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Considerations for amino acid analysis by liquid chromatography-tandem mass spectrometry: A tutorial review

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1. Introduction to amino acids

1.1. Amino acid structure and classification

Amino acids are a class of organic compounds containing a primary amine (-NH_2) or secondary amine (-NH), a carboxyl (-COOH) group, along with a variable side chain (Fig. 1). An amino acid with a secondary amine such as proline is sometimes referred to as an imino acid, however this terminology has become redundant [1].

Despite being called acids, amino acids are amphoteric due to the functional groups being able to donate or accept a proton. This also means that amino acids, under certain conditions (such as at physiological pH) are capable of becoming zwitterions and are perhaps the most common example of zwitterions in literature [2]. Amino acids can be further categorised into subgroups according to different criteria including polarity, chirality, biological function, and the position of the amine group in the side chain [3–6]. The position of the amine functional group on the side chain, separates amino acids into alpha (α-), beta (β-), gamma (γ-), delta (δ-) and so on amino acids [4] (Fig. 2).

Based on chirality, amino acids can be divided into dextrorotatory (D) or levorotatory (L) enantiomers (Fig. 2); this results from the spatial arrangement of the atoms within the amino acid, and despite having almost identical structures, the enantiomers can have different biological functions [7,8]. Absolute configuration (R or S) is rarely used in amino acid nomenclature since most L-amino acids are in an S configuration. There are a few exceptions however, where an L-amino acid is in an R configuration depending on the side chain (e.g. L-cysteine is in an R configuration [9]). Amino acids can also be characterised on polarity and pH due to the variation in the side chain groups. Amino acids with amines in their side chain with high enough pKa's to accept a proton in a neutral/physiological pH environment can be categorised as basic amino acids. Similarly, acidic amino acids are amino acids with carboxyl groups in their side chain with low enough pKa's to allow them to donate a proton at neutral/physiological pH [6]. While typically considered small polar molecules, amino acids can also be categorised based on their polarity, where they are divided into non-polar (hydrophobic) or polar (hydrophilic) [6]. Other categorisation schemes based on side chain variation include aromatic amino acids, which have an aromatic ring in their side chain, aliphatic amino acids which have a straight side chain composed of carbon (with the exception of methionine which is sometimes classified as aliphatic), and branched chain amino acids, which have an aliphatic straight side chain with a branched carbon (Fig. 2) [4,6].

Biological function and dietary requirement are also used to classify amino acids. The major biological role for amino acids is their use in the synthesis of proteins, however, not all amino acids are used in protein synthesis. There are total of 22 protein amino acids (21 in eukaryotes, 22 in prokaryotes) which are genetically coded for ribosomal protein synthesis [10]. All protein amino acids are α-amino acids and L enantiomers (excluding glycine, which does not have enantiomers) but are mixed when it comes to polarity and pH. Protein amino acids can further be broken up into two categories, essential and non-essential amino acids, however, this is species specific. Essential amino acids are required by an organism to function correctly but are not synthesised by that organism and are thus required to be supplied from external sources. Non-essential amino acids are the opposite, being synthesised by the organism and not requiring external sources [3].

The number of amino acids used in protein synthesis is greatly exceeded by the number of non-protein amino acids present in nature. Non-protein amino acids (or non-canonical amino acids) are amino acids that are not encoded for in protein synthesis. There are estimated to be more than 500 non-protein amino acids, some of which are naturally occurring and are produced by a wide array of organisms for a multitude of purposes (see Section 1.2). Others have been engineered for biotechnological applications [11,12] and some result from chemical modification of protein amino acids through processes such as oxidation [13]. Despite being called non-protein amino acids, it is important to note that due to their ability to mimic protein amino acids some can be mistakenly incorporated into peptides and proteins through ribosomal protein synthesis [14,15], or through special mechanisms such as non-ribosomal peptide synthesis [16]. As all protein amino acids are L enantiomers, D-amino acids can also be categorised as non-protein amino acids. Post-translational modification of amino acid residues in proteins and peptides can result in additional amino acid residues in polypeptide chains, which, despite not being present as free amino acids initially, upon hydrolysis they can be released and will be analysed in a post-translationally modified form [17].

As might be expected, there is a lot of overlap with these classifications since the criteria for each is completely independent, and thus, the choice of classification scheme is typically based on the field of research.

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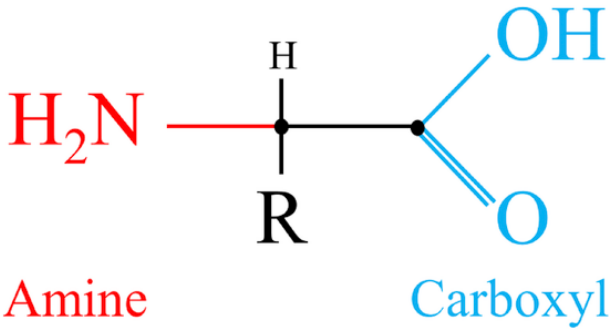

Fig. 1. General structure of α-amino acids with the R-group representing the variable side chain.

1.2. Importance of amino acids in research

Amino acids are sometimes referred to as the building blocks of life due to their important role in ribosomal protein synthesis. However their functions in biology are much more diverse [6]. Only 22 amino acids are used as building blocks of protein and many of these protein amino acids have secondary metabolic roles such as the role of methionine in cholesterol metabolism [18], glutamine's maintenance of pH homeostasis in the liver [19] and proline's role in modulating mitochondrial function [20]. Both non-protein and protein amino acids play important roles in human and animal metabolism; one of their most important roles is their function as neurotransmitters, examples include gamma-aminobutyric acid (GABA), glutamine and D-serine as GABA and N-methyl-D-aspartate (NMDA) receptors [21,22]. In addition, many are metabolic intermediates that are converted to other signalling molecules such as hormones [6]. Thus, because of these roles, amino acid levels in clinical samples can give indications of health status and can be used for the identification of diseases such as phenylketonuria [23]. Both non-protein and protein amino acids have been used as therapeutic agents. Levodopa (L-dopa) is a non-protein amino acid and a precursor to the neurotransmitter dopamine that is currently used for the symptomatic treatment of Parkinson's disease. L-dopa is used instead of dopamine as it is able to cross the blood brain barrier via amino acid transporters [14]. Other examples of potential therapeutic uses of amino acids include the use of L-theanine in schizophrenia [24] and L-serine as a treatment for motor neuron disease [25] and its possible protective role against a cyanobacterial neurotoxin [26]. Amino acids have also been engineered for biotechnological application, typically for use in therapeutic peptides [27].

One of the biggest areas of interest for amino acids is in dietary studies [28]. Amino acids are frequently sold as fitness supplements. These supplements can contain protein amino acids, sometimes selected protein amino acids most commonly the branched chain amino acids, and even some non-protein amino acids like norvaline [29]. The amino acid composition of animal feed is of huge importance to the agricultural industry to ensure that livestock receive the correct mixture of essential and non-essential amino acids for optimum growth and development [28].

Many non-protein amino acids produced by bacteria, algae and plants are known to evoke either acute or chronic toxic effects on other organisms [30,31]. The ability to synthesise these amino acids is believed to have evolved for allelopathy or aposematism. Acute toxicity of some of these non-protein amino acids like domoic acid have been associated with documented cases of sickness and fatalities in humans [32], whereas chronic toxicity caused by beta-methylamino-L-alanine (BMAA) and azetidine-2-carboxylic acid (Aze) are hypothesised to play a role in the epidemiology of certain diseases such as motor neuron disease and multiple sclerosis [33,34]. The analysis of non-protein amino acids can be complicated by the presence of isomers with similar physicochemical properties that are also present in the same sample matrices [35]. BMAA analysis, for example, is complicated by the presence of 3 isomers that can be produced by the same cyanobacterial species and are thus found together in environmental samples [35]. While this can be also be problematic for protein amino acids with proteogenic isomers such as leucine and isoleucine, it is more problematic with samples that contain a mixture of both non-protein and protein amino acids. These issues highlight the importance of the application of suitable analytical techniques to ensure correct identification. Due to this wide array of functions and importance to different fields of science, analysis of amino acids has become common practice in laboratories around the world. However, there are multiple methods of analysis available and each has advantages and disadvantages depending on the specific application.

1.3. Analysis of amino acids

Amino acids can be either free or protein or peptide-bound within samples. Free amino acids are amino acids that are in their native structure, while protein-bound amino acids are present in the polypeptide chain of peptides or proteins and are released via enzymatic diges-

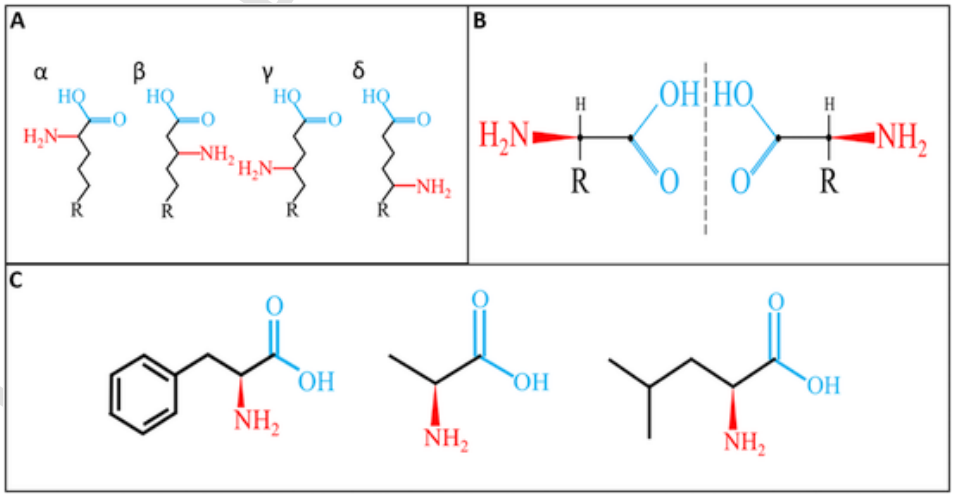

Fig. 2. Schematic diagrams of amino acids showing: (A) Alpha (α), beta (β), gamma (γ) and delta (δ) amino acids. (B) a comparison of amino acid enantiomers, D (left) and L (right). (C) from left to right, Phenylalanine, Alanine and Leucine, as examples of an aromatic, aliphatic straight chained and aliphatic branched chained amino acids.

tion [17] or chemical hydrolysis [36]. In some cases, amino acids released by hydrolysis could appear to be present within the polypeptide chain but might be strongly attached to a protein side chain [37].

Amino acids are often extracted from complex biological matrices containing multiple amino acids and often a variety of different metabolites, thus, some form of separation has to be applied before detection. Chromatographic separation methods are commonly used prior to amino acid analysis and these include thin-layer chromatography (TLC), liquid chromatography (LC) (including high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC)) and gas chromatography (GC). Non-chromatographic separation methods have also been used but less often, with the most prominent being capillary electrophoresis (CE). Detection methods include mass spectrometry (MS) and spectrophotometric detectors such as ultraviolet (UV), visible light (Vis) or fluorescence detection (FLD) [38,39]. There are two common approaches to analysing amino acids; they can either be identified in their native form, or in a derivatised form where chemical groups are added to assist in analysis [38]. Derivatisation can be carried out before (pre-column derivatisation) or after the chromatography prior to detection (post-column derivatisation) [38]. Chromatographic analysis of amino acids is complicated due to their polar nature requiring alternative chromatographic separation methods, most not being able to absorb light or have native fluorescence and being of low molecular weight. Derivatisation alleviates many of these issues.

TLC was traditionally used for the separation of native amino acids, requiring ninhydrin derivatisation for visualisation [39]. Aromatic amino acids can be detected using spectrophotometers, however, the remaining amino acids do not have the ability to absorb UV/Visible light nor have the capacity to fluoresce. Thus, when analysed via chromatography with spectrophotometric detection, most amino acids require derivatisation to make them detectable, with this being carried out either pre- or post-separation [38]. Unlike LC or GC, CE coupled with spectrophotometry doesn't require derivatisation to detect amino acids when the correct electrolyte solution is applied and a mix of direct and indirect absorbance detection is used, however derivatisation is still commonly applied [40,41]. Amino acid analysers (AAA) are another instrument used to analyse amino acids. These systems utilise ion-exchange liquid chromatography for separation with post-column derivatisation and spectrophotometric detection and quantification. Spectrophotometric detectors require complete chromatographic separation of analytes and this often require long run times.

Mass spectrometry has several advantages over spectrophotometry including higher sensitivity, selectivity, and throughput [42,43]. Further to this, tandem mass spectrometry adds another level of improvement in analyte identification through the fragmentation of molecules, resulting in higher specificity and reproducibility [42]. Spectrophotometry is unable to differentiate amino acids solely on detector signal thus strict chromatographic separation is essential to ensure the correct identification and quantification. Mass spectrometry, however, only requires amino acids of the same mass (constitutional isomers) to be baseline separated since overlap of amino acids with different masses does not affect qualification and quantification. Amino acid analysis via GC-MS and LC-MS yield comparable results but the use of LC-MS is more widespread then GC-MS due to GC-MS requiring more sample clean up steps prior to analysis [38,43]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection of amino acids for targeted and untargeted applications is preferred, especially with the rise in fields such as metabolomics. However, LC-MS/MS analysis of amino acids provides its own challenges due to their small size and zwitterionic nature. These challenges complicate all steps of method development from the extraction of the analytes, chromatographic separation, and MS detection. Depending on the purpose of the analysis these three factors might require special consideration. This tutorial review aims to be a guide and to provide an overview for scientists with an interest in amino acid analysis via LC-MS/MS. We outline the challenges and potential solutions associated with the three key steps in method development: sample preparation; chromatographic separation; and detection. Finally, we highlight current limitations and where these analyses may take us in the future.

2. Sample preparation

Amino acids are analysed in a variety of matrices including biological samples such as plant and animal tissues, microbial and cell cultures or clinical samples such as cerebrospinal fluid and plasma as well as environmental samples such as soils, sediments and marine and fresh water. The sample preparation will depend on the matrix and requires a number of considerations including accounting for extraction efficiency, extraction methods, hydrolysis of protein bound amino acids, and sample clean-up. For a general schematic of the steps involved in amino acid extraction, see Fig. 3.

2.1. Selection of internal standard

Selection of an appropriate internal standard (ISTD) is important as it can account for analyte loss during sample preparation and analysis (Fig. 3). ISTD addition before the sample extraction will account for losses during sample preparation if equilibration is achieved. ISTD addition after sample preparation normalises the analysis, accounting for changes in analyte behaviour due to matrix effects and alterations in instrument sensitivity and can be used for intra-run normalisation. Addition of an ISTD is essential for derivatisation, extraction or clean-up methods that might exacerbate analyte loss. Amino acid ISTDs are typically stable isotope labelled (SIL), using a deuterium or ^{13}C-containing analogue of the amino acid to be analysed. Non-protein amino acids such as norvaline and norleucine have also been used as ISTDs for protein amino acid methods [44]. While it is ideal to have an ISTD for every protein and non-protein amino acid analyte, a lack of commercial availability leads to custom orders that can be expensive and, as some amino acid analysis methods can consist of 20 or more analytes, it is not feasible to have an ISTD for every amino acid in these methods. Thus, most methods compromise, utilising one or a select few ISTDs to account for all amino acids being detected in the method [44].

2.2. Extraction of free amino acids and fraction separation

The method used to extract amino acids will be dependent on the matrix. Extraction of free amino acids in aqueous samples is relatively straightforward, solid phase extraction (SPE) is commonly applied for extraction and preconcentration [45] (see Section 2.3). Solid samples may be processed and weighed and the wet or dry weight used for normalisation, however wet samples are often freeze-dried to remove this variable when calculating concentrations of the analytes per mass of sample. The first step in extraction protocols for many biological samples is cell lysis, with some matrices such as tissue requiring homogenisation prior to lysis. Lysis is typically performed via physical methods including freeze/thaw cycles, physical disruption and sonication. Cell lysis is followed by the precipitation of proteins out of solution which will split the sample into two fractions; free amino acids in solution and protein-bound amino acids in the precipitate. The cell lysis can take place in the solvents used for protein precipitation and may include acids such as trichloroacetic acid (TCA) and perchloric acid (PCA), or organic solvents such as acetone, methanol and acetonitrile. While acid precipitation typically provides the best amino acid recoveries, some organic solvents such as acetone will precipitate out more molecules than TCA including some small peptides. Sedgwick and colleagues provided a good summary of recovery rates from a simple sample matrix (plasma) and data on how protein concentrations can affect amino

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acid extraction with particular solvents [46]. TCA is the most commonly used solvent, however multiple washes with different solvents may increase extraction efficiencies. Mixes of acids and organic solvents have also been used such as 10% (w/v) TCA in 90% acetone to utilise the benefits of both solutions in one step.

Metabolomic analyses typically encompass analytes able to solubilise in polar solvents, including amino acids. These methods can be simple methanol extractions or elaborate phase separation extractions such as the Matyash method (Methyl *tert*-butyl ether (MTBE)/methanol/water) that fractionate the sample into a polar (methanol/water) and non-polar (MTBE) phases [47]. However, to our knowledge, no studies have reported recovery rates with these extraction methods and thus it is uncertain how much loss may be incurred. An alternative extraction method for free amino acids from solid samples is supercritical fluid extraction (SFE) [48].

Regardless of the extraction method employed, once extracted, samples are usually dried then reconstituted in a low concentration of hydrochloric acid (HCl) (typically 10–100 mM). The solubilised amino acids can be further diluted into LC compatible solvents if being analysed in their native form, or solvents that are compatible with the selected derivatisation method. It is important when analysing native amino acids containing a thiol group (-SH) such as cysteine, to include a reducing agent such as dithiothreitol (DTT) to ensure no disulphide bonding will occur thus preventing dimer formation.

2.3. Solid phase extraction (SPE)

Solid phase extraction (SPE) may be used to isolate amino acids from matrices, providing a sample clean-up step and a means of analyte preconcentration [45]. SPE makes use of a solid adsorbent to either adsorb compounds of interest separating them from matrix compounds that may interfere with analysis, or by removing undesired components for analysis allowing the compounds of interest to pass through, with the former more commonly used in amino acid studies [45,49–51]. SPE can be carried out at a number of points throughout the extraction procedure as shown in Fig. 3. Reverse phase SPE may be used [49] but was reported to have poor retention of the more polar amino acids, and poor selectivity to allow for adequate sample clean-up [50]. The most commonly employed form of SPE used for amino acids is ion-exchange SPE [45,50,51]. Being zwitterionic, both cation and anion exchange SPE can be used to selectively bind amino acids. For most sample matrices SPE is optional, though it can greatly improve the quality of a sample when utilised for clean-up thus improving the overall analytical outcomes.

2.4. Hydrolysis of protein-bound amino acids

Hydrolysis is required to release amino acids from the peptide chains of the proteins. Depending on the application, the protein-bound fraction may be omitted from analysis, analysed separately, or com-

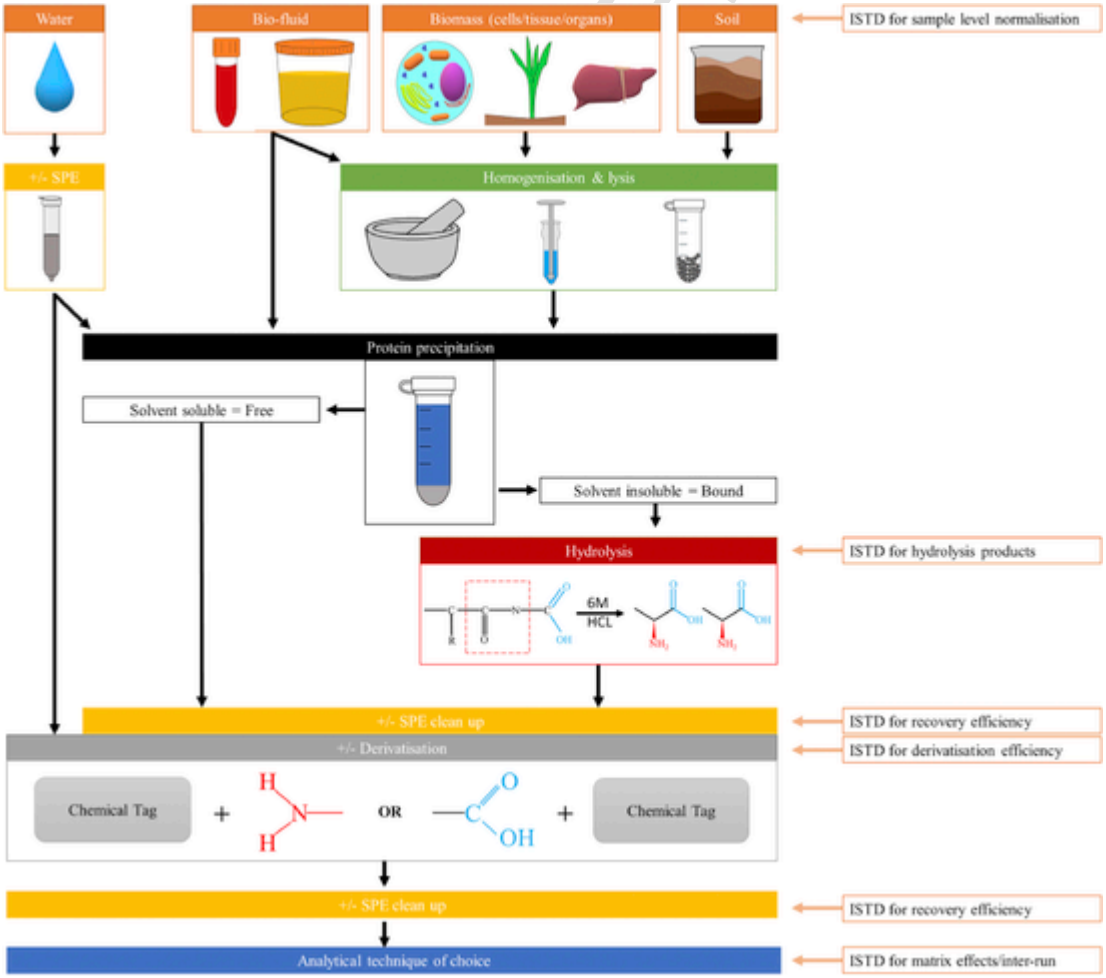

Fig. 3. Schematic representation of a standard amino acid analysis workflow, the introduction of an internal standard (ISTD) to the samples are denoted at each step with their effect correction.

bined with the free fraction for total amino acid quantification. It is generally assumed that the amino acids released from the protein fraction by hydrolysis are all amino acids that were incorporated into the polypeptide chain, however several studies into non-protein amino acids question if all amino acids released by hydrolysis were part of the protein backbone or if some could have been covalently bonded to an amino acid side chain [37]. Hydrolysis is typically performed at temperatures ranging from 110°C to 170°C with a highly concentrated acid such as 6 M HCl either in vapour or liquid form. A low oxygen environment is necessary for hydrolysis to prevent oxidation, thus it is often carried out under a vacuum or in the presence of an inert gas such as nitrogen or argon. Hydrolysis is often time consuming, taking 16–24 h to complete, however microwave irradiation has been shown to decrease these times to as little as 10 min [52]. Despite acid hydrolysis being the most commonly used method of hydrolysis, there is no single method that will completely cleave all peptide bonds in a sample to single amino acids completely and quantitatively. Some amino acids require the addition of reducing agents for complete hydrolysis, while others require longer hydrolysis times. For a more in-depth summation of different protein hydrolysis options, the reader is directed to the chapter 'Hydrolysis of Samples for Amino Acid Analysis' by Davidson which discusses the advantages and disadvantages of a range of hydrolysis methods including acid hydrolysis, alkaline hydrolysis and microwave irradiation [53]. Certain amino acids require modified conditions to prevent losses during the hydrolysis process, for example the addition of phenol will protect tyrosine residues from oxidation during acid hydrolysis and shortening hydrolysis times is required to preserve phosphorylated amino acids [46]. A less common method of amino acid release is enzymatic digestion of proteins by a protease [17]. Enzymatic digestion is rarely used as it is a more costly and complicated procedure with evidence suggesting amino acid contamination from the enzymes is possible due to self digestion [54]. Even with the use of a protease that cleaves at a wide array of amino acid residues, such as proteinase K, it typically doesn't result in complete hydrolysis and therefore multiple proteases are required [55]. Post-hydrolysis, samples are dried and dissolved in the same solvents as their free fraction counterparts in preparation for analysis or derivatisation.

2.5. Additional clean-up methods

The initial protein precipitation step should separate large biomolecules such as proteins, lipids, DNA and RNA from free amino acids, however small molecules including other metabolites, peptides and salts, which could interfere with analysis are often still present. The hydrolysed protein fraction might also contain other large biomolecules which have co-precipitated with the proteins such as polysaccharides in bacterial samples as well as insoluble molecules present after lysis such as components of the cell wall in plant samples. Further sample clean-up is not essential, especially if using targeted MS methods, however it may reduce the background signal or ion suppression during analysis, improving identification and quantification. The presence of these small molecules is an important factor in column and instrument maintenance as they may reduce the life of the column and dirty the MS interface. Filtration is an important final processing step for amino acid samples to remove any particulates. Inclusion of a guard column during LC-MS/MS will also further protect the column. Other clean-up methods include SPE [45,49–51], see Section 2.3 for more details on this technique. Offline HPLC fraction collection can also be employed [56]. These can be used to isolate amino acids from the sample, removing the other small molecules present and resulting in a cleaner sample for analysis.

3. Considerations for chromatographic separation

The choice of column for chromatographic separation of amino acids depends on whether the amino acids samples are in the native or derivatised form. Here we provide an overview outlining some chromatographic options and the most commonly used derivatisation methods for amino acids. The different forms of chromatography applied to amino acid studies are summarised in Table 1.

3.1. Analysis of underivatised amino acids

Analysis of native amino acids has the advantage of reducing the number of sample preparation steps and thereby reducing losses and analytical errors. However, native amino acid analysis via LC-MS/MS is problematic due to physiochemical properties of amino acids such

Table 1
Summary of types of LC used in amino acid analysis by LC-MS/MS outlining main advantages and disadvantages.

Type of LC	Advantages	Disadvantages	Section
Ion-exchange chromatography	Allows amino acids to be analysed in their native state.	Decrease in sensitivity due to ion suppression from high buffer concentration.	3.1.1
Ion-pairing chromatography	Allows amino acids to be analysed in their native state.	Decrease in sensitivity due to ion suppression from high buffer concentration.	3.1.2
Hydrophilic interaction liquid chromatography	Allows amino acids to be analysed in their native state.	Incorrect implementation results in poor peak shape, separation efficiency, and reproducibility. Requires specific knowledge for troubleshooting. Decrease in sensitivity due to ion suppression from high buffer concentration.	3.1.3
Aqueous normal phase chromatography	Allows amino acids to be analysed in their native state. Uses low buffer concentrations.	Limited studies on effectiveness.	3.1.4
Reverse phase	More sensitive than native analysis due to derivatisation. Ease in troubleshooting. Use of chiral derivatisation agents allow enantiomer separation.	Requires derivatisation. Additional sample preparation time required for derivatisation. Additional cost of reagents. A large range of derivatisation agents to select from.	3.2, 3.3
Chiral column chromatography	Allows enantiomer separation. Can be carried out with or without derivatisation.	Potential overlap between enantiomers and stereoisomers. Long run times. Expensive columns.	3.3.1
Two-dimensional (2D) liquid chromatography	Allows enantiomer separation. Can be carried out with or without derivatisation.	Disadvantages from the types of LC chosen remain. Requires specific knowledge for troubleshooting. Requires two columns, specific LC set-up and two compatible LC types.	3.3.23.3.1.1

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as small size and high polarity which result in long method development periods and potentially reduced MS sensitivity due to the high level of noise associated with their mass range. As a result, the majority of LC-MS/MS methods use derivatised methods. Here we describe the four types of liquid chromatography that can be coupled to a mass spectrometer to analyse native amino acids that take advantage of their native physiochemical properties.

3.1.1. Ion-exchange chromatography (IC)

Ion exchange chromatography (IC) takes advantage of the ionic properties of amino acids. Being amphoteric, under certain pH conditions amino acids can exist as zwitterions, cations or anions. As a result, both cation and anion exchange chromatography can be applied, however the former is more frequently used for separating amino acids. Amino acid analysers (AAA) use cation exchange chromatography with post-column ninhydrin derivatisation for spectrophotometric detection. IC separates via ionic interactions, with the stationary phase consisting of a matrix with oppositely charged ions to that of the analytes. The stationary phase is first conditioned with a low concentration solution containing ions with the same charge as the analytes prior to the sample being loaded. Once the sample is loaded, the analytes will displace the mobile phase ions and bind to the stationary phase through stronger ionic interactions. A change in pH of the mobile phase or an increase in the concentration of an ionic buffer will elute the analytes from the column. A shift in pH changes the charge state of the analytes, preventing them from interacting with the stationary phase, while increasing the concentration of the ionic buffer allows the ions in solution to out-compete and displace the analytes for interaction with the stationary phase. The choice of buffered salts in the mobile phase requires careful selection and optimisation as they can affect ionisation in the mass spectrometer and must be volatile. IC-MS has yet to be applied to amino acid studies however, its potential was demonstrated in a metabolomics proof of concept study [57].

3.1.2. Ion-pairing chromatography (IPC)

Ion-pairing chromatography (IPC) is another form of chromatography that takes advantage of the ionic properties of amino acids. IPC is a variant of reverse phase chromatography that uses non-polar stationary phases, typically C8 or C18, and a polar mobile phase. Generally, the highly polar amino acids show greater interactions with the mobile phase compared to the stationary phase and are not retained on the column. Retention is promoted via the addition of ion-paring agents to the mobile phase that allow the formation of an amino acid-ion-pairing agent complex that is then able to interact with the stationary phase [58]. Ion-pairing agents chosen must contain the opposite charge to the analyte to allow for the complex formation, and a hydrophobic group for interaction with the stationary phase. Ion-pairing agents used for amino acids in IPC-MS have included acids such as pentadecafluorooctanoic acid [58], or bases such as diisopropylethylamine [59]. A major disadvantage of IPC-MS is a reduction in limits of detection as a consequence of ion-suppression in the mass spectrometer source due to the ion-pairing reagents.

3.1.3. Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) is the most common form of chromatography used for analysing native amino acids. HILIC utilises a polar stationary phase with mobile phase conditions consisting of a non-polar solvent with a low amount of water. A partition is formed between the two mobile phase components, with a water layer being formed on the stationary phase. The hydrophilic interactions between this water layer and polar compounds allows for their retention. Thus, starting conditions for HILIC require some water to be present to allow for the formation of this water layer. Water is the strong eluent in HILIC chromatography and is increased during employment of gradient conditions. Hydrophilic partitioning is not the sole interaction that defines retention of analytes, with electrostatic interactions also playing an important role. For a complete overview on HILIC, Buszewsk and Noga [60] provide a comprehensive introduction to this technique. Common HILIC stationary phases used for the analysis of amino acids include amide, silica, and zwitterionic functional groups such as sulfobetaine. Acetonitrile (ACN) is the most common organic solvent used for amino acid separation with HILIC methods since methanol (MeOH) is typically too polar to allow for the phase partition and formation of the water layer on the stationary phase required to allow for adequate HILIC retention, however mobile phase selection is dependent on the stationary phase.

Method development and analysis can be problematic with HILIC with poor peak shape, separation efficiency, and reproducibility [60,61]. Higher flow rates may decrease peak width, however HILIC specific knowledge is often required with pH being one of the most important factors to consider with peak shape and retention heavily dependent on the pH and the buffer used [62]. Ammonium formate is the most commonly used buffer, typically at a concentration of 5–30 mM with the pH adjusted between pH 3 and pH 6 with the addition of formic acid, however some methods elect to use only formic acid [63]. Basic pH conditions are less often used but can also be employed with the use of certain high pH tolerant columns. Basic buffers include ammonium acetate at concentrations up to 30 mM and pH values ranging from 8 to 9 buffered with ammonium hydroxide. When preparing buffers such as ammonium formate, some water is required in the organic solvent to allow the buffer to completely solubilise in the organic solvent. Therefore, the majority of HILIC methods will have a low percentage of water in the organic solvent and/or a low percentage of organic in the aqueous solvent. There is no universal buffer or buffer combination that will work ideally for all amino acids and thus, a lot of method development is required to determine what pH and buffer concentration are most appropriate for the amino acids being analysed on a particular column. Depending on the stationary phase, some acidic or basic amino acids may have poor peak shape if not under ideal pH conditions, this can be seen with aspartic acid typically requiring a low pH (~3) for good peak shape. Another important consideration is the effect of the buffer and the pH on the ionisation of amino acids in the mass spectrometer as high buffer concentrations can lead to ion suppression. This usually leads to a compromise between ideal chromatography conditions and sufficient ion intensity in the mass spectrometer. Some methods use differing additives/buffers in each of the selected solvents to get sufficient chromatographic separation without sacrificing intensity in the mass spectrometer. One example of this is seen in Chen et al. [64] where the aqueous solvent is 0.1% formic acid, while the organic ACN has no additive.

The solvent used to reconstitute the sample following sample preparation may also contribute to poor peak shape with highly aqueous solutions often resulting in wide and/or double peaks [65]. The chosen sample diluent may present some difficulty for amino acids that are insoluble at high concentrations of organic solutions due to their polar properties. Ideally, samples should be reconstituted in the mobile phase starting conditions or a solution with an equivalent organic to aqueous ratio that may yield better ion intensities in the mass spectrometer. An example of this is methods opting for reconstitution of samples in MeOH instead of ACN to increase ionisation in the mass spectrometer despite ACN being used in the mobile phase. Greco and Letzel [62] and Heaton and McCalley [65] provide useful guides for troubleshooting HILIC methods.

3.1.4. Aqueous normal phase chromatography (ANP)

Aqueous normal phase chromatography (ANP) is a variant of normal phase chromatography that can be seen as an alternative to HILIC. ANP is used to separate both polar and non-polar compounds by em-

ploying similar stationary phases and mobile phases to HILIC, however unlike HILIC it typically uses low concentrations of buffers. Thus, the high buffer concentrations that can result in ion suppression in MS that are common in many HILIC methods are absent. Currently, ANP has only been used for amino acid analysis via LC-MS/MS in one study by Hellmuth et al. [66] with the employment of a silica hydride-based stationary phase.

3.2. Derivatisation and analysis of derivatised amino acids

Derivatisation is employed to change the physicochemical properties of an analyte and make it more suited to the analytical technique. Due to the complexity of analysing amino acids in their native state, derivatisation allows for a reduction in both time spent on method development and sample analysis with the drawback of increased sample preparation time. Currently all forms of derivatisation for LC-MS/MS are pre-column, with no post-column methods reported at the time of writing. During derivatisation, the desired derivatisation agent (or tag) reacts with either the amine or carboxyl group of the amino acids or in some cases, both. While most amino acids will only have one additional functional group following derivatisation, those with additional amines or carboxyl groups in their side chain may gain multiple tags. It is important to allow the derivatisation reaction to go to completion because if the reaction is incomplete not all amino acids in a sample will be completely derivatised, which can result in multiple species for those amino acids with multiple reaction sites.

The addition of large carbon-based functional groups shifts the polarity of amino acids and allows them to interact with reverse phase liquid chromatography stationary phases. Reverse phase utilises non-polar stationary phases and polar mobile phases to retain and separate analytes based on their physical and chemical properties. C18 columns are the most common choice of stationary phase, however C8 columns have also been used [67], with the mobile phase containing combinations of water and either MeOH or ACN. Acidic buffers such as ammonium formate and formic acid are used at a pH range of 2.5–7. Basic buffers have been used sparingly with one study using ammonium bicarbonate at pH 9.5 [68]. Beyond changing the physicochemical properties of the amino acids to allow for reverse-phase chromatographic separation, derivatisation increases the overall sensitivity through increasing the size of the precursor and product ions, moving them away from the low-mass 'noise' region typical in electrospray mass spectrometry, and thus derivatisation methods on average have lower limits of detection when compared to HILIC methods [69,70]. However, there are multiple types of derivatisation reagents each with their own advantages and disadvantages that are sold by a variety of vendors, complicating selection for those new to the field.

3.2.1. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatisation

6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), sold as AccQ-Tag by Waters, will react with primary and secondary amines (Fig. 4) and will give an additional mass of 170 Da for each AQC molecule added to the amino acid. When analysed by tandem mass spectrometry, all AQC derivatised amino acids will have a common fragment ion of the AQC tag (171 *m/z*) and a fragment of the amino acid's original mass. The protocol for ACQ derivatisation is quick and straight forward with preparation of reagents taking a maximum time of 15 min, with the following reaction taking approximately 10 min. The reaction can be carried out in an auto sampler vial with an insert and is ready for injection when the reaction is complete. There are no additional clean-up methods included in the Waters AccQ-tag kit. While no evaporation is required, the manufactures instructions do require a 1 in 10 dilution of the sample in the reagents which may be problematic for low abundance analytes. AQC derivatised amino acids are relativity stable for up to 3 weeks at room temperature in the dark, allowing samples to be prepared in advance rather than on the day of analysis [61].

3.2.2. Propyl chloroformate (PCF) derivatisation

Propyl chloroformate (PCF) will react with both primary and secondary amines and the carboxyl group of the amino acid (Fig. 5). Each addition from this derivatisation agent at an amine group will add 86 Da while each addition at the carboxyl group will add 42 Da to the amino acid mass. PCF is also used for amino acid analysis via GC-MS [71]. PCF derivatisation kits are sold by Phenomenex as EZ:faast™. These kits come with an additional SPE sorbent tip for sample clean up and thus results in a cleaner sample at the cost of increasing the duration of sample preparation. The PCF derivatisation protocol requires additional time and more steps when compared to other methods, the reagents are more toxic, and the sample requires evaporation to remove volatile reagents before reconstitution in HPLC compatible solvents. However, the benefit of the additional clean up may be essential for some matrices. As the PCF derivatisation protocol is more hands on, especially if the SPE clean up is applied, total derivatisation time is highly variable and dependant on the analysts. These samples are stable for one day when stored at room temperature, however, stability is increased when stored at 4°C, with the longest time reported being two days [72]. Thus, for runs with a high number of samples, it is suggested to lower the temperature of the autosampler if possible.

3.2.3. 9-fluorenylmethyloxycarbonyl chloride (Fmoc) derivatisation

9-fluorenylmethyloxycarbonyl chloride (Fmoc) reacts with both primary and secondary amines (Fig. 6). The derivatisation protocol is relatively simple and quick (approximately 20 min). The Fmoc reaction adds 222 Da to the amino acid per reaction site. Fmoc derivatisation has been reported to potentially form multiple reaction species for a single amino acid and can have a loss of 25–50% for certain amino acids due to some being partly extracted into the organic phase [73]. Fmoc is stable at room temperature for at least three days [74] with no information on whether lower temperatures increase the stability of the sample, however, standards for Fmoc amino acids can be purchased for testing the derivatisation efficacy and are recommended to be refrigerated for a longer shelf life. Jámbor and Molnár-Perl (2006) [73] dis-

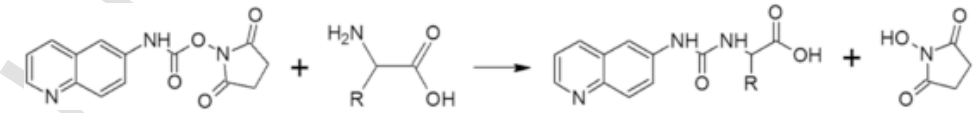

Fig. 4. Example AQC derivatisation reaction with a primary amino acid.

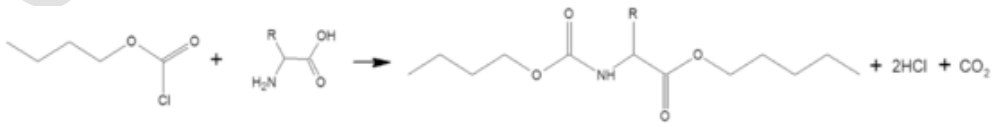

Fig. 5. Example of Propyl chloroformate derivatisation reaction with an amino acid containing both a primary amine and a carboxyl group.

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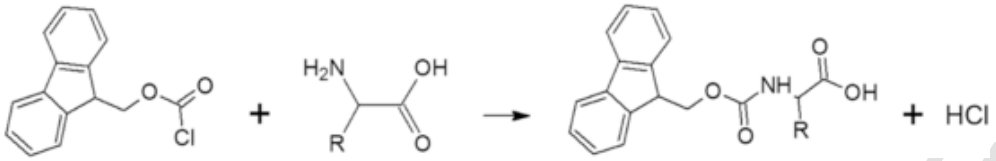

Fig. 6. Example of Fmoc derivatisation reaction with a primary amino acid.

cuss the issues associated with the method and how to optimise derivatisation conditions. Fmoc reagents and standards can be purchased from Sigma–Aldrich and Thermo Fisher Scientific.

3.2.4. Isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT)

Isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT) are techniques that were originally developed for proteomics that have been adapted for use as a type of amino acid derivatisation. While both function identically, iTRAQ has been used in more amino acid studies then TMT. iTRAQ and TMT (collectively referred to as tags) will react with primary or secondary amines on an amino acid, however there are two TMT variants that have yet to be applied to amino acid studies, with one that reacts to carboxyl groups and the other to sulfhydryl groups. Tags are sold in multiple kits and the mass added depends on what kit is purchased. iTRAQ will add an additional 144 Da when purchased in the 4plex (Fig. 7) and an additional 304 Da for the 8plex (structure currently not disclosed) [75].

There are currently multiple TMT kits available that react with amines available, however, all but one share identical structures and mass, adding 229 Da to each reaction site (Fig. 8), with only the 16plex (titled TMTpro) having a different structure, adding 304 Da per reaction side (Fig. 9).

The tags themselves are divided into three regions, a reporter ion, balance group and amine specific reactive group. Balance and reporter ion groups can differ between kits, with the structure of the amine specific reaction group staying the same for all amine-reactant variants of TMT and iTRAQ. Within the kit, while tags are isobaric in mass and structure, there is variation between carbon 12 and 13 and nitrogen 14 and 15 atoms in the reporter ion and balancer groups amongst reagents for both TMT and iTRAQ, with iTRAQ also having variation between oxygen isotopes on the balancer group. These tags intended use is to spike a sample with standards that have been derivatised with different isotopically labelled reagent so that quantification can be based on the ratio between the known standards and the unknown samples [71]. Larger plexed kits can be used to include a complete standard curve in a sample for quantification. Tagged amino acids can still be quantified from an external calibration curve by derivatising the samples and standards with the same reagent.

The derivatisation protocol is simple, but the reaction between the amino acids and reagents is longer than the other conventional derivatisation methods (30 min). Sample preparation time is further extended as the samples are typically dried and reconstituted in the solution containing the different isotopically labelled standards. iTRAQ is sold by SCIEX and Sigma–Aldrich with SCIEX also selling aTRAQ, a variation of the 4plex iTRAQ whose sole intended use is amino acid analysis. Compared to the 4plex kit, aTRAQ only includes two reagent tags [76]. TMT is sold exclusively by Thermo Fisher Scientific. A disadvantage for some of the reagents is that the small difference in reporter masses requires the usage of high resolution MS such as an Orbitrap or quadrupole time of flight (QTOF) spectrophotometer. However, tags that are significantly different in mass (such as those included in aTRAQ with a difference of 8 Da), can be differentiated using less selective instruments such as a triple quadrupole MS. One of the most significant disadvantages of these methods is the cost associated with purchasing the reagents, as they are typically twice the price of other derivatisation kits. However, this cost can be offset by the lessened requirement of instrument time if the ability to use an internal standard curve is utilised.

3.2.5. Comparison studies and alternative derivatisation methods

Currently, no single study compares all of the common derivatisation methods, however, there are some that do make some comparisons or compare them to alternative methods [69,70,74]. These studies typically agree that HILIC-MS/MS and amino acid analysers are less sensitive than the derivatisation methods. GC-MS methods are mostly comparable but may struggle with the analysis of specific amino acids. The best derivatisation method is most likely related to the sample matrix, and the particular amino acids that are being analysed.

Alternative types of amino acid derivatisation have been used for LC-MS/MS; however, these are rarer than the methods described above. Advantages and disadvantages of these methods are listed in Table 2. Some have been developed specifically for LC-MS but have yet to become commercially available. Others do not come in a kit and re-

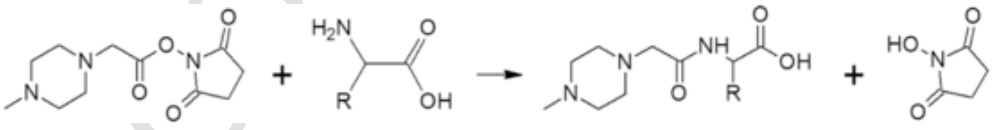

Fig. 7. Example of 4plex iTRAQ derivatisation reaction with a primary amino acid.

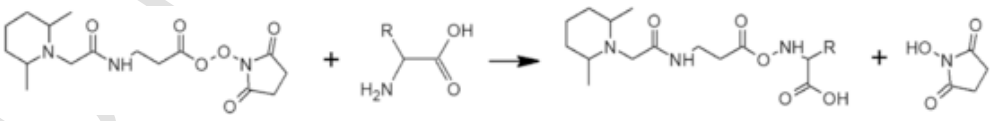

Fig. 8. Example of TMT derivatisation reaction with a primary amino acid.

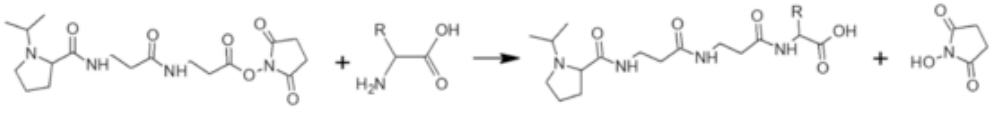

Fig. 9. Example of 16plex TMT derivatisation reaction with a primary amino acid.

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Table 2
Some of the less common derivatisation methods used in LC-MS/MS outlining main advantages and disadvantages. Chemical names and abbreviations are as follows 2,5-dioxopyrrolidin-1-yl Ntri(pyrrolidino)phosphoranylideneamino carbamate (FOSF), p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS), dibenzyl ethyl ethoxymethylene malonate (DBEMM), o-phthalaldehyde (OPA), dibenzyl ethoxymethylene malonate (DEEMM), 5-(DimethylAmino)Naphthalene-1-SulfonYL chloride (DNS) and 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)/7-Chloro-4-nitrobenzoxadiazole (NBD-Cl).

Derivatisation	Reaction site	Advantages	Disadvantages
Butanol [74]	Carboxyl	Doesn't interfere with the protonation of the amine.	Poor reverse phase retention. Small mass increase.
TAHS [78]	Amine	Designed specifically for MS analysis.	Requires reagent synthesis. Poor chromatographic separation.
DEEMM [77,79]	Amine	Detectable by UV.	Long reaction time (50 min). No kit available only individual reagents.
DBEMM [80]	Amine	Designed specifically for MS analysis. High ionisation efficiency.	Requires reagent synthesis.
DNS [77]	Amine	Fluorescent. Simple derivatisation protocol.	Low stability.
OPA [81]	Amine	Detectable by UV.	Low stability.
FOSF [77]	Amine	Designed specifically for MS analysis.	Requires reagent synthesis. Poor chromatographic separation.
NBD-F/Cl [82]	Amine	Fast derivatisation time (5 min).	No kit available only individual reagents.

quire individual standards to be purchased, and a few are predominantly used for alternative detection techniques like spectrophotometry and are rarely used for LC-MS/MS. Interestingly, while not used as extensively, comparisons with the standard derivatisation methods yield comparable results [74,77].

3.3. Chiral analysis

Chiral analysis of amino acids is often overlooked, with most native and derivatised chromatographic methods opting not to separate enantiomers. While D-amino acids are typically in lower abundance in most sample types than their L counterparts [8], there is increasing interest in the analysis of D-amino acids due to their wide array of important biological functions (see Section 1). Chiral separation in some research fields, such as determining the nutritional information of some food products or identification of certain genetic diseases, is becoming a necessity. A recent review by Bastings et al. [83] highlights some of the physiological processes of D-amino acids and their implications in different diseases, with a focus on cancer. However, unlike standard amino acid analysis, options for chiral analysis are more limited; mostly due to the need for specialist column types or derivatisation methods that, while designed for enantiomer separation, may not be the most efficient or straight-forward methods available for amino acid analysis.

3.3.1. Chiral column chromatography (CCC)

Chiral column chromatography (CCC) utilises columns packed with chiral stationary phases (CSPs) for the separation of enantiomers. A CSP is composed of a single enantiomer of a chiral molecule and can be packed into a wide array of different chromatography columns including GC and LC columns. With the stationary phase being comprised of a single enantiomer, enantiomers of the same amino acid passing through the column will have slightly different interactions with the stationary phase, allowing their chromatographic separation. These columns are manufactured for use in differing variants of LC, including reverse phase, normal phase, and ion exchange. While several chiral columns with different CSPs have been shown to be capable of amino acid enantiomer separation in LC [84], only some of these phases are MS compatible. This is due to solvents that are not suitable for MS being required to separate enantiomers, for example hexane in normal phase chromatography [85]. Stationary phases that have been used to separate native amino acid enantiomers in LC-MS include, teicoplanin [86] and crown ether [87] which have the ability to separate out up to 18 [87] and 19 [85] enantiomers respectively in a single run. CCC can also be applied to derivatised amino acids under reverse phase conditions with chiral stationary phases including *tert*-butyl carbamate modified quinine and quinidine [88]. However, these studies are limited, with the use of chiral derivatisation agents being preferred due to higher sensitivity by MS and better cost efficiency. Common reverse phase and HILIC mobile phases may be used dependent on the CSP, with organic solvents including MeOH, ACN, ethanol, or mixes of the three, used alongside an aqueous phase. The aqueous phase may use acidic buffers such as formic acid and trifluoroacetic acid to assist in ionisation [86,87]. Basic buffers are used sparingly with one study utilising ammonium acetate [88].

CCC of native amino acids is similar to HILIC due to the necessary high organic content of the mobile phase, however, the mechanism behind the retention is distinct [87]. Isocratic methods are often preferred since gradients may result in the loss of enantiomer separation. CCC has several shortcomings which prevent it from being used more commonly. Methods that use high organic content have been reported to have similar issues to HILIC including peak distortion and long equilibration times [87]. Many factors need to be considered upon selection of a column, as there is not a specific phase that will sufficiently separate all amino acid enantiomers. Column selection needs to be based on which CSP will separate the amino acid/s enantiomers of interest. Additionally, long method development periods are typically required to test which mobile phase combinations, selected buffers and column oven temperatures will allow enantiomer separation for all the compounds of interest [87]. While one set of conditions may be ideal for particular amino acid enantiomers, it may not be ideal for others [86]. Amino acids with constitutional isomers add a further level of complication to the analyses. Their analysis is difficult using standard chromatography since the number of isomers requiring separation has doubled [86]. However, focusing on one pair of enantiomers can simplify this. Multiple studies have shown the potential to separate a large number of amino acid enantiomers, with Desai and Armstrong separating 19 amino acid enantiomers [85]. These methods typically fail to separate at least one pair of desired enantiomers [86,87] or have overlap between constitutional isomers and their enantiomers [85]. As with other underivatised methods, native CCC will also be less sensitive relative to derivatised methods [89]. Another shortcoming of CCC is the higher cost of the column in comparison to traditional LC columns.

3.3.1.1. Two-dimensional (2D) liquid chromatography Two-dimensional LC (2D-LC) is a technique that takes advantage of orthogonal separation mechanisms by utilising two columns to increase resolution between peaks that are typically difficult to resolve by one-dimensional LC (1D-LC). Inline 2D-LC is typically performed in one of two ways; the first and most straightforward involves all of the eluate from the first column entering the second column; the second method involves selection of a peak or part of a peak through "heart-cutting", and this fraction is then sent through to the second column. Amino acid analysis by 2D-LC allows the separation of individual amino acids in the first dimension followed by enantiomers in the second. 2D-LC has been

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used sparingly for the analysis of derivatised amino acid enantiomers, typically through the employment of a C18 column in the first dimension followed by a CCC in the second [90,91]. Native amino acid analysis has not been conducted, however, is theoretically possible with the use of a traditional LC variant, such as HILIC, in the first dimension followed by CCC in the second. The benefit of using 2D-LC is the ability to utilise the strengths of two different forms of LC in a single run. This can overcome the shortcomings of some forms of chromatography such as CCC which may struggle with the separation of the amino acid constitutional isomers [85]. However, the individual problems associated with each form of chromatography or column will transition to 2D-LC. Implementation of 2D-LC is more complicated than 1D-LC, requiring increased complexity to the instrument set up and some 2D-LC specific knowledge for method development. Phase incompatibility, reduced sensitivity and increased analysis time can all occur with 2D-LC [92]. Increased cost can also be associated with 2D-LC with the need to purchase more than one column, and if using the heart-cutting method, multiple pumps. 2D-LC also increases the analysis time compared to 1D-LC [91]. For more information about the forms of 2D-LC and their benefits and shortcomings the reader is directed to Pirok et al. [92].

3.3.2. Chiral derivatisation agents (CDA)

Due to small differences in the physiochemical properties of amino acid enantiomers, several derivatisation methods have been developed to amplify these differences using agents collectively known as chiral derivatisation agents (CDA). The assurance of enantiomeric purity (>99%) of derivatisation reagents is essential when performing these forms of derivatisation as impurities may give false positives [93,94]. Another essential requirement for a CDA is that the derivatisation process will not lead to racemisation [95]. Once derivatised, as with the other conventional forms of derivatisation, reverse phase liquid chromatography is the separation method of choice. For an overview of reverse phase chromatography including commonly used buffers and solvents used in derivatised amino acid analysis, see Section 3.2. While specific types of derivatisation have been developed for chiral analysis, standard derivatisation methods such as ACQ have been utilised with CCC (see Section 3.3.1) to allow for enantiomer separation of amino acids [88].

3.3.2.1. Marfey's reagent/(1-fluoro-2-4-dinitrophenyl-5-L-alanine amide) (FDAA) 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA), more commonly referred to as Marfey's reagent, can react with primary and secondary amines (Fig. 10), phenols, and thiols [94,96], adding 252 Da to the amino acid for each tag. Some amino acids such, as asparagine, will only react with one tag despite having more than one amine in their structure [97], while others will react with one tag per potential site such as lysine which receives two tags [97]. This difference in tagging can occur for multiple reasons including steric hindrance or interference from nearby chemical groups inhibiting the derivatisation reaction and has been reported in multiple studies [94]. This typically results in the amine closest to alpha carbon being the only site for derivatisation in multiple amine containing amino acids. Due to this, prediction of the mass increase from derivatisation is more complicated than in standard methods.The derivatisation reagent is synthesised from two precursors, 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and L-alanine amide [94]. When an L-amino acid reacts with the reagent, intermolecular hydrogen bonding occurs between the hydrogen in the carboxyl group of the derivatised L-amino acid and the carbonyl group of the L-alanine-amide (Fig. 10). If a D-amino acid reacts with the reagent, the hydrogen bonding is absent leading to a gap and no interactions between the two groups. This gap reduces the polarity of the D amino acid derivatives, resulting in the D-amino acid derivatives having stronger interactions with reverse phase columns. Resulting in the D-amino acid derivatives retaining longer than their L-counterparts and allowing for chromatographic separation of enantiomers [94]. Originally this derivatisation method was developed for α amino acids, however, the separation of β amino acids enantiomers has also been reported [98]. While the derivatisation procedure is simple, the reaction time for Marfey's reagent is longer than most derivatisation methods, with reaction times reported ranging from 90 min [98] to overnight [97] for completion. For some amino acids these long reaction times are essential, even in excess of the reagent, for even partial derivatisation. Derivatives will be stable for up to a week at ambient temperature or up to a month if frozen [99].The biggest limitation of Marfey's reagent is the potential occurrence of multiple derivative species for certain amino acids [98]. Some amino acids including lysine and histidine, may simultaneously form both a single tagged derivative and a multi-tagged derivative for each enantiomer. This will increase the number of compounds requiring monitoring and separation in a method, while also reducing the concentration of these amino acids in a sample. This is due to one amino acid enantiomer being split into two forms, effectively raising the limit of detection for these derivatives. This and other derivatisation issues can be alleviated by the inclusion of an ISTD for normalisation or modifying the reaction conditions, such as adding excess reagent, increasing temperatures, lengthening reaction times, increasing the rate of reaction via the inclusion of dimethylsulfoxide (DMSO) or utilisation of microwave-assisted derivatisation [98]. However, these conditions may lead to racemisation or the decomposition of Marfey's reagent [98]. Marfey's reagent is available for purchase from both Sigma–Aldrich and Thermo Fisher Scientific. While Marfey's reagent is synthesised with DFDNB and L-alanine amide, multiple vari-

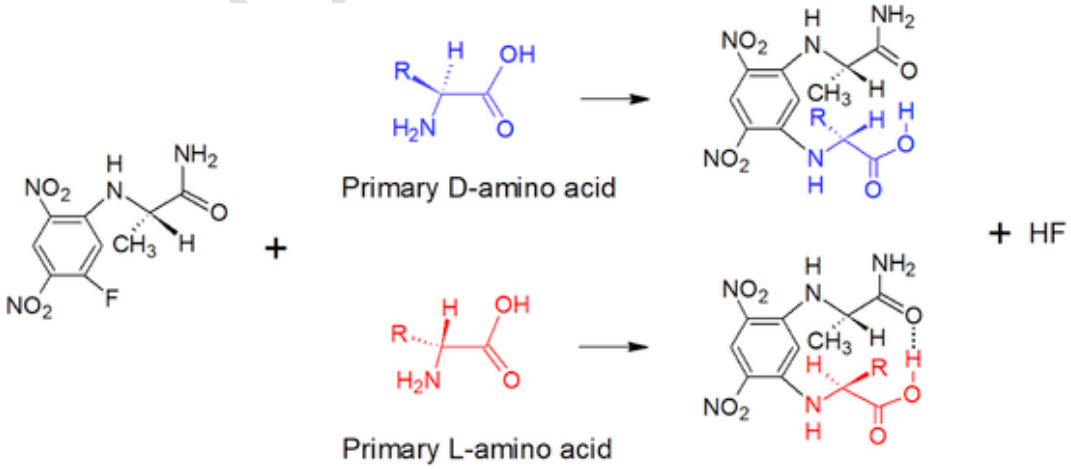

Fig. 10. Marfey's reagent derivatisation reaction with primary D and L amino acids.

ants have been synthesised and their use reported in the literature. Replacing the L-alanine amide with alternative amino acid amides has had varying degrees of success [98]. One of the more well-known and widely used alternatives is 1-fluoro-2-4-dinitrophenyl-5-L-valinamide (FDVA) which is also available for purchase. These variants are not limited to only L-enantiomer amino acid amides as with Marfey's reagent with D-alanine amide having also been used in some studies (D-FDAA or D-Marfey's reagent) [100]. For a more in-depth overview of this derivatisation technique and its variants, the reader is directed to two reviews written by Bhushan and Brückner [94,98].

3.3.2.2. 1-(9-fluorenyl)-ethyl chloroformate (FLEC) 1-(9-fluorenyl)-ethyl chloroformate (FLEC) primarily reacts with primary and secondary amines (Fig. 11) however, under alternative reaction conditions can react with hydroxyl groups [93]. The hydroxyl derivatives have yet to be utilised in amino acid analysis. FLEC derivatisation will add 236 Da to each site of derivatisation. LC-MS studies using FLEC report a common fragment ion of 193 *m/z* for all FLEC derivatised molecules [101]. Structurally, FLEC is quite similar to FMOC with the only difference being the methyl group on the acyl moiety. As FMOC is non-chiral, the introduction of this methyl group allows for chirality of the molecule. This chirality contributes to its ability to resolve enantiomers in LC and CE, while also splitting FLEC into two alternative forms, (+)-FLEC and (−)-FLEC (Fig. 12). While both forms have been used for amino acid derivatisation (+)-FLEC is more commonly used. Retention of enantiomeric species on reverse phase columns is dependent on which variant is used. (+)-FLEC will increase the retention of L-Amino acids [102], with the opposite occurring if (−)-FLEC is employed. The reported times required for FLEC derivatisation are highly variable, with some studies stopping the reaction after 2–5 min [103,104] while other studies allow the reaction to proceed for an hour [105]. FLEC derivatives are stable for 5 days at room temperature and a month if frozen [106,107]. The biggest disadvantage of FLEC is the long chromatographic methods required for separation [93]. Some methods have analysis times of more than 100 min [93], however, Moldovan et al. [105] separated 18 out of the 19 protein amino acids tested within 30 min via employment of a C18 column with a diphenyl stationary phase. Both variants of FLEC are commercially available for purchase. For more information about FLEC and its applications which extend beyond amino acid analysis it is suggested to read Moldovan et al. [93].

3.3.2.3. N-(4-Nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE) N-(4-Nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE) will react with primary and secondary amines, phenols and thiols (Fig. 13) with each tag adding 249 Da to the amino acid. As with FDAA, its reaction with multiple amines (primary or secondary) is conditional (see Section 3.3.3.1) thus, the prediction of the derivatised mass is more complicated compared to other derivatisation methods. Like FLEC, (S)-NIFE is a chiral molecule, which contributes to its ability to separate enantiomers [96]. However unlike FLEC, the enantiomer of (S)-NIFE has yet to be used for enantiomer separation. (S)-NIFE derivatised L-amino acids will elute before (S)-NIFE derivatised D-amino acids [96]. MS methods analysing (S)-NIFE derivatives have reported the occurrence of two common fragment ions, 224 *m/z* and 120 *m/z* [108]. (S)-NIFE derivatisation time is short, taking only 15–20 min and derivatised samples are stable for up to up to 9 days [68] with refrigeration increasing storage times to several weeks [109]. The major drawback of (S)-NIFE derivatisation is shared with FDAA in that it has the potential to form multiple species for some amino acids. Like FDAA, excess reagent will largely elevate this problem however aromatic amino acids with a phenol group such as tyrosine will form multiple derivative species even in the presence of excess of the derivatising agent and thus may need to be accounted for with an ISTD [95]. SNIFE derivatives where shown to be twice as sensitive in an MS when compared to their Marfey's reagent counterparts however had poorer enantiomer separation for certain amino acids [96]. (S)-NIFE is sold by Cayman Chemical and Santa Cruz Biotechnology.

3.3.2.4. Alternative chiral derivatisation agents Marfey's reagent, FLEC and (S)-NIFE are the most common CDAs used in chiral amino acid analysis however there are other CDAs that have been developed and employed in a limited number of studies. These other CDAs were mostly used in older LC–UV studies, and include 2,3,4,6-tetra-O-acetyl-β-d-glucopyranosyl isothiocyanate (GITC), and O-phthaldialdehyde and N-isobutyryl-L-cysteine (OPA/IBLC). GITC will only react with primary amines (Fig. 14) and gives an additional mass of 389 Da. Reported reaction times have a high variance between studies ranging from 10 min [96] to 60 min [110]. Separation of enantiomers with GITC has been reported to be less effective when compared to the more commonly used Marfey's reagent [96]. Its greatest disadvantage is the instability of the derivatives produced, degrading within 24 h and its inability to react with certain amino acids due to steric hindrance [96]. OPA/IBLC will only react with primary amines (Fig. 15) and will add 289 Da to the amino acid. The derivatisation reaction for OPA/IBLC is straightforward, only taking 20 min. OPA/IBLC is a combination of two reagents, OPA and IBLC, paired together to allow for enantiomer separation. Multiple forms of this derivatisation exist such as the D enantiomers (OPA/IBDC) and alternatives that replace the IBLC with other compounds such as N-acetyl-L-cysteine (NAC) (OPA/NAC) [103]. As GITC and all alternative forms of OPA/IBLC only react with primary amines, amino acids with secondary amines will not be derivatised. One study overcame this via first derivatising with OPA/NAC and then with FLEC to ensure the derivatisation of both primary and secondary amine containing amino acids [102]. For both methods, generally the L-amino acid derivatives will elute before their D-amino acid counterparts in reverse phase chromatography [96]. A comparison study by Hess et al. [96] compared FDAA, (S)-NIFE, GITC and OPA/IBLC in LC-MS found that GITC and OPA/IBLC was four times more sensitive then FDAA and two times more sensitive then (S)-NIFE.

4. Choosing the correct detector

4.1. Overview

Mass spectrometry (MS) is a powerful analytical technique that is used to measure the mass-to-charge ratio (*m/z*) of ions, thus giving mass and structural information on the analyte being detected. It has an advantage over the spectrophotometric detectors previously used for amino acid analysis that were unable to differentiate molecules and relied on strict chromatographic separation to allow retention times to be compared to standards. Chromatographic separation is less problematic with MS detectors since the *m/z* resolving power can differentiate between co-eluting species unless they are constitutional isomers, such as leucine and isoleucine or enantiomers [111]. To increase specificity and overcome the low mass resolution of quadrupole analysers, tan-

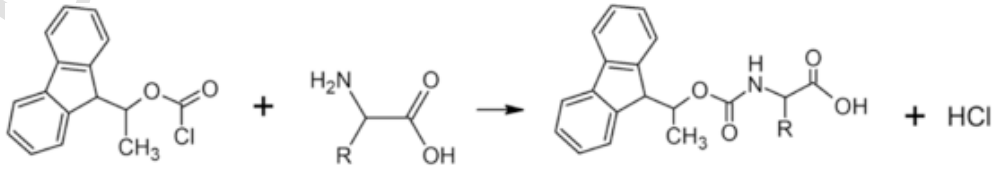

Fig. 11. FLEC derivatisation reaction with a primary amino acid.

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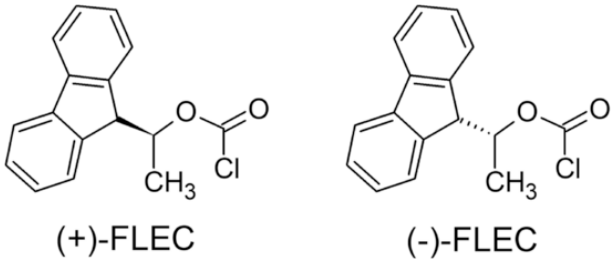

Fig. 12. The two enantiomers of FLEC.

dem mass spectrometry (MS/MS) has mostly replaced traditional non-fragmentation MS. MS/MS fragments the analytes allowing for the detection of either the fragments or both the intact and fragment mass of the analyte/s. This fragmentation of molecules is achieved via collision-induced dissociation (CID) which occurs in the collision cell of the MS, however the exact type of CID employed depends on the model of the MS [112].

While most amino acids will fragment uniquely, some structurally similar amino acids may produce common fragment ions, for a comprehensive study on α-amino acid fragmentation by CID the reader is directed to Zhang et al. [113] or Rogalewicz et al. [114]. LC-MS/MS uses a combination of retention time and fragmentation to provide a robust technique for the identification and quantification of small molecules. Historically, amino acid analysis by MS and MS/MS has been troublesome due to low ionisation efficiency and a tendency to fragment in the source (in-source fragmentation). Thus, there are multiple factors that need to be taken into account when determining the correct mass spectrometer and conditions to be applied to amino acid analysis.

The first consideration that needs to be made is what type of ionisation source will be used to ionise the amino acids. Multiple mass spectrometry ionisation processes have been applied to amino acids including electron ionisation (EI), chemical ionisation (CI), microwave-induced plasma ionisation (MIPI), electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) [115,116]. EI and CI are typically reserved for GC-MS studies [116]. MIPI was applied to native amino acids in a single study [115] whereas ESI and APCI have been applied to native and derivatised amino acids in numerous LC-MS/MS studies [69,117,118]. There are no universal MS parameters for native or derivatised amino acids. Due to structural variation, each amino acid will have different optimal parameters, thus, most methods compromise and use parameters that, while not being optimal for individual amino acids, will sufficiently ionise all amino acids being analysed. There is a lot to consider when optimising a method or selecting the type of ion source or instrument that is best suited for the particular amino acid analysis. Automated optimisation can be utilised with the use of standards to shorted the method development time. However, as some automated programs do not optimise all MS parameters, some manual optimisation may still need to be performed. This function is dependent on the MS model and vendor.

4.2. Native amino acid analysis

The analysis of native amino acids while requiring less sample preparation, is more labour intensive when it comes to optimising parameters for maximal signal in the mass spectrometer. Amino acids are considered fragile analytes, and thus in-source fragmentation is a common problem that many analysts can encounter in initial method development and this, coupled with low ionisation efficiencies, means that mass spectrometry optimisation is essential for native amino acid analysis. It is crucial to start optimisation with highly concentrated standards (recommended concentration range from 0.5 μg/ml to 1 μg/mL) as the default parameters for some mass spectrometers may not be able to detect low levels of standards.

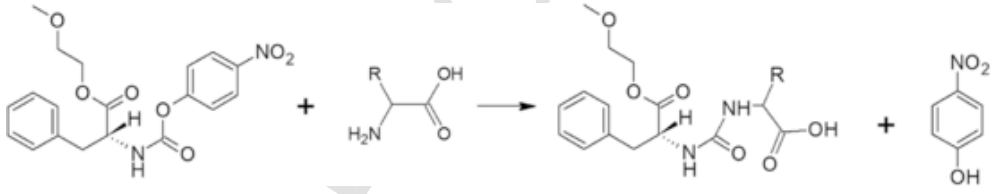

Fig. 13. (S)-NIFE derivatisation reaction with a primary amino acid.

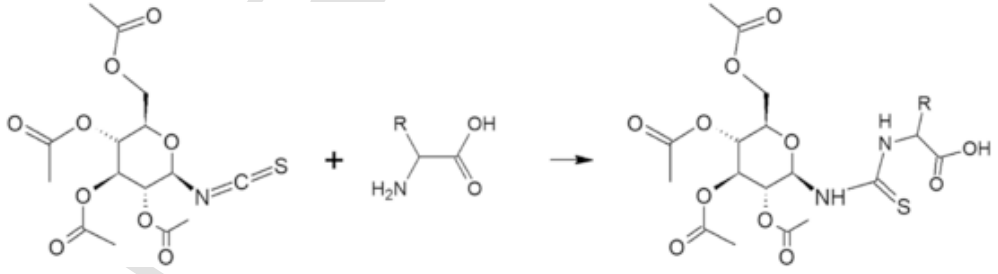

Fig. 14. GITC derivatisation reaction with a primary amino acid.

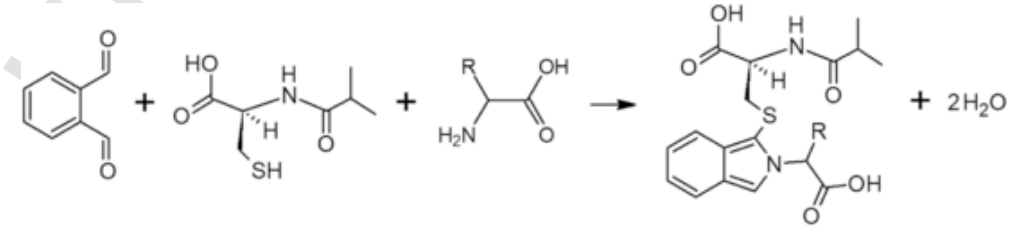

Fig. 15. OPA/IBLC derivatisation reaction with a primary amino acid.

ESI is the most widely used ionisation method for native amino acid studies [51,119–123], with APCI being employed in fewer of studies [117,124]. Comparisons between the two show mixed results as to which one is better suited to amino acid analysis [117,124], resulting in most methods continuing to use the more common ESI. One ion-pairing study utilised MIPI ionisation and made a comparison to ESI. Amino acid intensities in general were higher in MIPI however individual results varied for each amino acid analysed and for which ion-pairing agent was used [115]. Being zwitterionic, amino acids can be analysed in both positive and negative mode. The majority of studies use positive mode when analysing amino acids with only a small number of metabolic studies demonstrating the potential for analysis in untargeted negative mode [125] and one vendor technical note demonstrating its applications in targeted mass spectrometry [126]. Several metabolomic studies have detected amino acids in negative mode, however the majority state that intensities for amino acids and metabolites are generally higher in positive mode [127,128].

There are inconsistencies in the literature in source conditions used for amino acid analysis. Many state that native amino acids prefer weaker or softer source conditions; meaning conditions that are considered lower than the default parameters (i.e. lower voltages, temperatures etc.). These weaker conditions are employed to prevent or lessen in-source fragmentation [51,119,120]. However, an equal number of methods use moderate source conditions with a comparable effect [121–123]. Source conditions vary heavily with temperatures ranging from 150°C to 300°C and capillary voltages ranging from 0.8 Kv to 5.5 Kv demonstrating that source conditions need to be developed on a case by case basis, as different mass spectrometer models will require different settings for optimal amino acid intensities. Therefore, the optimal parameters from one machine may not be transferable to another.

Though source conditions are important to increase native amino acid intensities, equally important parameters include those responsible for the transfer of amino acids through the mass spectrometer. These can include other voltages or parameters applied after the capillary that are responsible for correct transmission of ions, since it is not uncommon for these parameters to cause in-source fragmentation. An example of this can be seen in most methods using the Agilent QTOF-MS with the fragmentor voltage. The fragmentor voltage is the voltage applied in-between the capillary and skimmer, and in many amino acid methods this is lowered to allow for better transmission of amino acid ions [51,129]. While this is only one example, it highlights that some higher energy systems may fail to detect amino acids without first lowering or softening of ion transmission conditions.

Buffers play an important role in both the chromatographic retention of analytes and analyte ionisation in the MS. As with source parameters there is no universal buffer for all amino acids, and while a buffer might increase an amino acid's ionisation it might not provide the chromatographic separation the analyst requires. Thus, depending on the study, the composition of the buffer may not be chosen for its effectiveness in ionisation but might be selected during the development of the chromatography. This is especially apparent when separating isomers, and generally requires a compromise on buffer conditions that yield ideal suitable chromatographic separation as well as sufficient MS intensity. For methods that don't require strict chromatographic separation like those with few non-isobaric amino acids, buffers can instead be chosen for ionisation merit.

4.3. Derivatised amino acid analysis

Compared to native amino acid analysis, derivatised amino acid analysis requires substantially less MS method development. Derivatised amino acids are usually analysed by ESI since the benefits of APCI and MIPI don't apply to the less polar compounds, however there are derivatising agents specifically designed for APCI such as p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS) [118]. Both positive and negative mode have been utilised for derivatised amino acid analysis. Similar to native amino acid methods, positive mode is employed more frequently [61,70,130]. One study utilising dibenzyl ethyl ethoxymethylene malonate (DBEMM), o-phthalaldehyde (OPA), dibenzyl ethoxymethylene malonate (DEEMM) derivatisation suggested that negative mode may help with minimizing matrix effects due to a reduction in background noise [118]. Many default MS profiles will readily detect derivatised amino acids, nevertheless, optimisation will greatly improve sensitivity. Optimised derivatisation methods greatly surpass optimised native methods with regard to limits of detection, limits of quantification and linearity. Like native amino acids, published derivatisation methods use a large variety of in source conditions with no clear trends to report [61,70,130], even among the same derivatisation methods [61,131]. Therefore, as with native amino acid analysis, source conditions should also be selected on a case by case basis, depending on what MS and derivatisation method are being employed.

4.4. Targeted analysis

If the goal is to analyse individual or a selection of known amino acids, targeted analysis will yield the best results. Targeted analysis has most frequently been applied to toxic non-protein amino acid monitoring [132] or for detection of the protein amino acids [133]. The ideal MS/MS for targeted analysis is a triple quadrupole mass spectrometer. Triple quadrupole mass spectrometers (TQMS) specialise in selected reaction monitoring (SRM), alternatively referred to as multiple reaction monitoring (MRM). SRM will only monitor specific pre-defined ion transitions to the exclusion of all other ions. This means that SRMs are highly specific and sensitive, offering the lowest limits of detection available. While SRMs are not unique to TQMS, they are best suited due to the added ion filtering from the third quadrupole. To increase confidence in identification multiple SRM transitions (the combination of quadrupole 1 (Q1) and quadrupole 3 (Q3) masses being monitored) can be established for each amino acid [132], however some methods opt to only have one SRM for each amino acid. A common trend that is seen in many derivatisation methods is only setting up one SRM for each amino acid, with the fragment selected being the fragment corresponding to the derivatisation tag as its usually the most intense [61,130]. To increase the number of available ion transitions, it is essential to carry out instrument optimisation of the intact mass before setting up SRMs. This is especially important to allow for the maximum number of potential fragments to be observed for native amino acids which have a low ionisation efficiency. This may be essential to analyse isobaric amino acids, as identification of a unique fragment ion can greatly boost the confidence in identification.

Another factor that needs to be taken into consideration is the dwell times for all the monitored transitions, especially for methods that acquire a large number over a short period of time. Dwell time is the time taken to acquire a specific SRM transition. In general, higher dwell times improve sensitivity per transition by increasing the number of ions that reach the detector, however this may reduce the number of data points across a chromatographic elution peak. The reduction in data points becomes especially problematic if switching between multiple SRMs as it may lead to poor peak shape. Poor peak shape will affect integration leading to irreproducible quantification and may also decrease sensitivity as the signal-to-noise is also affected. This can be resolved by decreasing the number of SRM transitions monitored over a set time by further optimisation of the chromatography and/or by scheduling SRMs to be monitored during the specific time window when the analyte is eluting from the column rather than over the entire run. Instruments that are capable of parallel reaction monitoring (PRM) such as the quadruple Orbitrap and QTOF do not have this is-

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sue. Whereas a SRM transition monitors one fragment, PRM will monitor all fragments from a selected parent mass, and thus has an advantage over SRMs as it can monitor more fragments from an individual amino acid without increasing the workload of the instrument [134].

4.5. Untargeted analysis

The rise in 'omics' fields such as metabolomics will see rapid growth in the untargeted analysis of amino acids to obtain more information from a sample. Untargeted approaches are utilised to their fullest when the analyst wishes to acquire more information than just a select number of amino acids. When conducting untargeted analysis high resolution mass spectrometers (HRMSs) are employed, typically a QTOF or a quadrupole Orbitrap mass spectrometer. Some fragments for smaller amino acids such as glycine may be undetectable with a quadrupole Orbitrap mass spectrometer due to their mass range not going low enough, this is not an issue for derivatised amino acids. A Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) is also applicable however has largely been replaced by Orbitraps and has only been used in a limited number of studies [135,136]. The advantages and disadvantages of untargeted analysis are the inverse of targeted analysis; it allows for the expanded acquisition of information in a sample that includes the amino acids, with a trade-off in decreased sensitivity since the increase in ions transmitted will result in higher noise.

HRMS can be operated in two different modes for the acquisition of fragmentation data; data-dependent acquisition (DDA) and data-independent acquisition (DIA) [137,138]. DDA will select a fixed number of precursor ions (typically the most abundant ions in a spectra, referred to as top N) to fragment individually and sequentially, with small isolation windows of typically ± 0.5 Da on the selected precursor ion, thus giving information about what fragment ions originate from what precursor ion. The method of DIA varies between the types of HRMSs and the vendors who supply them. DDA is often not appropriate for underivatised amino acids as they may not represent the most abundant ions in the sample matrix. This issue can be alleviated with further clean-up of a sample with techniques such as SPE, or by including the *m/z* of the desired amino acid/s in an inclusion list. Alternatively, the mass range can be limited to only include the amino acid masses [51]. However, the limiting of the mass range hinders the greatest strength of untargeted analysis which is the acquisition of non-amino acid data. DDA can be used for derivatised amino acids which have increased ionisation efficiency and intensities in the mass spectrometer. Care is still required as other molecules, such as peptides or other metabolites, can also react with the derivatisation tags and therefore have increased ionisation/intensities.

DIA can overcome these issues and is potentially the better of the two approaches for amino acid analysis due to all ions being fragmented regardless of signal intensities, thus acquiring amino acid fragmentation while not sacrificing the acquisition of data pertaining to other molecules. However, being a relatively new technique many older MS models are unable to perform DIA. As the main benefit of untargeted acquisition methods is to acquire more data than just that of the amino acids in a sample, one alternative for untargeted amino acid methods is to utilise metabolomics methods. A comparison study by Klepacki et al. (2016) [139] highlights how a metabolomic method compares to a traditional targeted amino acid method showing that there are inconsistencies between the two with the metabolomic method being inferior to the targeted amino acid method since it is unable to detect select amino acids or separate them from the noise.

5. Limitations and future directions

5.1. Limitations

The potential applications for amino acid analysis via LC-MS/MS are quite broad, however some current limitations have prevented the technique from being utilised to its highest potential. The biggest hurdle to furthering the application of LC-MS/MS to amino acid analysis is the need for a straightforward and highly sensitive method for amino acid derivatisation. While native analysis is possible, it currently has inferior sensitivity compared to derivatised analysis and requires a higher level of expertise. Derivatisation is more user-friendly but involves increased sample preparation time and increased cost, with most derivatisation reagents sold as expensive kits. Another limitation and oversight in the field is enantiomer analysis. Currently most methods do not discriminate between enantiomers, potentially reporting them as the same molecule. Some studies do separate enantiomers using chromatography, typically through the employment of specific techniques (See Section 3.3 2.4), however, this is far from the common practice. Analysing both D and L amino acids is becoming increasingly important as D-amino acids are shown to have crucial biological roles (see Section 1.2) [140].

5.2. Alternative chromatography options

One of the major drawbacks of native amino acid analysis is the difficulty in developing sensitive and reliable chromatography. Alternative chromatographic techniques may both shorten analysis time, simplify sample preparation or optimisation, and avoid issues that plague traditional chromatography. There has been a growing interest in supercritical fluid chromatography (SFC) as an alternative method to HILIC. Supercritical fluids are substances (most commonly CO_2) that at a certain temperature and pressure reach a critical point where a distinction between gases and liquids is non-existent. SFC has several advantages over traditional chromatography including increased flow rates, more effective separation, especially of chiral molecules, and a reduction in matrix effects [141], however due to the extremely non-polar nature of CO_2, the analysis of polar molecules may be difficult. The addition of small percentages of organic modifiers to the supercritical fluid however can alleviate this to some extent.

Wolrab et al. [124] demonstrated the potential of SFC-MS/MS for the analysis of the protein amino acids, however, serine and glycine could not be detected, which may indicate that this technique might not be easily applicable to all amino acids. Further development of currently underutilised chromatographic techniques coupled to MS, such as chiral column chromatography (CCC), ion exchange chromatography (IC) and aqueous normal phase chromatography (ANP), have the potential to yield better results than the native amino acid chromatographic methods currently used in LC-MS/MS. CCC has shown promise in separating enantiomers, while the low buffer concentration of ANP may make it a suitable alternative to replace the more popular HILIC that generally uses high buffer concentrations that can lead to ion suppression [66]. Development of non-chromatographic separation techniques might also be a viable alternative to those currently used as capillary electrophoresis (CE) has shown great potential in the separation of enantiomers [142]. A combination of different techniques may also yield improved results. Raimbault et al. [143] combined SFC and HPLC (referred to as unified chromatography) with a CSP to achieve enantioselectivity for 16 of the protein amino acids. This method required further improvement as they were unable to detect cysteine, could not separate stereoisomers and reported poor peak shape for some amino acids. However, a later study by Raimbault et al. [144] using the same unified chromatography and stationary phase which didn't focus on enantiomer separation improved the poor peak shape and the separa-

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tion of stereoisomers by broadening the gradient and using methanesulfonic acid (MSA) as a mobile phase additive.

5.3. Advancements in mass spectrometry (Ion mobility spectrometry-MS/MS)

Further advancement in MS technology and capabilities is another avenue that might overcome the current limitations of amino acid analysis by LC-MS/MS. An increased interest in metabolomics has resulted in the development of new mass spectrometers designed with small molecule analysis in mind, and thus could potentially reduce the time required to optimise acquisition methods for amino acids. Differing enantiomers and constitutional isomers are problematic using current MS methods unless specific chromatography is used to separate these compounds. Ion mobility spectrometry (IMS) coupled to MS is becoming a common technique that adds an extra dimension of separation to LC-MS/MS methods. Four main variants of IMS are used with MS, these being traveling wave ion mobility spectrometry (TWIMS), drift-time ion mobility spectrometry (DTIMS), trapped ion mobility spectrometry (TIMS) and differential mobility spectrometry (DMS) (otherwise known as field asymmetric-waveform ion-mobility spectrometry (FAIMS)). TWIMS measures the movement of an ion through a gas filled cell that has a direct current (DC) and radio-frequency (RF) voltages applied to a series of ring electrodes throughout which causes these ions to travel in a wave. Similarly, DTIMS measures ion drift through a gas filled tube without an applied voltage. TIMS measures the mobility of ions inversely to that of DTIMS and TWIMS; instead of ions traveling through a gas filled cell, the ions are held in place with the gas moving though the cell. DTIMS, TWIMS and TIMS measure the mobility of ions by a collision cross-section (CCS) value. DMS does not give a CCS value as the RF field distorts the ion conformation. DMS utilises static DC and asymmetric RF waveforms applied between two electrodes; the ion will then move toward one of the electrodes. By changing the DC voltage (compensation voltage or CV), an ion will travel between the two electrodes to the detector. Only ions with a specific mobility will pass through to the detector filtering out other ions. Thus, unlike DTIMS and TWIMS which transmits all ions, DMS only transmits desired ions resulting in a lower sensitivity compared to the other two IMS variants and is therefore usually reserved for targeted studies.

In principle, enantiomers and constitutional isomers have the same mass and will have almost identical retention time and fragmentation patterns in traditional LC-MS/MS. In IMS however their ion mobility may be different, thus adding another layer of separation and qualification to the analysis without the need for extensive chromatography optimisation. However, mobility values aren't fixed and certain parameters such as the voltages applied, the carrier gas in the IMS cell and gas modifiers (low organic solvents) can be modified to improve the separation of similar compounds [145].

The majority of IMS-MS/MS analyses of native amino acids have employed DMS. DMS was able to successfully separate isoleucine and leucine [146], and a more recent investigation of 20 of the protein amino acids demonstrated its use in targeted amino acid analysis by adding CV as a third criteria for identification for more accurate qualification [147]. TWIMS has also been used in native amino acid studies, with Jones et al. [148] reporting multiple CCS values for each of the 20 protein amino acids tested under different carrier gas and voltage conditions. While constitutional isomer separation has been demonstrated, there has been a lack of studies on the use of IMS for the analysis of amino acid enantiomers. A study by Dwivedi et al. utilised a chiral modifier to demonstrate limited enantiomeric separation of several amino acids [149]. There have also been multiple studies which have used the formation of amino acid-metal-chiral reference complexes to increase the separation of differing enantiomers in TWIMS [150,151]. Studies on the use of IMS for the analysis of derivatised amino acids are scarce, with one report on (S)-NIFE derivatised amino acid analysis via LC-TWIMS-MS/MS [68]. Another TWIMS study utilised FMOC in conjunction with the complexes mentioned above and found that while there was slight enantiomer differentiation, it was not increased compared to the corresponding native amino acid results of the same study [150]. FLEC derivatisation with TIMS was able to achieve partial to complete separation for seventeen of the twenty one amino acids examined [152]. IMS is increasingly being sold as an add-on by many MS Vendors, with some MS models having inbuilt IMS capabilities.

5.4. Applications of amino acid analysis by LC-MS/MS

Currently, amino acid analysis via LC-MS/MS is primarily a tool for research; however, there is excellent potential in applying it to clinical and environmental testing applications. Amino acid analysis via LC-MS/MS is becoming more prevalent in environmental testing laboratories with the increase in discovery of toxic non-protein amino acids. The best method for the detection of several toxic amino acids is LC-MS/MS [70,153]. If regulations for levels of toxic amino acids in the environment come into force, there will be an increased need to analyse them with the most accurate and sensitive method available. In addition, LC-MS/MS also has great potential in diagnostic applications in a clinical setting. Currently, its biggest role is its use in the detection of inborn errors of metabolism (IEM); genetically inherited diseases that cause irregularities to the body's metabolism. One example of an IEM diagnosed with LC-MS/MS amino acid analysis is phenylketonuria (PKU), which is asymptomatic at birth but if left untreated can lead to a multitude of disabilities. As PKU results in a decrease in the metabolism of phenylalanine, newborn screening programs utilise amino acid analysis via LC-MS/MS to detect the condition early so treatment can be applied to the newborn as soon as possible allowing the individual to have a normal healthy life if a strict diet is maintained continuously [23]. This demonstrates the potential of amino acid analysis via LC-MS/MS in a clinical setting however currently LC-MS/MS still has many issues to overcome before becoming common practice in a clinical setting. An overview of why LC-MS isn't more widely utilised in the clinic and its current limitations is provided by Vogeser and Seger (2010) [154]. Amino acid analysis is an important component of metabolomics however metabolomics is still an emerging field compared to other omics fields [155]. While metabolomic methods currently are inferior at detecting amino acids compared to amino acid specific methods, further developments in metabolomics may one day render amino acid specific methods obsolete.

6. Concluding statements

LC-MS/MS is a powerful technique for the analysis of amino acids however it is limited by the most sensitive methods requiring sample derivatisation, and the limited options that allow the analysis of amino acid enantiomers. While native amino acid analysis can be achieved, it requires substantially more method development and lacks the sensitivity of derivatisation methods. Studies showing the importance of chiral analysis have been increasing, having importance to a large array of different fields. However, like native amino acid analysis, chiral analysis is riddled with complications typically being more costly and requiring specific resources and knowledge to allow for the successful analysis of amino acid enantiomers. However, with the rise in interest in small molecule quantification and the development/application of new or underused technologies, these issues may soon no longer be limiting factors and will further push the applications of amino acid analysis via LC-MS/MS into more widespread applications.

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Declaration of competing interest

The authors declare no conflict of interest.

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References

[1] A D McNaught, A D McNaught, Compendium of Chemical Terminology, 1669, Blackwell Science Oxford, 1997.
[2] R Barret, 2 - Importance and evaluation of the pKa, in: R Barret (Ed.), Therapeutical Chemistry, Elsevier, 2018, pp. 21–51.
[3] P J Reeds, Dispensable and indispensable amino acids for humans, J. Nutr. 130 (7) (2000) 1835S–1840S.
[4] P O Larsen, 6 - Physical and chemical properties of amino acids, in: B J Miflin (Ed.), Amino Acids and Derivatives, Academic Press, 1980, pp. 225–269.
[5] K J Rodgers, Non-protein amino acids and neurodegeneration: the enemy within, Exp. Neurol. 253 (2014) 192–196.
[6] N V Bhagavan, C-E Ha, Chapter 3 – Amino acids, in: N V Bhagavan, C-E Ha (Eds.), Essentials of Medical Biochemistry, second ed., Academic Press, San Diego, 2015, pp. 21–29.
[7] Y Kiriyama, H Nochi, D-amino acids in the nervous and endocrine systems, Scientifica 2016 (2016) 9.
[8] D V Grishin, D D Zhdanov, M V Pokrovskaya, N N Sokolov, D-amino acids in nature, agriculture and biomedicine, Front. Life Sci. 13 (1) (2020) 11–22.
[9] J M Berg, J L Tymoczko, L Stryer, Biochemistry, 38, fifth ed., WH Freeman, New York, 2006, p. 76 (894).
[10] S K Kuncha, S P Kruparani, R Sankaranarayanan, Chiral checkpoints during protein biosynthesis, J. Biol. Chem. (2019).
[11] I Wagner, H Musso, New naturally occurring amino acids, Angew Chem. Int. Ed. Engl. 22 (11) (1983) 816–828.
[12] W Leuchtenberger, K Huthmacher, K Drauz, Biotechnological production of amino acids and derivatives: current status and prospects, Appl. Microbiol. Biotechnol. 69 (1) (2005) 1–8.
[13] T J Bullwinkle, N M Reynolds, M Raina, A Moghal, E Matsa, A Rajkovic, H Kayadibi, F Fazlollahi, C Ryan, N Howitz, K F Faull, B A Lazazzera, M Ibba, Oxidation of cellular amino acid pools leads to cytotoxic mistranslation of the genetic code, Elife 3 (2014) e02501.
[14] S W Chan, R A Dunlop, A Rowe, K L Double, K J Rodgers, L-DOPA is incorporated into brain proteins of patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro, Exp. Neurol. 238 (1) (2012) 29–37.
[15] K J Rodgers, N Shiozawa, Misincorporation of amino acid analogues into proteins by biosynthesis, Int. J. Biochem. Cell Biol. 40 (8) (2008) 1452–1466.
[16] R D Süssmuth, A Mainz, Nonribosomal peptide synthesis—principles and prospects, Angew. Chem. Int. Ed. 56 (14) (2017) 3770–3821.
[17] J J Galligan, P J Kingsley, O R Wauchope, M M Mitchener, J M Camarillo, J A Wepy, P S Harris, K S Fritz, L J Marnett, Quantitative analysis and discovery of lysine and arginine modifications, Anal. Chem. 89 (2) (2017) 1299–1306.
[18] F Hirche, A Schroder, B Knoth, G I Stangl, K Eder, Effect of dietary methionine on plasma and liver cholesterol concentrations in rats and expression of hepatic genes involved in cholesterol metabolism, Br. J. Nutr. 95 (5) (2006) 879–888.
[19] L Taylor, N P Curthoys, Glutamine metabolism: role in acid-base balance*, Biochem. Mol. Biol. Educ. 32 (5) (2004) 291–304.
[20] L Szabados, A Savouré, Proline: a multifunctional amino acid, Trends Plant Sci. 15 (2) (2010) 89–97.
[21] D T Balu, A C Basu, J P Corradi, A M Cacace, J T Coyle, The NMDA receptor co-agonists, D-serine and glycine, regulate neuronal dendritic architecture in the somatosensory cortex, Neurobiol. Dis. 45 (2) (2012) 671–682.
[22] L K Bak, A Schousboe, H S Waagepetersen, The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer, J. Neurochem. 98 (3) (2006) 641–653.
[23] J Qu, Z P Wu, G A Luo, M X Liu, C D Yang, Newborn screening of phenylketonuria by LC/MS/MS, Chem. J. Chinese U. 21 (2000).
[24] F Lopes Sakamoto, R Metzker Pereira Ribeiro, A Amador Bueno, H Oliveira Santos, Psychotropic effects of L-theanine and its clinical properties: from the management of anxiety and stress to a potential use in schizophrenia, Pharmacol. Res. 147 (2019) 104395.
[25] T D Levine, R G Miller, W G Bradley, D H Moore, D S Saperstein, L E Flynn, J S Katz, D A Forshew, J S Metcalf, S A Banack, P A Cox, Phase I clinical trial of safety of L-serine for ALS patients, Amyotroph. Lateral Scler. Frontotemporal Degener. 18 (1–2) (2017) 107–111.
[26] B J Main, R A Dunlop, K J Rodgers, The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress in vitro, Toxicon 109 (Suppl. C) (2016) 7–12.
[27] S Chen, R Gopalakrishnan, T Schaer, F Marger, R Hovius, D Bertrand, F Pojer, C Heinis, Dithiol amino acids can structurally shape and enhance the ligand-binding properties of polypeptides, Nat. Chem. 6 (2014) 1009.
[28] A Karau, I Grayson, Amino acids in human and animal nutrition, Adv. Biochem. Eng. Biotechnol. 143 (2014) 189–228.
[29] K Samardzic, K J Rodgers, Cytotoxicity and mitochondrial dysfunction caused by the dietary supplement l-norvaline, Toxicol. In Vitro 56 (2019) 163–171.
[30] K Rodgers, K Samardzic, B Main, Toxic Nonprotein Amino Acids, 2017, pp. 263–285.
[31] K J Rodgers, B J Main, K Samardzic, Cyanobacterial neurotoxins: their occurrence and mechanisms of toxicity, Neurotox. Res. 33 (1) (2018) 168–177.
[32] D V S Rao, M A Quilliam, R Pocklington, Domoic acid—a neurotoxic amino acid produced by the marine Diatom Nitzschia pungens in culture, Can. J. Fish. Aquat. Sci. 45 (12) (1988) 2076–2079.
[33] E Rubenstein, Misincorporation of the proline analog azetidine-2-carboxylic acid in the pathogenesis of multiple sclerosis: a hypothesis, J. Neuropathol. Exp. Neurol. 67 (11) (2008) 1035–1040.
[34] N Torbick, B Ziniti, E Stommel, E Linder, A Andrew, T Caller, J Haney, W Bradley, P L Henegan, X Shi, Assessing cyanobacterial harmful algal blooms as risk factors for amyotrophic lateral sclerosis, Neurotox. Res. 33 (1) (2018) 199–212.
[35] L Jiang, B Aigret, W M De Borggraeve, Z Spacil, L L Ilag, Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples, Anal. Bioanal. Chem. 403 (6) (2012) 1719–1730.
[36] S Moore, W H Stein, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem. 176 (1) (1948) 367–388.
[37] J Regueiro, N Negreira, A Carreira-Casais, C Pérez-Lamela, J Simal-Gándara, Dietary exposure and neurotoxicity of the environmental free and bound toxin β-N-methylamino-L-alanine, Food Res. Int. 100 (Part 1) (2017) 1–13.
[38] S M Rutherfurd, G S Gilani, Amino Acid Analysis, Current Protocols in Protein Science, 2009 (Chapter 11), Unit 11.9.
[39] S A Bhawani, M N Mohamad Ibrahim, O Sulaiman, R Hashim, A Mohammad, S Hena, Thin-layer chromatography of amino acids: a review, J. Liq. Chromatogr. Relat. Technol. 35 (11) (2012) 1497–1516.
[40] G D Zunic, S Spasic, Z Jelic-Ivanovic, Capillary electrophoresis of free amino acids in physiological fluids without derivatization employing direct or indirect absorbance detection, Methods Mol. Biol. 828 (2012) 243–254.
[41] M Castro-Puyana, A L Crego, M L Marina, C Garcia-Ruiz, CE methods for the determination of non-protein amino acids in foods, Electrophoresis 28 (22) (2007) 4031–4045.
[42] I Aretz, D Meierhofer, Advantages and pitfalls of mass spectrometry based metabolome profiling in systems biology, Int. J. Mol. Sci. 17 (5) (2016) 632.
[43] H Kaspar, K Dettmer, Q Chan, S Daniels, S Nimkar, M L Daviglus, J Stamler, P Elliott, P J Oefner, Urinary amino acid analysis: a comparison of iTRAQ-LC-MS/MS, GC-MS, and amino acid analyzer, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (20–21) (2009) 1838–1846.
[44] J Matysiak, P Dereziński, A Klupczyńska, J Matysiak, E Kaczmarek, Z J Kokot, Effects of a honeybee sting on the serum free amino acid profile in humans, PLoS One 9 (7) (2014) e103533.
[45] Z T How, F Busetti, K L Linge, I Kristiana, C A Joll, J W A Charrois, Analysis of free amino acids in natural waters by liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1370 (2014) 135–146.
[46] G W Sedgwick, T W Fenton, J R Thompson, Effect of protein precipitating agents on the recovery of plasma free amino acids, Can. J. Anim. Sci. 71 (3) (1991) 953–957.
[47] J Sostare, R Di Guida, J Kirwan, K Chalal, E Palmer, W B Dunn, M R Viant, Comparison of modified Matyash method to conventional solvent systems for polar metabolite and lipid extractions, Anal. Chim. Acta 1037 (2018) 301–315.
[48] E Arnaiz, J Bernal, M T Martin, M J Nozal, J L Bernal, L Toribio, Supercritical fluid extraction of free amino acids from broccoli leaves, J. Chromatogr. A 1250 (2012) 49–53.
[49] L Wang, R Xu, B Hu, W Li, Y Sun, Y Tu, X Zeng, Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction, Food Chem. 123 (4) (2010) 1259–1266.
[50] X Tang, Y Gu, J Nie, S Fan, C Wang, Quantification of amino acids in rat urine by solid-phase extraction and liquid chromatography/electrospray tandem mass spectrometry: application to radiation injury rat model, J. Liq. Chromatogr. Relat. Technol. 37 (7) (2014) 951–973.
[51] M Armstrong, K Jonscher, N A Reisdorph, Analysis of 25 underivatized amino acids in human plasma using ion-pairing reversed-phase liquid chromatography/time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 21 (16) (2007) 2717–2726.
[52] J R Lill, E S Ingle, P S Liu, V Pham, W N Sandoval, Microwave-assisted proteomics, Mass Spectrom. Rev. 26 (5) (2007) 657–671.
[53] I Davidson, Hydrolysis of samples for amino acid analysis, in: B J Smith (Ed.), Protein Sequencing Protocols, Humana Press, Totowa, NJ, 2003, pp. 111–122.
[54] A P Williams, Amino acids determination, in: B Caballero (Ed.), Encyclopedia of Food Sciences and Nutrition, second ed., Academic Press, Oxford, 2003, pp. 192–197.
[55] R L Hill, W R Schmidt, The complete enzymic hydrolysis of proteins, J. Biol. Chem. 237 (1962) 389–396.
[56] X Wang, H Wu, R Luo, D Xia, Z Jiang, H Han, Separation and detection of free D- and L-amino acids in tea by off-line two-dimensional liquid chromatography, Anal. Methods 9 (43) (2017) 6131–6138.
[57] M Schwaiger, E Rampler, G Hermann, W Miklos, W Berger, G Koellensperger, Anion-exchange chromatography coupled to high-resolution mass spectrometry: a powerful tool for merging targeted and non-targeted metabolomics, Anal. Chem. 89 (14) (2017) 7667–7674.

UNCORRECTED PROOF

[58] P Chaimbault, K Petritis, C Elfakir, M Dreux, Determination of 20 underivatized proteinic amino acids by ion-pairing chromatography and pneumatically assisted electrospray mass spectrometry, J. Chromatogr. A 855 (1) (1999) 191–202.

[59] L Guo, A J Worth, C Mesaros, N W Snyder, J D Glickson, I A Blair, Diisopropylethylamine/hexafluoroisopropanol-mediated ion-pairing ultra-high-performance liquid chromatography/mass spectrometry for phosphate and carboxylate metabolite analysis: utility for studying cellular metabolism, Rapid Commun. Mass Spectrom. 30 (16) (2016) 1835–1845.

[60] B Buszewski, S Noga, Hydrophilic interaction liquid chromatography (HILIC) – a powerful separation technique, Anal. Bioanal. Chem. 402 (1) (2012) 231–247.

[61] C Salazar, J M Armenta, V Shulaev, An UPLC-ESI-MS/MS assay using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: application to screening of *Arabidopsis thaliana* mutants, Metabolites 2 (3) (2012) 398–428.

[62] G Greco, T Letzel, Main interactions and influences of the chromatographic parameters in HILIC separations, J. Chromatogr. Sci. 51 (7) (2013) 684–693.

[63] Y Prokopenko, V Jakštas, V Žvikas, V Georgiyants, L Ivanauskas, Hilic MS/MS determination of amino acids in herbs of *Fumaria schleicheri* L., *Ocimum basilicum* L., and leaves of *Corylus avellana* L, Nat. Prod. Res. 33 (13) (2019) 1961–1963.

[64] Z Chen, F Lin, X Ye, Y Dong, L Hu, A Huang, Simultaneous determination of five essential amino acids in plasma of hyperlipidemic subjects by UPLC-MS/MS, Lipids Health Dis. 19 (1) (2020) 52.

[65] J C Heaton, D V McCalley, Some factors that can lead to poor peak shape in hydrophilic interaction chromatography, and possibilities for their remediation, J. Chromatogr. A 1427 (2016) 37–44.

[66] C Hellmuth, B Koletzko, W Peissner, Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 879 (1) (2011) 83–89.

[67] D J Dietzen, A L Weindel, M O Carayannopoulos, M Landt, E T Normansell, T E Reimschisel, C H Smith, Rapid comprehensive amino acid analysis by liquid chromatography/tandem mass spectrometry: comparison to cation exchange with post-column ninhydrin detection, Rapid Commun. Mass Spectrom. 22 (22) (2008) 3481–3488.

[68] H Tian, N Zheng, S Li, Y Zhang, S Zhao, F Wen, J Wang, Characterization of chiral amino acids from different milk origins using ultra-performance liquid chromatography coupled to ion-mobility mass spectrometry, Sci. Rep. 7 (2017) 46289.

[69] P Krumpochova, B Bruyneel, D Molenaar, A Koukou, M Wuhrer, W M A Niessen, M Giera, Amino acid analysis using chromatography–mass spectrometry: an inter platform comparison study, J. Pharmaceut. Biomed. Anal. 114 (2015) 398–407.

[70] T C Baker, F J M Tymm, S J Murch, Assessing environmental exposure to beta-N-Methylamino-L-Alanine (BMAA) in complex sample matrices: a comparison of the three most popular LC-MS/MS methods, Neurotox. Res. (2017).

[71] K Dettmer, A P Stevens, S R Fagerer, H Kaspar, P J Oefner, Amino acid analysis in physiological samples by GC–MS with propyl chloroformate derivatization and iTRAQ–LC–MS/MS, in: M A Alterman, P Hunziker (Eds.), Amino Acid Analysis: Methods and Protocols, Humana Press, Totowa, NJ, 2012, pp. 165–181.

[72] A A Badawy, The EZ:Faast family of amino acid analysis kits: application of the GC-FID kit for rapid determination of plasma tryptophan and other amino acids, Methods Mol. Biol. 2030 (2019) 119–130.

[73] A Jámbor, I Molnár-Perl, Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride: literature overview and further study, J. Chromatogr. A 1216 (15) (2009) 3064–3077.

[74] P Uutela, R A Ketola, P Piepponen, R Kostiainen, Comparison of different amino acid derivatives and analysis of rat brain microdialysates by liquid chromatography tandem mass spectrometry, Anal. Chim. Acta 633 (2) (2009) 223–231.

[75] S Aggarwal, A Yadav, Dissecting the iTRAQ Data Analysis, 2015, pp. 277–291.

[76] F Boemer, M Deberg, R Schoos, E Baise, H Amory, G Gault, J Carlier, Y Gaillard, C Marcillaud-Pitel, D Votion, Quantification of hypoglycin A in serum using aTRAQ® assay, J. Chromatogr. B 997 (2015) 75–80.

[77] R Rebane, M-L Oldekop, K Herodes, Comparison of amino acid derivatization reagents for LC–ESI-MS analysis. Introducing a novel phosphazene-based derivatization reagent, J. Chromatogr. B 904 (2012) 99–106.

[78] K Shimbo, A Yahashi, K Hirayama, M Nakazawa, H Miyano, Multifunctional and highly sensitive precolumn reagents for amino acids in liquid chromatography/tandem mass spectrometry, Anal. Chem. 81 (13) (2009) 5172–5179.

[79] M Alaiz, J L Navarro, J Girón, E Vioque, Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate, J. Chromatogr. A 591 (1) (1992) 181–186.

[80] R Rebane, T Rodima, A Kutt, K Herodes, Development of amino acid derivatization reagents for liquid chromatography electrospray ionization mass spectrometric analysis and ionization efficiency measurements, J. Chromatogr. A 1390 (2015) 62–70.

[81] M Song, T-J Hang, C Wang, L Yang, A-D Wen, Precolumn derivatization LC–MS/MS method for the determination and pharmacokinetic study of glucosamine in human plasma and urine, J. Pharm. Anal. 2 (1) (2012) 19–28.

[82] A Elbashir, F O Suliman, H Aboul-Enein, The application of 7-chloro-4-nitrobenzoxadiazole and 4-fluoro-7-nitro-2,1,3-benzoxadiazole for the analysis of amines and amino acids using high-performance liquid chromatography, Gazi Univ. J. Sci. 24 (2011) 679–697.

[83] J Bastings, H M van Eijk, S W Olde Damink, S S Rensen, D-Amino acids in health and disease: a focus on cancer, Nutrients 11 (9) (2019).

[84] S C Moldoveanu, V David, Chapter 10 – Stationary phases and columns for chiral chromatography, in: S C Moldoveanu, V David (Eds.), Selection of the HPLC Method in Chemical Analysis, Elsevier, Boston, 2017, pp. 363–376.

[85] M J Desai, D W Armstrong, Analysis of native amino acid and peptide enantiomers by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, J. Mass Spectrom. 39 (2) (2004) 177–187.

[86] K Petritis, A Valleix, C Elfakir, M Dreux, Simultaneous analysis of underivatized chiral amino acids by liquid chromatography–ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase, J. Chromatogr. A 913 (1) (2001) 331–340.

[87] Y Konya, M Taniguchi, M Furuno, Y Nakano, N Tanaka, E Fukusaki, Mechanistic study on the high-selectivity enantioseparation of amino acids using a chiral crown ether-bonded stationary phase and acidic, highly organic mobile phase by liquid chromatography/time-of-flight mass spectrometry, J. Chromatogr. A 1578 (2018) 35–44.

[88] R Hellinger, J Horak, W Lindner, Enantioseparation of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate tagged amino acids and other zwitterionic compounds on cinchona-based chiral stationary phases, Anal. Bioanal. Chem. 405 (25) (2013) 8105–8120.

[89] C Fontanarosa, F Pane, N Sepe, G Pinto, M Trifuoggi, M Squillace, F Errico, A Usiello, P Pucci, A Amoresano, Quantitative determination of free D-Asp, L-Asp and N-methyl-D-aspartate in mouse brain tissues by chiral separation and multiple reaction monitoring tandem mass spectrometry, PLoS One 12 (6) (2017) e0179748.

[90] C Ishii, T Akita, M Mita, T Ide, K Hamase, Development of an online two-dimensional high-performance liquid chromatographic system in combination with tandem mass spectrometric detection for enantiomeric analysis of free amino acids in human physiological fluid, J. Chromatogr. A 1570 (2018) 91–98.

[91] K Hamase, A Morikawa, T Ohgusu, W Lindner, K Zaitsu, Comprehensive analysis of branched aliphatic D-amino acids in mammals using an integrated multi-loop two-dimensional column-switching high-performance liquid chromatographic system combining reversed-phase and enantioselective columns, J. Chromatogr. A 1143 (1–2) (2007) 105–111.

[92] B W J Pirok, D R Stoll, P J Schoenmakers, Recent developments in two-dimensional liquid chromatography: fundamental improvements for practical applications, Anal. Chem. 91 (1) (2019) 240–263.

[93] R-C Moldovan, E Bodoki, A-C Servais, J Crommen, R Oprean, M Fillet, (+) or (−)-1-(9-fluorenyl)ethyl chloroformate as chiral derivatizing agent: a review, J. Chromatogr. A 1513 (2017) 1–17.

[94] R Bhushan, H Bruckner, Marfey's reagent for chiral amino acid analysis: a review, Amino Acids 27 (3–4) (2004) 231–247.

[95] W F Visser, N M Verhoeven-Duif, R Ophoff, S Bakker, L W Klomp, R Berger, T J de Koning, A sensitive and simple ultra-high-performance-liquid chromatography-tandem mass spectrometry based method for the quantification of D-amino acids in body fluids, J. Chromatogr. A 1218 (40) (2011) 7130–7136.

[96] S Hess, K R Gustafson, D J Milanowski, E Alvira, M A Lipton, L K Pannell, Chirality determination of unusual amino acids using precolumn derivatization and liquid chromatography-electrospray ionization mass spectrometry, J. Chromatogr. A 1035 (2) (2004) 211–219.

[97] N J Ayon, A D Sharma, W G Gutheil, LC-MS/MS-Based separation and quantification of Marfey's reagent derivatized proteinogenic amino acid DL-stereoisomers, J. Am. Soc. Mass Spectrom. 30 (3) (2019) 448–458.

[98] R Bhushan, H Bruckner, Use of Marfey's reagent and analogs for chiral amino acid analysis: assessment and applications to natural products and biological systems, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879 (29) (2011) 3148–3161.

[99] J Walker, The Protein Protocols Handbook, 2009.

[100] S Wei, W Zhang, Z Ji, Structure and antibacterial activity of ambobactin, a new telomycin-like cyclic depsipeptide antibiotic produced by *Streptomyces ambofaciens* F3 20 (9) (2015) 16278–16289.

[101] A Prior, R C Moldovan, J Crommen, A C Servais, M Fillet, G J de Jong, G W Somsen, Enantioselective capillary electrophoresis-mass spectrometry of amino acids in cerebrospinal fluid using a chiral derivatizing agent and volatile surfactant, Anal. Chim. Acta 940 (2016) 150–158.

[102] T Yokoyama, M Tokuda, M Amano, K Mikami, Simultaneous determination of primary and secondary D-and L-amino acids by reversed-phase high-performance liquid chromatography using pre-column derivatization with two-step labelling method, Biosci. Biotechnol. Biochem. 81 (2017) 1681–1686.

[103] T Yokoyama, M Tokuda, M Amano, K Mikami, Simultaneous determination of primary and secondary D- and L-amino acids by reversed-phase high-performance liquid chromatography using pre-column derivatization with two-step labelling method, Biosci. Biotechnol. Biochem. 81 (9) (2017) 1681–1686.

[104] R R Walters, S C Buist, Improved enantioselective method for the determination of the enantiomers of reboxetine in plasma by solid-phase extraction, chiral derivatization, and column-switching high-performance liquid chromatography with fluorescence detection, J. Chromatogr. A 828 (1) (1998) 167–176.

[105] R-C Moldovan, E Bodoki, A-C Servais, J Crommen, R Oprean, M Fillet, Selectivity evaluation of phenyl based stationary phases for the analysis of amino acid diastereomers by liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1590 (2019) 80–87.

[106] E Okuma, H Abe, Simultaneous determination of D- and L-amino acids in the nervous tissues of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl. 660 (2) (1994) 243–250.

UNCORRECTED PROOF

[107] Y Bergqvist, M Doverskog, J Al Kabbani, High-performance liquid chromatographic determination of (SR)- and (RS)-enantiomers of mefloquine in plasma and capillary blood sampled on paper after derivatization with (−)-1-(9-fluorenyl)ethyl chloroformate, J. Chromatogr. B Biomed. Sci. Appl. 652 (1) (1994) 73–81.
[108] M Danielsen, C Nebel, K T Dalsgaard, Simultaneous determination of L- and D-amino acids in proteins: a sensitive method using hydrolysis in deuterated acid and liquid chromatography–tandem mass spectrometry analysis, Foods 9 (3) (2020).
[109] A Peter, E Vekes, G Toth, D Tourwe, F Borremans, Application of a new chiral derivatizing agent to the enantioseparation of secondary amino acids, J. Chromatogr. A 948 (1–2) (2002) 283–294.
[110] N Nimura, H Ogura, T Kinoshita, Reversed-phase liquid chromatographic resolution of amino acid enantiomers by derivatization with 2,3,4,6-tetra-O-acetyl-β-d-glucopyranosyl isothiocyanate, J. Chromatogr. A 202 (3) (1980) 375–379.
[111] F Berthias, B Maatoug, G L Glish, F Moussa, P Maitre, Resolution and assignment of differential ion mobility spectra of sarcosine and isomers, J. Am. Soc. Mass Spectrom. 29 (4) (2018) 752–760.
[112] M P Jedrychowski, E L Huttlin, W Haas, M E Sowa, R Rad, S P Gygi, Evaluation of HCD- and CID-type fragmentation within their respective detection platforms for murine phosphoproteomics, Mol. Cell. Proteomics 10 (12) (2011) M111.009910.
[113] P Zhang, W Chan, I L Ang, R Wei, M M T Lam, K M K Lei, T C W Poon, Revisiting fragmentation reactions of protonated α-amino acids by high-resolution electrospray ionization tandem mass spectrometry with collision-induced dissociation, Sci. Rep. 9 (1) (2019) 6453.
[114] F Rogalewicz, Y Hoppilliard, G Ohanessian, Fragmentation mechanisms of α-amino acids protonated under electrospray ionization: a collisional activation and ab initio theoretical study 11 dedicated to Bob Squires for his many seminal contributions to mass spectrometry and ion chemistry, Int. J. Mass Spectrom. 195–196 (2000) 565–590.
[115] J-Y Kwon, M Moini, Analysis of underivatized amino acid mixtures using high performance liquid chromatography/dual oscillating nebulizer atmospheric pressure microwave induced plasma ionization-mass spectrometry, J. Am. Soc. Mass Spectrom. 12 (1) (2001) 117–122.
[116] P Dallakian, H Budzikiewicz, Gas chromatography—chemical ionization mass spectrometry in amino acid analysis of pyoverdins, J. Chromatogr. A 787 (1) (1997) 195–203.
[117] M J Desai, D W Armstrong, Analysis of native amino acid and peptide enantiomers by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, J. Mass Spectrom. 39 (2) (2004) 177–187.
[118] M L Oldekop, R Rebane, K Herodes, Dependence of matrix effect on ionization polarity during LC-ESI-MS analysis of derivatized amino acids in some natural samples, Eur. J. Mass Spectrom. 23 (5) (2017) 245–253.
[119] R Joyce, V Kuziene, X Zou, X Wang, F Pullen, R L Loo, Development and validation of an ultra-performance liquid chromatography quadrupole time of flight mass spectrometry method for rapid quantification of free amino acids in human urine, Amino Acids 48 (1) (2016) 219–234.
[120] H C M T Prinsen, B G M Schiebergen-Bronkhorst, M W Roeleveld, J J M Jans, M G M de Sain-van der Velden, G Visser, P M van Hasselt, N M Verhoeven-Duif, Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry, J. Inherit. Metab. Dis. 39 (5) (2016) 651–660.
[121] A Le, A Ng, T Kwan, K Cusmano-Ozog, T M Cowan, A rapid, sensitive method for quantitative analysis of underivatized amino acids by liquid chromatography-tandem mass spectrometry (LC-MS/MS), J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 944 (2014) 166–174.
[122] T Nemkov, A D'Alessandro, K C Hansen, Three-minute method for amino acid analysis by UHPLC and high-resolution quadrupole orbitrap mass spectrometry, Amino Acids 47 (11) (2015) 2345–2357.
[123] M Z Peng, Y N Cai, Y X Shao, L Zhao, M Y Jiang, Y T Lin, X Yin, H Y Sheng, L Liu, Simultaneous quantification of 48 plasma amino acids by liquid chromatography-tandem mass spectrometry to investigate urea cycle disorders, Clin. Chim. Acta 495 (2019) 406–416.
[124] D Wolrab, P Fruhauf, C Gerner, Direct coupling of supercritical fluid chromatography with tandem mass spectrometry for the analysis of amino acids and related compounds: comparing electrospray ionization and atmospheric pressure chemical ionization, Anal. Chim. Acta 981 (2017) 106–115.
[125] H Ghosson, A Schwarzenberg, F Jamois, J-C Yvin, Simultaneous untargeted and targeted metabolomics profiling of underivatized primary metabolites in sulfur-deficient barley by ultra-high performance liquid chromatography-quadrupole/time-of-flight mass spectrometry, Plant Methods 14 (2018) 62.
[126] J H, T C, A K, A B, A B, Analysis of Underivatized Amino Acids by LC/MS for Bioreactor Cell Culture Monitoring, 2018.
[127] M Dickinson, M Parker, F Schoutsen, A J Charlton, A rapid method for the determination of free alpha-amino acids in pea (*Pisum sativum*) using ultra high performance liquid chromatography-high resolution accurate mass-mass spectrometry, Analyt. Methods 6 (7) (2014) 2395–2398.
[128] Y Ge, Y Tang, S Guo, X Liu, Z Zhu, L Zhang, P Liu, S Ding, X Lin, R Lin, J A Duan, Simultaneous quantitation of free amino acids, nucleosides and nucleobases in *Sipunculus nudus* by ultra-high performance liquid chromatography with triple quadrupole mass spectrometry, Molecules 21 (4) (2016) 408.
[129] S Samy, J Robinson, M D Hays, An advanced LC-MS (Q-TOF) technique for the detection of amino acids in atmospheric aerosols, Anal. Bioanal. Chem. 401 (10) (2011) 3103–3113.
[130] Z An, T Hu, Y Lv, P Li, L Liu, Targeted amino acid and related amines analysis based on iTRAQ®-LC-MS/MS for discovering potential hepatotoxicity biomarkers, J. Pharmaceut. Biomed. Anal. 178 (2020) 112812.
[131] J M Armenta, D F Cortes, J M Pisciotta, J L Shuman, K Blakeslee, D Rasoloson, O Ogunbiyi, D J Sullivan Jr., V Shulaev, Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ.Tag ultra performance liquid chromatography-electrospray ionization-MS/MS with multiple reaction monitoring, Anal. Chem. 82 (2) (2010) 548–558.
[132] L Berntzon, L O Ronnevi, B Bergman, J Eriksson, Detection of BMAA in the human central nervous system, Neuroscience 292 (2015) 137–147.
[133] X Zhang, H Chen, D Wu, W Gu, X Sun, J Chen, Q Wu, Determination of free amino acids in three species of Duckweed (Lemnaceae), J. Food Qual. 2018 (2018) 15.
[134] N Rauniyar, Parallel reaction monitoring: a targeted experiment performed using high resolution and high mass accuracy mass spectrometry, Int. J. Mol. Sci. 16 (12) (2015) 28566–28581.
[135] F Pingitore, Y Tang, G H Kruppa, J D Keasling, Analysis of amino acid isotopomers using FT-ICR MS, Anal. Chem. 79 (6) (2007) 2483–2490.
[136] C Muller, I Dietz, D Tziotis, F Moritz, J Rupp, P Schmitt-Kopplin, Molecular cartography in acute *Chlamydia pneumoniae* infections – a non-targeted metabolomics approach, Anal. Bioanal. Chem. 405 (15) (2013) 5119–5131.
[137] F Koopmans, J T C Ho, A B Smit, K W Li, Comparative analyses of data independent acquisition mass spectrometric approaches: DIA, WiSIM-DIA, and untargeted DIA, Proteomics 18 (1) (2018) 1700304.
[138] M R Heaven, A J Funk, A L Cobbs, W D Haffey, J L Norris, R E McCullumsmith, K D Greis, Systematic evaluation of data-independent acquisition for sensitive and reproducible proteomics - a prototype design for a single injection assay, J. Mass Spectrom. 51 (1) (2016) 1–11.
[139] J Klepacki, J Klawitter, J Klawitter, A Karimpour-Fard, J Thurman, G Ingle, D Patel, U Christians, Amino acids in a targeted versus a non-targeted metabolomics LC-MS/MS assay. Are the results consistent?, Clin. Biochem. 49 (13–14) (2016) 955–961.
[140] G Genchi, An overview on D-amino acids, Amino Acids 49 (9) (2017) 1521–1533.
[141] S Wang, P Qi, S Di, J Wang, S Wu, X Wang, Z Wang, Q Wang, X Wang, C Zhao, Q Li, Significant role of supercritical fluid chromatography - mass spectrometry in improving the matrix effect and analytical efficiency during multi-pesticides residue analysis of complex chrysanthemum samples, Anal. Chim. Acta 1074 (2019) 108–116.
[142] F Kitagawa, K Otsuka, Recent progress in capillary electrophoretic analysis of amino acid enantiomers, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879 (29) (2011) 3078–3095.
[143] A Raimbault, M Dorebska, C West, A chiral unified chromatography–mass spectrometry method to analyze free amino acids, Anal. Bioanal. Chem. 411 (19) (2019) 4909–4917.
[144] A Raimbault, A Noireau, C West, Analysis of free amino acids with unified chromatography-mass spectrometry—application to food supplements, J. Chromatogr. A 1616 (2020) 460772.
[145] K L Davidson, M F Bush, Effects of drift gas selection on the ambient-temperature, ion mobility mass spectrometry analysis of amino acids, Anal. Chem. 89 (3) (2017) 2017–2023.
[146] D A Barnett, B Ells, R Guevremont, R W Purves, Separation of leucine and isoleucine by electrospray ionization–high field asymmetric waveform ion mobility spectrometry–mass spectrometry, J. Am. Soc. Mass Spectrom. 10 (12) (1999) 1279–1284.
[147] D G Beach, E S Kerrin, S D Giddings, M A Quilliam, P McCarron, Differential mobility-mass spectrometry double spike isotope dilution study of release of β-methylaminoalanine and proteinogenic amino acids during biological sample hydrolysis, Sci. Rep. 8 (1) (2018) 117.
[148] Anupriya, C A Jones, D V Dearden, Collision cross sections for 20 protonated amino acids: Fourier transform ion cyclotron resonance and ion mobility results, J. Am. Soc. Mass Spectrom. 27 (8) (2016) 1366–1375.
[149] P Dwivedi, C Wu, L M Matz, B H Clowers, W F Siems, H H Hill, Gas-phase chiral separations by ion mobility spectrometry, Anal. Chem. 78 (24) (2006) 8200–8206.
[150] V Domalain, M Hubert-Roux, V Tognetti, L Joubert, C Lange, J Rouden, C Afonso, Enantiomeric differentiation of aromatic amino acids using traveling wave ion mobility-mass spectrometry, Chem. Sci. 5 (2014) 3234.
[151] A Mie, M Jornten-Karlsson, B O Axelsson, A Ray, C T Reimann, Enantiomer separation of amino acids by complexation with chiral reference compounds and high-field asymmetric waveform ion mobility spectrometry: preliminary results and possible limitations, Anal. Chem. 79 (7) (2007) 2850–2858.
[152] R Pérez-Míguez, B Bruyneel, M Castro-Puyana, M L Marina, G W Somsen, E Domínguez-Vega, Chiral discrimination of DL-amino acids by trapped ion mobility spectrometry after derivatization with (+)-1-(9-Fluorenyl)ethyl chloroformate, Anal. Chem. 91 (5) (2019) 3277–3285.
[153] S Chen, X Zhang, Z Yan, Y Hu, Y Lu, Development and application of immunoaffinity column purification and ultrahigh performance liquid chromatography-tandem mass spectrometry for determination of domoic acid in shellfish, Toxins 11 (2) (2019) 83.
[154] M Vogeser, C Seger, Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory, Clin. Chem. 56 (8) (2010) 1234–1244.
[155] H Gika, I Wilson, G Theodoridis, Metabolomics: an Analytical Perspective, 2018.

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