallin : 55-65 TVLD ⁵⁸ SGISEVR peptide	<i>N</i> -(<i>tert</i> -butyloxycarbonyl)-L-cysteine (BOC) and <i>O</i> -phthaldialdehyde (OPA) mixture	LC/MS	Novapack ODS (3.9 x 300 mm ; 4 μ m)	[66]
D- and L-aspartic and iso-aspartic acids	In human β B2-crystallin : MASD ⁴ HQTQAGKPKQSLNPK WD ⁸³ SWTSSR RTD ⁹² SLSSLRPIK TD ⁹² SLSSLRPIK IRD ¹⁹² MQWHQR D ¹⁹² MQWHQR peptides	<i>N</i> -(<i>tert</i> -butyloxycarbonyl)-L-cysteine (BOC) and <i>O</i> -phthaldialdehyde (OPA) mixture	HPLC/Fluo	Novapack ODS (3.9 x 300 mm ; 4 μ m)	[67]
D- and L-aspartic and iso-aspartic acids	In bovine α A-crystallin : TVLD ⁵⁸ SGISEVR FVIFLD ⁷⁶ VK HFSPED ⁸⁴ LTVK IPSGVD ¹⁵¹ AGHSER peptides In bovine α B-crystallin : APSWID ⁶² TGLSEMR VLGD ⁹⁶ VIEVHGK peptides	2,5-Dioxopyrrolidin-1-yl-1-(4,6- dimethoxy-1,3,5-triazine-2-yl) pyrrolidine- 2-carboxylate [DMT-(R)-Pro-OSu]	UPLC/MS/MS	BEH C18 (2.1 x 150 mm, 1.7 μ m)	[68]
<i>Ion mobility spectrometry</i>					
D- and L-aspartic and iso-aspartic acids D- and L-serine D- and L-glutamic acid	In human α B-crystallin: ⁵⁷ APSWFDTGLSEMR ⁶⁹ in human β B1-crystallin: ²⁰² GYQYLLEPGDFR ²¹³ in human α A-crystallin: ⁷⁹ HFSPEDLTVK ⁸⁸ peptides		TWIM-MS		[57]