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TRENDS

Advances in amino acid analysis

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Abstract Amino acids are important targets for metabolic profiling. For decades, amino acid analysis has been accomplished by either cation-exchange or reversed-phase liquid chromatography coupled to UV absorbance or fluorescence detection of pre-column or post-column-derivatized amino acids. Recent years have seen great progress in the development of direct-infusion or hyphenated mass spectrometry in the analysis of free amino acids in physiological fluids, because mass spectrometry not only matches optical detection in sensitivity, but also offers superior selectivity. The advent of cryo-probes has also brought NMR spectroscopy within the detection limits required for the analysis of free amino acids. But there is still room for further improvement, including expansion of the analyte spectrum, reduction of sample preparation and analysis time, automation, and synthesis of affordable isotope standards.

Keywords Amino acids · Quantitative analysis · Chromatography · Mass spectrometry · NMR spectroscopy · Derivatization

Introduction

Amino acids are not only the basic structural units of proteins, they are also a source of energy and serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide. Further, glutamate and the non-protein amino acid γ-aminobutyric acid (GABA) play important roles as neurotransmitters, and carnitine is used in intracellular lipid transport.

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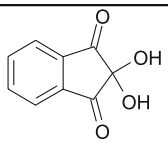
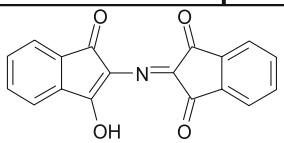
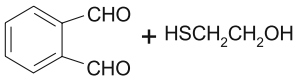
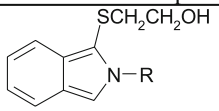
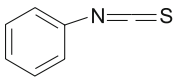
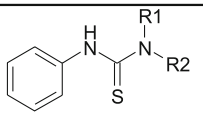
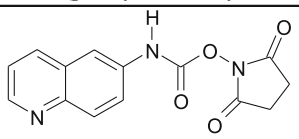
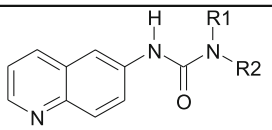
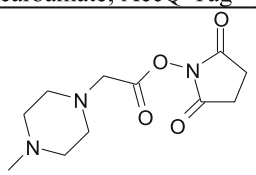
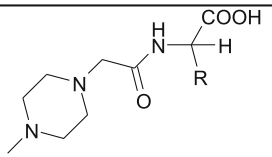
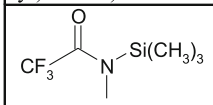
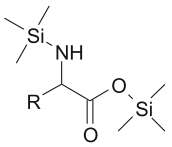
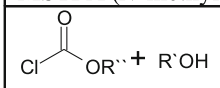
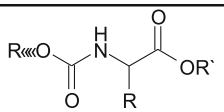
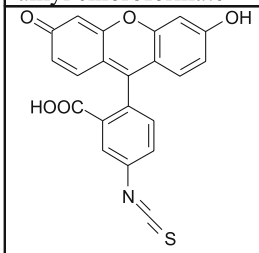
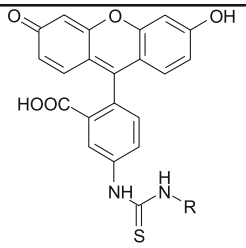
Quantitative analysis of amino acids is required in several fields, including clinical diagnostics of inborn errors of amino acid metabolism, biomedical research, bio-engineering, and food science. Different methods for analysis of amino acids have been developed and commercialized. Still, efforts to improve existing methodology with regard to speed of analysis, robustness, reproducibility, and sensitivity are ongoing and have been driven by a shift in application from the analysis of protein hydrolysates to the analysis of free amino acids in various biological matrices. The aim of this Trend article is to briefly revisit established methods and to present new approaches to amino acid analysis, albeit excluding the analysis of amino acid enantiomers, which will be covered separately.

Sample preparation

Analysis of free amino acids in biological samples often requires protein removal prior to analysis, for example by precipitation with acid or organic solvent, and ultrafiltration. The most common method is precipitation with sulfosalicylic acid [1].

Amino acids are highly polar analytes and, therefore, not suitable for conventional reversed-phase high-performance liquid chromatographic (RP-HPLC) [2] or gas chromatographic (GC) analysis. Capillary electrophoresis (CE) does not require derivatization, but sensitivity of CE-UV analysis can be increased by introduction of a UV-active label. Therefore, a derivatization step is often employed. Most reagents used react with the amino group. Some reagents react only with primary amines, but ideally secondary amines, such as proline and hydroxyproline, are also covered. Another option is to derivatize the carboxy function. The most common derivatization reagents are listed in Table 1 and their use will be discussed in the following sections.

Table 1 Chemical structures of derivatization reagents and derivatives or detected compounds

Structure of the reagent	Structure of derivatives or detected compound
 ninhydrin	 Ruhemann's Purple
 OPA (o-phthalaldehyde)	
 PITC (phenylisothiocyanate; Pico•Tag®)	
 AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; AccQ•Tag™)	
 iTRAQ™ (2,5-dioxopyrrolidin-1-yl-2-(4-methylpiperazin-1-yl)acetate)	
 MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide)	
 alkyl chloroformate	
 FITC (fluorescein isothiocyanate)	

Liquid chromatographic methods coupled with optical detection

Liquid chromatography coupled with optical detection has been used widely for amino acid analysis. The two general approaches are ion-exchange chromatography followed by post-column derivatization or pre-column derivatization preceding RP-HPLC. The gold standard method is cation-exchange chromatography using a lithium buffer system followed by post-column derivatization with ninhydrin and UV detection. The reaction of ninhydrin with amino acids containing a primary amine generates Ruhemann's purple, which is UV-active (λ_{max} 570 nm). Secondary amines, such as proline, produce a yellow product (λ_{max} 440 nm). The eluate is monitored at 440 and 570 nm, respectively. Linearity ranges, typically, from 5–2500 μmol L^{-1}. Routinely, 38 amino acids are separated in about 2 h. Disadvantages of the method are the long runtime, the instability of ninhydrin, crosstalk by analytes other than amino acids that may react with ninhydrin in complex biological samples, and the need for protein precipitation, which impedes complete automation [3].

Derivatization with *o*-phthalaldehyde (OPA) has been used both post column after cation-exchange chromatography and pre column coupled with RP-HPLC. OPA reacts with amino compounds in the presence of a thiol such as mercaptoethanol to form a fluorescent derivative. The OPA derivatives can be detected by UV absorbance at 340 nm, fluorimetry at excitation and emission wavelengths of 340 nm and 450 nm, respectively, and/or amperometry for those OPA derivatives with little or no fluorescent activity [4].

Alternative reagents for precolumn derivatization of free amino groups are phenylisothiocyanate (PITC), dimethylamino-azobenzenesulfonyl chloride (DABS-Cl), 9-fluorenylmethylchloroformate (FMOC-Cl) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [5]. The derivatization time varies between 1 min for OPA and 20 min for PITC. Depending on the number of the analytes, chromatographic run time ranges from 13 min for 23 compounds to 95 min for 38 compounds.

Based on the coupling reaction of the well-known Edman degradation, the reaction of phenylisothiocyanate (PITC) with both primary and secondary amino acids produces phenylthiocarbamyl derivatives that are amenable to RP-HPLC and UV detection at 254 nm. This is the basis for the Pico·Tag method commercialized by Waters (Milford, MA, USA). More recently, Waters introduced AccQ·Tag. This exploits pre-column derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), which converts both primary and secondary amino acids into stable fluorescent derivatives that are amenable to UV-absorbance, fluorescence, electrochemical, and mass spectrometric detection [6]. Faster analysis and improved resolution have been realized with the AccQ·Tag Ultra UPLC method that employs columns packed with 1.7-μm particles. It is 3–5 times faster than conventional HPLC, and baseline separation of all proteinogenic amino acids is achieved in less than 10 min. In a direct comparison of Pico·Tag HPLC and AccQ·Tag Ultra UPLC, the latter separated 16 amino acids in 8 min compared with 23 min for the former [7].

A drawback of optical detection is its lack of analyte specificity. This gives rise to uncertainties in the analysis of complex biological samples that may contain other non-protein amino acids or compounds with an amino function that have similar or identical retention behavior. This may be avoided by the use of mass spectrometry, which enables identification of co-eluting compounds unless they are isobaric and/or have identical fragmentation patterns. Optical detection systems are also not suited to distinguishing between isotopes and, therefore, cannot be used for flux analysis in organisms fed with stable-isotope-labeled substrates. The advantages of LC coupled to optical detection are good reproducibility, comparatively inexpensive equipment, and high sensitivity in the low picomole range.

Ion-pair liquid chromatography-tandem mass spectrometry (IP-LC-MS-MS)

It is feasible to separate non-derivatized amino acids by ion pairing (IP) on RP C_{18} HPLC columns. The use of volatile IP reagents such as perfluorocarboxylic acids allows the hyphenation of LC to electrospray ionization mass spectrometry (ESI-MS). Using tridecafluoroheptanoic acid (TDFHA), Piraud et al. [8] measured 76 amino acids in less than 20 min by RP-HPLC coupled to tandem mass spectrometry operated in multiple-reaction-monitoring (MRM) mode. The quantification of 16 amino acids was validated using their stable-isotope-labeled analogs as internal standards. By coupling TDFHA-RP-HPLC to time-of-flight mass spectrometry, Armstrong et al. [2] quantified 25 amino acids in human plasma over a linear dynamic range of 1.56 to 400 μmol L^{-1}. Further gains in sensitivity are feasible depending on amino acid, type of mass spectrometer, and nature and concentration of IP reagent. De Person et al. [9] achieved limits of detection in the range 0.0003–9 μmol L^{-1} in a comparative study of the perfluorinated carboxylic acids TFA (C_2), HFBA (C_4), NFPA (C_5), TDFHA (C_7), and PDFOA (C_8).

HILIC-MS

Hydrophilic-interaction chromatography (HILIC) employs a polar stationary phase, such as bare silica, amide,

hydroxyl, cyano, amino, and ion-exchange columns, in combination with RP-type solvent systems for separation of polar analytes. Gradient elution is started with a high percentage of organic solvent, typically acetonitrile, and the retained compounds are eluted by increasing the water-content of the mobile phase. Langrock et al. [10] demonstrated the separation of 16 proteinogenic amino acids in 25 min using an amide column coupled to ESI-MS-MS. Detection was carried out using a neutral loss scan of formic acid. Separation of all hydroxyproline isomers present in collagen hydrolysates was achieved. Detection limits were below 50 pmol for the hydroxyproline isomers.

CE-MS

Amino acids are chargeable analytes and, therefore, amenable to CE separation without prior derivatization. However, if optical detection is employed, derivatization is needed to improve sensitivity. Labeling can be carried out with FMOC, NDA, OPA, or FITC [11]. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been used to analyze free amino acids in cerebrospinal fluid [12]. The amino acids were derivatized with FITC prior to analysis and the separation was completed within 22 min. Detection limits were in the low nanomolar range. Light-emitting diodes (LED) are replacing conventional gas lasers for CE-LIF. LEDs are very stable and provide high intensity at low cost [13]. Soga et al. [14] analyzed urinary amino acids without derivatization by capillary electrophoresis-electrospray ionization triple-quadrupole mass spectrometry. The method was validated for 32 amino acids with LODs between 0.1 and 14 μmol L^{-1} and a linear dynamic range of approximately 10–200 μmol L^{-1}. The relatively high LODs are due to the low injection volumes applied in CE.

GC-MS

GC offers high resolving power and can be interfaced efficiently to MS. But amino acids have to be derivatized prior to GC. The derivatization procedure employed most commonly is silylation, whereby an active hydrogen is replaced by an alkylsilyl group, primarily trimethylsilyl, using *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) or *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA). GC analysis of silylated amino acids is feasible, but not all derivatives are stable; e.g., arginine decomposes to ornithine and glutamic acid rearranges to form pyroglutamic acid. Moreover, both reagents and derivatives are sensitive to moisture [15].

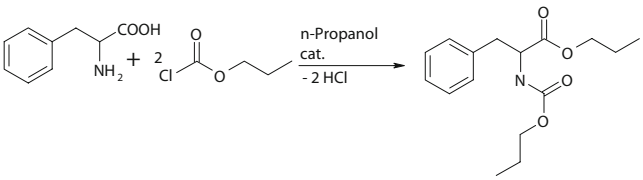


Fig. 1 Reaction scheme for the derivatization of phenylalanine with propyl chloroformate

Alternatives to silylation include acylation/esterification using various anhydride/alcohol combinations such as pentafluoropropyl anhydride and isopropanol [16], or indirect alkylation via chloroformates in the presence of an alcohol and pyridine [17]. In the latter reaction, which is depicted in Fig. 1 for phenylalanine, carboxyl groups are converted directly to esters and amino groups to carbamates. Using the alkyl chloroformate reaction, amino acids can be derivatized directly in aqueous solution without prior removal of proteins. The amino acids react very quickly and the derivatives can be extracted with an organic solvent. From the organic phase an aliquot can be injected directly into the GC-MS [18, 19]. By applying this approach, a fast and fully automated quantitative GC-MS method for analysis of amino acids in physiological fluids was developed using 19 stable isotope-labeled amino acids as internal standards to correct for loss of analyte during sample preparation, incomplete derivatization, and variation of ionization efficiency. Separation of 34 amino acids was achieved within 10 min, as shown in Fig. 2. Limits of detection (LOD) and lower limits of quantification (LLOQ) were in the ranges 0.03–12 μmol L^{-1} and 0.3–30 μmol L^{-1}, respectively. For most amino acids, the calibration range was linear up to 2000 μmol L^{-1} [20]. Fluorinated alcohols yield even more volatile compounds and, consequently, improved MS response in the femtomole to low picomole range [21, 22]. Further, fluoroalkyl chloroformates have expanded the spectrum of amino acids amenable to GC-MS analysis, including 1- and 3-methylhistidine [22] that had not been detected previously using alkyl chloroformate derivatization.

iTRAQ-LC-MS-MS

Applied Biosystems (Foster City, CA, USA) introduced a kit for quantification of 42 physiological amino acids and related compounds based on the iTRAQ chemistry originally developed for the quantification of peptides [23] using LC-MS-MS. The analytes are derivatized with a reactive ester to introduce an isobaric tag. The tag contains a cleavable reporter ion, which can be detected upon collision-induced dissociation in MS-MS mode. These reporter ions differ by one mass unit and can be used to quantify multiplexed

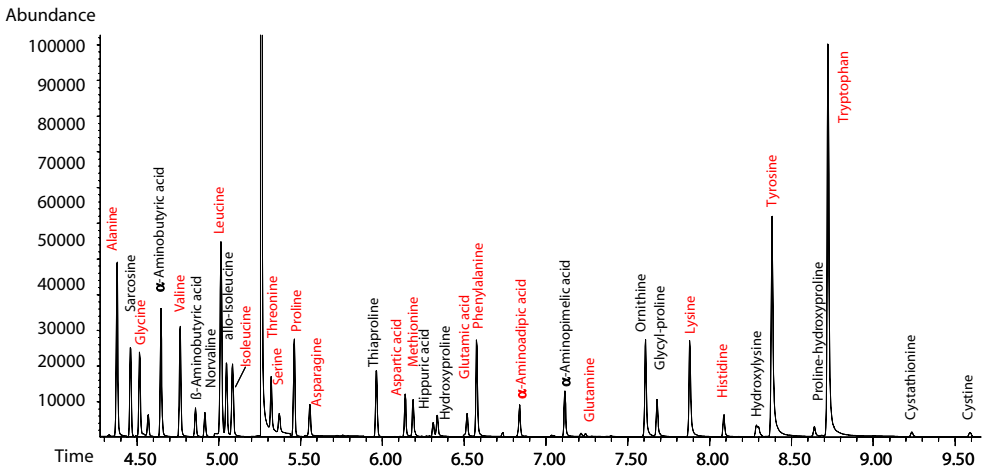


Fig. 2 Typical GC-MS chromatogram from analysis of an amino acid standard on a 15 m × 0.25 mm I.D. ZB-AAA column after derivatization with propyl chloroformate. Amino acids printed in *red* were quantified using the corresponding stable-isotope-labeled amino acid as internal standards for quantification

biological samples. For analysis of free amino acids, the biological sample is labeled with the tag containing the reporter ion *m/z* 115. Before analysis, the sample is mixed with an amino acid standard solution labeled with the reporter ion *m/z* 114. Because the two derivatives of one amino acid have the same mass, they elute at the same retention time and experience the same matrix effects during ESI ionization. Quantitative determination is achieved by multiple-reaction-monitoring (MRM) using one transition each from the precursor to the reporter ions for every amino acid and its corresponding internal standard. Consequently, each amino acid is quantified on the basis of the ratio of the *m/z* 115 ion to the *m/z* 114-reporter ion. The main advantage of iTRAQ-LC-MS-MS is the availability of an internal standard for each analyte. Disadvantages are insufficient recovery of amino acids with sulfur-containing groups, for example methionine and cysteine, high reagent cost, and somewhat imprecise quantification because of the large number of transitions and the resulting insufficient acquisition of data points in a single LC-MS-MS run. The latter may be remedied by scheduled MRMs that limit the number of transitions acquired at any given time.

Direct-infusion tandem mass spectrometry

Direct-infusion MS-MS is used routinely in the analysis of blood and urinary amino acids in the screening of newborns for inherited metabolic disorders such as phenylketonuria. Blood and urine samples are typically collected on filter paper. Disks of the defined size are punched out and amino acids are extracted with methanol containing stable-isotope-labeled amino acids, followed by conversion into the corresponding butyl esters using hydrochloric acid in *n*-butanol [24]. Analysis is performed using direct-infusion MS-MS, which enables analysis of a large number of samples. Additionally, fatty acid and organic acid disorders can be detected in one brief analysis. However, isobaric amino acids, such as leucine, isoleucine, and *allo*-isoleucine or alanine and sarcosine cannot be distinguished.

Identification of analytes in direct-infusion MS may also be accomplished on the basis of accurate mass using mass analyzers with high resolving power, such as time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FT-ICR), and Orbitrap mass spectrometers. This even enables unambiguous identification of amino acids with identical nominal masses such as glutamine and lysine by matching their observed accurate masses to their theoretical masses of 146.06912 and 146.10551 Da, respectively [25].

NMR

The main advantage of NMR is its ability to detect all proton-containing metabolites in a sample simultaneously. Physiological fluids such as urine can be directly analyzed with only limited preparation, and sensitivity does not depend on chemical properties of the analytes such as pK_a or hydrophobicity. NMR is very reproducible and signals scale linearly with metabolite concentrations, which enables reliable quantification. The main drawback of the method is its limited sensitivity compared with mass spectrometry. However, with the use of the newly developed cryo-probes limits of detection in the low μmol L^{-1} range are obtained. Due to the large number of metabolites typically present in biological samples, however, significant overlap of amino acid signals with other signals is commonly observed in 1D ^{1}H NMR spectra. A mathematical solution to this problem is to fit overlapped signals with modeled peaks [26]. Alternatively, multidimensional NMR such as 2D ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectra may be used to separate overlapping metabolite signals in a second heteronuclear dimension [27]. Figure 3 shows a ^{1}H-^{13}C HSQC spectrum of human urine. The metabolites that are easily quantified are marked. In many instances, it is advantageous to combine the results obtained by different

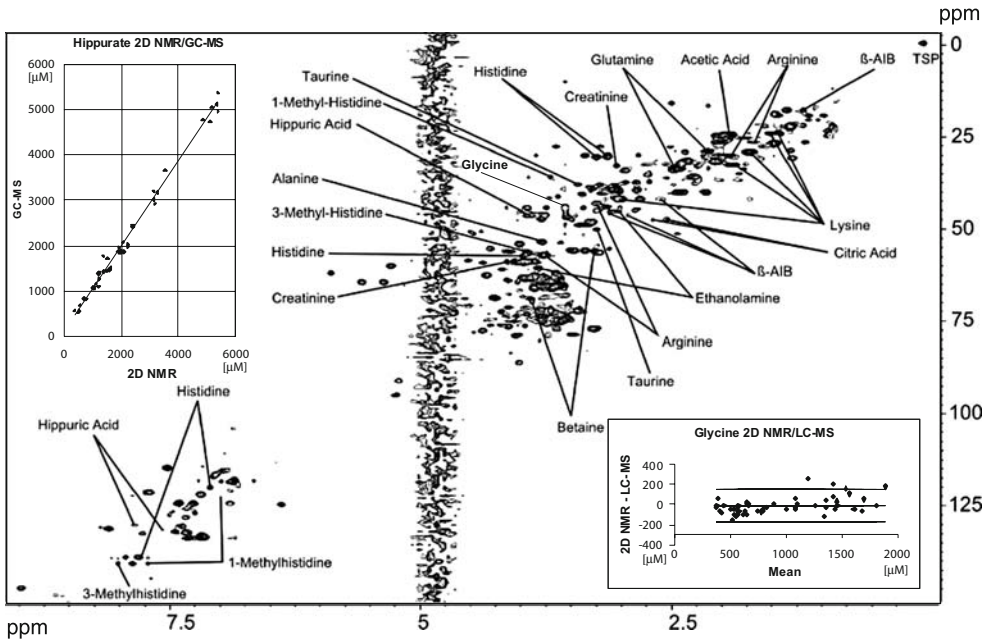


Fig. 3 ^{1}H-^{13}C HSQC spectrum of human urine obtained from a patient suffering from aminoaciduria. The spectrum was acquired at 600 MHz on a Bruker Avance III NMR spectrometer equipped with a cryo-probe. Some highly abundant metabolites that are easily quantified by NMR are marked. The *inset in the upper left corner* shows, for hippurate, the correlation between quantitative 2D NMR and gas chromatography-mass spectrometry (GC-MS) data obtained for 50 urine samples from healthy volunteers. The r^2 value is 0.99 and for the regression line a slope of 0.936 and an axis intercept of 115 µmol L^{-1} were obtained. The *inset in the lower right corner* shows a Bland-Altman plot between 2D NMR and liquid chromatography-mass spectrometry (LC-MS) data for urinary glycine for the same 50 samples

methods such as NMR and mass spectrometry. Therefore, it is important to compare the accuracy of the different approaches. The inset in the upper left corner of Fig. 3 shows the good correlation between 2D NMR and GC-MS quantitative data for hippurate measured in 50 human urine samples. The second inset in the lower right corner of Fig. 3 displays a Bland-Altman plot of 2D NMR and LC-MS data for urinary glycine in the same 50 samples. Only a small mean difference is observed between the two methods, and similar good agreement was obtained for other metabolites.

As mentioned above, some intensity loss is observed on going from 1D ^{1}H spectra to 2D ^{1}H-^{13}C HSQC spectra. One way of regaining this intensity loss due to the low natural abundance of ^{13}C is to chemically *N*-acetylate amino acids with ^{13}C-labeled acetic anhydride [28].

Comparison of methods for amino acid analysis

The methods available for analysis of amino acids are compared in Table 2. The major advantage of NMR is that physiological fluids may be analyzed directly, albeit at the expense of sensitivity. Gains in sensitivity are feasible, but require *N*-acetylation of the amino acids with ^{13}C-labeled acetic anhydride. Another disadvantage is the large sample volume required but, because of the non-destructive nature of NMR, samples may be subjected to further testing. The need for the acquisition of 2D spectra limits throughput, but this is balanced by the ability of NMR to detect proton and carbon-containing metabolites other than amino acids. Protein precipitation is required for all LC and CE methods, irrespective of the detection method used, which renders complete automation difficult. Liquid chromatographic methods coupled with optical detection are well established and highly reproducible. However, classical pre-column and post-column derivatization procedures employing OPA or ninhydrin suffer from long chromatographic run times, which render them poorly suited to large clinical and epidemiological studies. Another drawback shared by all methods based on optical detection is their lack of analyte specificity compared with mass spectrometry. The latter, however, is subject to matrix and ion-suppression effects that impair quantitative accuracy and necessitate the use of stable-isotope-labeled internal standards. Nevertheless, MS-based methods will prevail in the future. HILIC-MS and CE-MS enable direct analysis of amino acids without prior derivatization, but they suffer from low throughput and comparatively poor reliability. Ion-pair LC-MS has been applied to the analysis of both native and iTRAQ-labeled amino acids. The most important benefit of iTRAQ-LC-MS-MS compared with other MS-based methods is the availability of internal standards for all the analytes. But iTRAQ-LC-MS-MS has a number of disadvantages includ-

Table 2 Comparison of selected approaches for analysis of amino acids

Method	Advantages	Disadvantages	LOD or LOQ	Ref.
LC-methods coupled with optical detection	Highly reproducible Inexpensive equipment Good linearity over a broad range	Protein precipitation and derivatization necessary Lack of analyte specificity Co-eluting substances cannot be distinguished Not applicable to flux analysis	UV: 5 μmol L^{-1} (LOQ)	3–6
UPLC-MS	Fast separation Good resolution	Protein precipitation necessary High pressure requires special equipment Limited number of amino acids covered Ion suppression	1.3–5.3 μmol L^{-1} (LOQ)	7
IP-LC-MS-MS	Derivatization not necessary Large number of analytes covered Good resolution for polar amino acids	Protein precipitation necessary Ion suppression Contamination of analytical system with IP reagent	0.0003–9 μmol L^{-1} (LOD)	2, 8, 9
HILIC	Derivatization not necessary Compatible with MS Well-suited to polar compounds	Protein precipitation necessary Poor reproducibility Ion suppression in case of MS detection	5 μmol L^{-1} (LOD) 10 μmol L^{-1} (LOQ)	10
CE-MS	Derivatization not necessary Low sample consumption	Protein precipitation necessary Only low injection volume possible	0.1–14 μmol L^{-1} (LOD)	14
GC-MS	Robust method Highly reproducible Good resolution Fast separation	Derivatization necessary Not suitable for thermolabile amino acid derivatives	0.03–12 μmol L^{-1} (LOD) 0.3–30 μmol L^{-1} (LOQ)	20
iTRAQ	Fast separation Availability of internal standards for each analyte	Protein precipitation necessary Poor recovery of sulfur containing amino acids Difficult to automate	2–10 μmol L^{-1} (LOQ)	Unpublished own data
Direct-infusion MS-MS	No separation needed High throughput	Extraction and derivatization required Isobaric amino acids cannot be resolved	Not reported	24, 25
NMR	No separation and derivatization needed Robust quantification Minimal sample preparation	Insufficient sensitivity, LOD can be lowered by derivatization with 1,1′-$^{13}C_2$ acetic anhydride Long analysis time	2D: 20–312 μmol L^{-1} (LOD)	Unpublished own data

ing somewhat poor reproducibility, because of the large number of transitions that have to be acquired, which may be alleviated in the future by scheduled multiple-reaction-monitoring, the inability to accurately measure sulfur-containing amino acids, the difficulty of automating sample preparation, and high reagent costs. GC-MS is a very robust method with excellent reproducibility of retention times. Especially with alkyl chloroformate derivatization excellent reproducibility of quantitative data has been observed and the method can be automated easily, thus enabling high sample throughput. However, thermo-labile derivatives cannot be measured. Finally, direct flow injection analysis with ESI-MS-MS or high-resolution MS offers high throughput and is now widely used for screening of newborns for inborn errors of metabolism. The one major limitation is the inability to resolve isobaric amino acids.

Outlook

There is room for new or improved methodology for amino acid analysis, including expansion of the analyte spectrum covered, reduction of sample-preparation and analysis time, improved sensitivity, and good robustness and reproducibility. Due to high selectivity and sensitivity, MS is expected to play a key role provided that stable-isotope-

labeled standards, which are a prerequisite for robust quantification, become readily and cheaply available. Reduced sample pre-treatment is another important aspect for facilitating automation and improving robustness and sample throughput, which are essential in epidemiological studies with large sample numbers.

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