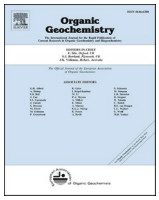
Organic Geochemistry 164 (2022) 104345

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem



Review Article

Practical considerations for amino acid isotope analysis

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ARTICLE INFO

Associate Editor–**Julian Sachs**

Keywords:
Amino acids
Compound-specific isotope analysis
Position-specific isotope analysis
Acid hydrolysis
Derivatization
Separation
Isotope ratio mass spectrometry
Orbitrap™
Nuclear magnetic resonance

ABSTRACT

Over the last few decades, isotopic analysis of amino acids at the compound- and position-specific levels has been rapidly advancing across diverse fields. As these techniques progress, evaluation of isotopic fractionation associated with sample workup is essential. This critical review of analytical methods through the lens of isotope geochemistry provides a benchmark for researchers across disciplines seeking to make compound- and position-specific amino acid isotope measurements. We focus on preparation, acid hydrolysis, clean-up, derivatization, separation, and C, H, N, and S isotope measurement. Despite substantial customizability across these steps, the following general recommendations should maximize recovery while minimizing isotopic fractionation. Samples should be freeze-dried and stored anoxically at ≤ -20 °C prior to conventional acid hydrolysis (6N HCl, 110 °C, 20–24 h, anoxic), which suffices for many residues. Both gas and liquid chromatographic (GC and LC, respectively) techniques are well-established and separate about 15 amino acids; LC bypasses the need for derivatization, while GC provides higher sensitivity. When derivatization is needed, *n*-acetyl and alkoxycarbonyl esters provide the most reproducible C isotope ratios. For compound-specific analyses, online GC–IRMS and LC–IRMS systems offer the easiest workflow, but EA–IRMS enables potential multi-element isotope analysis. Emerging techniques like high-resolution mass spectrometry are also promising for multi-element analysis and recover position-specific isotopic information. Looking forward to the next decade of innovation, isotope geochemists and ecologists can improve amino acid isotope analysis by focusing on streamlining multi-element analysis and standardizing calibration practices across laboratories.

1. Introduction

Stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{2}H/^{1}H$, and $^{34}S/^{32}S$) of amino acids record details of biosynthesis, enabling interrogation of environmental and physiological processes. This review covers common steps within the workflow for amino acid isotope analysis, including protein hydrolysis, derivatization, chromatographic separation, and isotope ratio detection (Fig. 1). While these steps build on earlier studies

Abbreviations: ala, alanine; arg, arginine; asn, asparagine; asp, aspartic acid; asx, asparagine + aspartic acid; cys, cysteine; his, histidine; gln, glutamine; glu, glutamic acid; glx, glutamine + glutamic acid; gly, glycine; ile, isoleucine; leu, leucine; lys, lysine; met, methionine; phe, phenylalanine; pro, proline; ser, serine; thr, threonine; trp, tryptophan; tyr, tyrosine; val, valine; HCl, hydrochloric acid; HF, hydrofluoric acid; DCM, dichloromethane; TFAA, trifluoroacetic anhydride; HFB, heptafluorobutyric anhydride; PFP, pentafluoropropionic anhydride; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; MTBSTFA, methyltributylsilyl tetrafluoroacetamide; NPME, *n*-pivaloyl methyl ester; NPNP, *n*-pivaloyl n-propyl ester; NPIP, *n*-pivaloyl isopropyl ester; TFA-IP, trifluoroacetyl isopropyl ester; TFA-ME, trifluoroacetyl methyl ester; PFP-IP, pentafluoropropionyl isopropyl ester; HFB-IB, heptafluorobutyryl isobutyl ester; NANP, *n*-acetyl *n*-propyl ester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester; MOC ME, methoxycarbonyl methyl ester; EOC EE, ethoxycarbonyl ethyl ester; TMS, trimethylsilyl; t-BDMS, *tert*-butyldimethylsilyl; IRMS, isotope ratio mass spectrometry; GC–IRMS, gas chromatography–IRMS; LC–IRMS, liquid chromatography–IRMS; HPLC, high performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; IC, ion chromatography; CE, capillary electrophoresis; PT-CF–IRMS, purge-and-trap continuous-flow IRMS; FIA-NR–IRMS, flow injection analysis reaction with ninhydrin–IRMS; SWiM–IRMS, spooling wire micro-combustion–IRMS; EA–IRMS, elemental analysis–IRMS; TCEA–IRMS, thermal conversion-EA–IRMS; NMR, nuclear magnetic resonance; KIE, kinetic isotope effect; VPDB, Vienna Pee Dee Belemnite; PES, polyethersulfone; PVDF, polyvinylidenedifluoride; SD, standard deviation; USGS, United States Geological Survey; IAEA, International Atomic Energy Agency.

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https://doi.org/10.1016/j.orggeochem.2021.104345
Received 26 February 2021; Received in revised form 10 November 2021; Accepted 19 November 2021
Available online 28 December 2021

quantifying amino acids (e.g., Homer, 1915; Tristram, 1939; Rees, 1946), no review has covered amino acid isotope analysis in full, but rather have provided overviews of C and N isotope analysis that emphasize data interpretation and ecological applications (Ohkouchi et al., 2017; McMahon and Newsome, 2019; Whiteman et al., 2019). Here we review published techniques encompassing sample preparation through isotope ratio measurement for C, N, H, and S (Fig. 1). We highlight (1) where nonquantitative reactions and/or incomplete separations can fractionate isotopes, and (2) established and emerging analytical options available to isotope geochemists that enable compound-specific and position-specific isotope analysis of amino acids.

1.1. Terminology

Natural-abundance stable isotope compositions are typically reported in delta (δ) notation (Urey, 1948; McKinney et al., 1950) to highlight small variations between samples. A δ value is the relative difference in isotope ratio (R) between a sample and standard (Eq. 1), commonly expressed in parts per thousand (per mil, ‰). The heavy (i.e., rare) isotope is placed in the numerator of R by convention.

$$\delta = \frac{R_{sample} - R_{standard}}{R_{standard}} = \frac{R_{sample}}{R_{standard}} - 1 \quad (1)$$

Isotopic substitutions alter the bond energies and physical properties of molecules, causing them to react at different rates or partition differently between phases. These physical phenomena are "isotope effects," defined as the change in some chemical or physical parameter (kinetic rate constant, equilibrium constant, vapor pressure, etc.) due to isotopic substitution. A kinetic isotope effect (KIE) represents the ratio of reaction rate constants between two isotopologues (versions of molecules with differing isotopic substitutions); a "normal" KIE describes a reaction in which the lighter isotope reacts faster (Hayes, 2002). Isotope effects are considered primary at the reacting atomic position(s), and secondary at non-reacting positions (Hayes, 2002).

Isotope effects result in measurable differences in isotopic composition between products, pools, materials, etc., called "fractionations." Isotope fractionations are commonly expressed as fractionation factors (α), the ratio of isotope ratios between two pools of interest (Hayes, 2002). For example, given the generic reaction A → B, the isotopic fractionation would be described as:

$$\alpha_{B-A} = \frac{R_B}{R_A} \quad (2)$$

There is no consensus on whether reactant or product belongs in the numerator of α. Throughout this review, we use the convention shown in Eq. (2), which for a normal KIE results in $\alpha < 1$. For convenience, α is occasionally expressed as an ε value, in ‰ (Eq. (3)). For further review on notation and calculations, we refer the readers to Hayes (2002) and Coplen (2011).

$$\varepsilon = (\alpha - 1) \times 10^3 \quad (3)$$

2. Sample storage and preparation

Sample storage conditions should be selected to minimize amino acid degradation and contamination, ideally achieved by storing freeze-dried or frozen samples in clean plastic or baked glassware with an anoxic headspace. Higher storage temperatures (> –20 °C) may promote

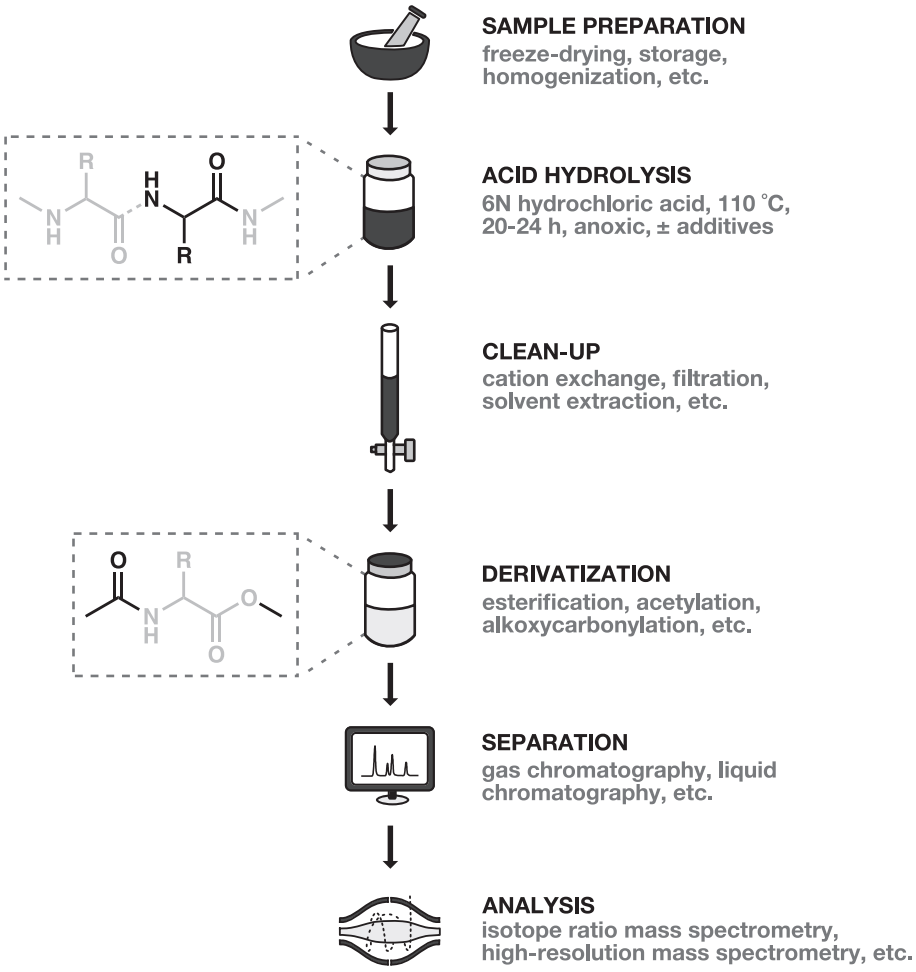

Fig. 1. A typical workflow for amino acid isotope analysis, highlighting the major preparatory and analytical steps examined in this review paper. We cover sample preparation, acid hydrolysis, clean-up, derivatization, separation, and isotopic analysis. Sample preparation includes drying and homogenization steps, and, less commonly, clean-up steps prior to hydrolysis. Peptide-bound amino acids can be released by acid hydrolysis and are typically followed by additional clean-up steps depending on the sample matrix. Amino acids must be derivatized to decrease their polarity and make them amenable to separation by gas chromatography. Samples can also be separated without derivatization via liquid chromatography. Finally, isotope measurements are achieved via a variety of established and emerging techniques including isotope ratio mass spectrometry (IRMS), high-resolution mass spectrometry (e.g., Orbitrap), or nuclear magnetic resonance (NMR). Insets show peptide bonds cleaved during acid hydrolysis and example derivative groups added during derivatization.

decomposition (Laegeler et al., 1974; Rutherfurd and Gilani, 2009; Whiteman et al., 2019), while oxic conditions can degrade cysteine, methionine, and tryptophan (Hunt, 1985). There is no consensus on whether plastic or glass containers are better for amino acid recovery. Because dust, fingerprints, sweat, and reagents may introduce contaminant amino acids (Ozols, 1990; Henrichs, 1991; McCoy et al., 2019, and references therein), any glassware, foil, or glass fiber filters that will contact samples should be baked (e.g., at 450 °C for 8 h; Molero et al., 2011; Larsen et al., 2013; Unger and Holzgrabe, 2018; Whiteman et al., 2019). Most samples should be homogenized – e.g., by mortar and pestle, bead beater, or cryogenic grinding – before or after storage to increase efficiency of acid hydrolysis.

While general storage recommendations can be made, pretreatment is more sample-dependent as geochemists process diverse sample matrices ranging from hard rocks to soft tissues. The goal of pretreatment is to remove large, non-amino acid components that interfere with measurement (e.g., through co-elutions or column overloading) and cannot be eliminated at a later stage. However, as each additional step may lower recovery or fractionate isotopes, we recommend minimizing pretreatments and monitoring procedural blanks and external standards. Procedural blanks are controls that do not contain the sample matrix and are subjected to the entire workflow, including pretreatment; external standards are well-characterized materials (e.g., proteins like bovine serum albumin or amino acids with known isotope ratios) that are processed alongside samples. Additional pretreatment steps are highly matrix-specific and should be carefully assessed, but could include surface rinsing, mechanical abrasion, or deeper cleaning (e.g., Hare et al., 1991; Johnson et al., 1998; Schiff et al., 2014); solvent extraction to remove lipids from fatty tissues (Bligh and Dyer, 1959; Newsome et al., 2018; Whiteman et al., 2019); demineralization with hydrofluoric acid (Cheng, 1975; Gélinas et al., 2001; Ingalls et al., 2003; Nunn and Keil, 2006); or decarbonation with hydrochloric acid (e.g., Hare et al., 1991; Johnson et al., 1998; Schiff et al., 2014). Protein extraction prior to hydrolysis, though tempting to avoid interfering components, is not recommended, as this procedure is labor-intensive and introduces bias by preferentially removing hydrophilic or hydrophobic peptides (Nguyen and Harvey, 1998; Wang et al., 2003; Nunn and Keil, 2006; Niu et al., 2018).

3. Acid hydrolysis

The goal of acid hydrolysis is to liberate proteinogenic amino acids into their "free" (non-peptide-bound) forms (Fig. 2) with maximum recovery and minimal isotopic fractionation. Studies have largely converged on heating samples in 6 N HCl for 20–24 h at 110 °C under anoxic conditions (e.g., flushed with N_2 gas; Moore and Stein, 1963;

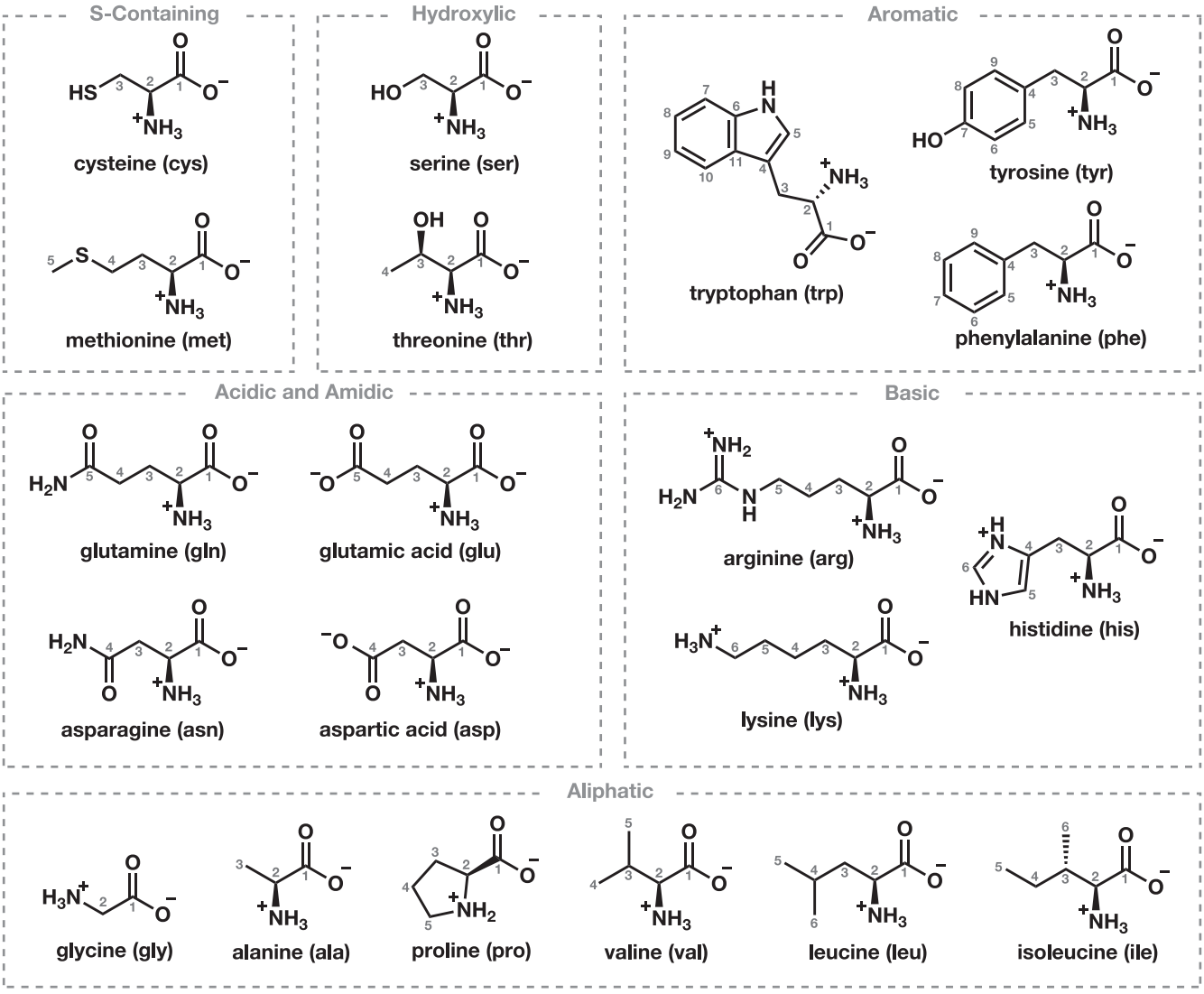

Fig. 2. Structures of 20 common proteinogenic amino acids in zwitterion form at pH 7. Residues are grouped by side-chain chemistries. Numbering for amino acid C sites (e.g., C-1) used throughout this review is shown.

Fountoulakis and Lahm, 1998; Rutherfurd and Gilani, 2009; Fogel et al., 2016), hereafter referred to as "conventional hydrolysis." We recommend this method for most applications, as it results in high recoveries and negligible fractionation of many amino acids with minimal additional chemical workups (Fig. 3). Here we discuss this conventional hydrolysis method and its variations, including different durations and additives that stabilize certain amino acid side-chains. We also discuss fast hydrolysis alternatives, including microwave and vapor-phase methods. It is important to emphasize that no single hydrolysis method recovers all amino acids (Fig. 4); in particular, no current method prevents quantitative deamidation of asparagine and glutamine to aspartic acid and glutamic acid (Supplementary Fig. S2; Supplementary Section 1.2; Hill, 1965; Wright, 1991, Rutherfurd and Gilani, 2009). Yields and isotopic compositions are therefore commonly reported as asx (asparagine + aspartic acid) and glx (glutamine + glutamic acid). Loss mechanisms are influenced by amino acid side-chain chemistry (e.g., hydroxyl groups on serine and threonine; see Supplementary Section 1), protein composition (e.g., the proportion of S-containing amino acids), and hydrolysis method (e.g., duration, temperature). Sample matrix effects also likely influence yields, but studies thus far have focused on loss and isotopic fractionations of pure standards or synthetic polypeptides and cannot account for the complexity of geochemically relevant samples.

3.1. Conventional hydrolysis

Conventional hydrolysis (6 N HCl, 110 °C, 20–24 h, anoxic conditions) results in consistently high yields for 13 of the 20 amino acids (Figs. 3 and 4; Supplementary Table S1), including all aliphatic and basic amino acids. Lengthening or shortening hydrolysis duration reduces the total number of stable residues but can maximize yields of particular amino acids (Fig. 4). For example, extended hydrolyses (>24 h) improve recoveries of most aliphatic amino acids, especially valine, leucine, and isoleucine (Darragh and Moughan, 2005) at the expense of other amino acids (Hirs et al., 1954; Smith and Stockell, 1954; Darragh et al., 1996). Meanwhile, shortened hydrolysis times (<20 h) optimize yields of serine and threonine (Rowan et al., 1992; Albin et al., 2000), with maximum recovery between ~10 and 16 h (Gardner, 1981; Gehrke et al., 1985; Rutherfurd, 2009). Although phenylalanine and tyrosine are stable regardless of hydrolysis length, tryptophan has low and variable yields (0–55%; Fig. 3; Supplementary Table S1; Keutmann and Potts, 1969; Matsubara and Sasaki, 1969; Mondino and Bongiovanni, 1970; Hunt, 1985; Manneberg et al., 1995, Rutherfurd and Gilani, 2009). Like tryptophan, sulfur-containing and amidic amino acids are unstable regardless of hydrolysis length (Fig. 4; Hunt 1985).

3.2. Isotopic fractionation

Each amino acid experiences different mechanisms and magnitudes of loss during conventional hydrolysis, which can lead to isotopic fractionation. We review what is known about isotopic fractionation during hydrolysis and peptide bond cleavage; mechanistic details concerning amino acid losses are presented in Supplementary Section 1. Studies of pure amino acid standards subjected to hydrolysis conditions revealed minimal C isotope changes (Demmelmair and Schmidt, 1993; Metges and Daenzer, 2000), even for amino acids with significant losses like serine and methionine. This is further supported by Jim et al. (2003), who found no detectable C isotope fractionation upon hydrolysis of synthetic alanine, serine, glutamic acid, phenylalanine, leucine, or proline polypeptides. Nitrogen isotopes may be more susceptible, especially in aliphatic amino acids. Bada et al. (1989) observed ~20‰ ^{15}N-enrichment of residual, unhydrolyzed collagen, and Silfer et al. (1992) observed temperature-dependent normal ^{15}N-KIEs of 0.9960–0.9975 for residual diglycine. We also caution interpreting δ^{15}N values of asx and glx due to the loss of amide-N from asparagine and glutamine. Although few studies have investigated H isotopes of amino acids, deuterated and tritiated hydrolysis experiments suggest H isotope exchange with the aqueous medium is significant for tyrosine (C-3 atomic site; Fig. 2), aspartic acid (C-3 site), and glutamic acid (C-4 site; Hill and Leach, 1964; Fogel et al., 2016). Studies of S isotopes are also limited but indicate a ^{34}S-KIE of 0.985 associated with oxic degradation of cysteine during conventional hydrolysis (Phillips et al., 2021). Importantly, the lack of geochemically-relevant sample matrices in all the aforementioned studies limits the scope of conclusions that can be drawn. Moving forward, a more comprehensive characterization of isotope fractionations accompanying protein hydrolysis in complex matrices like sediments and soils is needed.

3.3. Alternative hydrolysis methods

Variations on conventional hydrolysis offer some specific advantages, including: (1) protection of certain amino acids through chemical additives and/or (2) much faster hydrolysis times via microwave or vapor-phase methods. For additives, the use of β-mercaptoethanol appears most promising, as it increases the number of stable residues from 13 to 17 (Fig. 4; Hunt, 1985; Ng et al., 1987). We especially recommend the use of β-mercaptoethanol for studies of tryptophan, as this is the only method that can reproducibly recover this residue. Phenol is another common additive as it mitigates halogenation of aromatic residues, but it does not stabilize other amino acids so is not recommended for general use. Addition of the oxidizing agent performic acid is one of the few hydrolysis methods that recovers cysteine, but it destroys several aromatic and hydroxylic residues (Fig. 4; Hunt, 1985; Rutherfurd and Gilani, 2009). Microwave-assisted and vapor-phase methods achieve

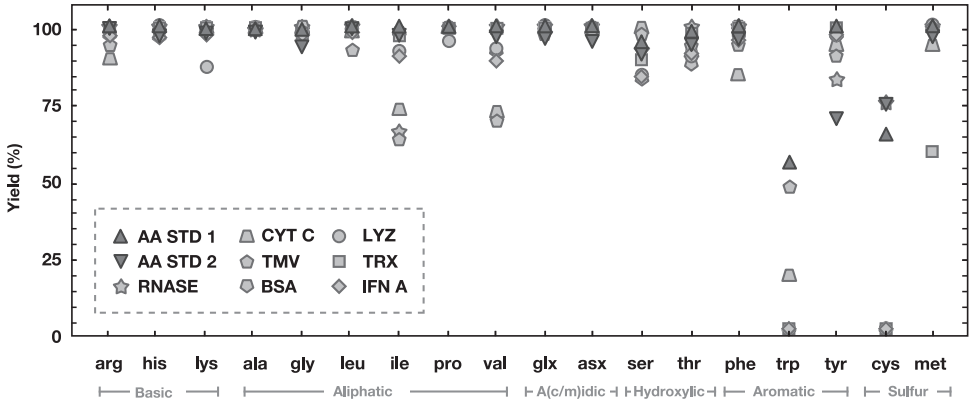

Fig. 3. Yields of free amino acid standard mixtures (dark gray triangles) and of amino acids from proteins (light gray shapes) after conventional acid hydrolysis (6 N HCl, 110 °C, 20–24 h, anoxic conditions). Corresponding yield data can be found in Supplementary Table S1. Abbreviations and references: AA STDs, amino acid standard 1 (Mondino and Bongiovanni, 1970) and amino acid standard 2 (Keutmann and Potts, 1969); RNASE, Ribonuclease (Keutmann and Potts, 1969); CYT C, Cytochrome *c* (Matsubara and Sasaki, 1969); TMV, Tobacco mosaic virus (Matsubara and Sasaki, 1969); BSA, Bovine serum albumin (Manneberg et al., 1995); LYZ, Lysozyme (Manneberg et al., 1995); TRX, Thioredoxin (Manneberg et al., 1995); IFN A, Interferon A (Manneberg et al., 1995).

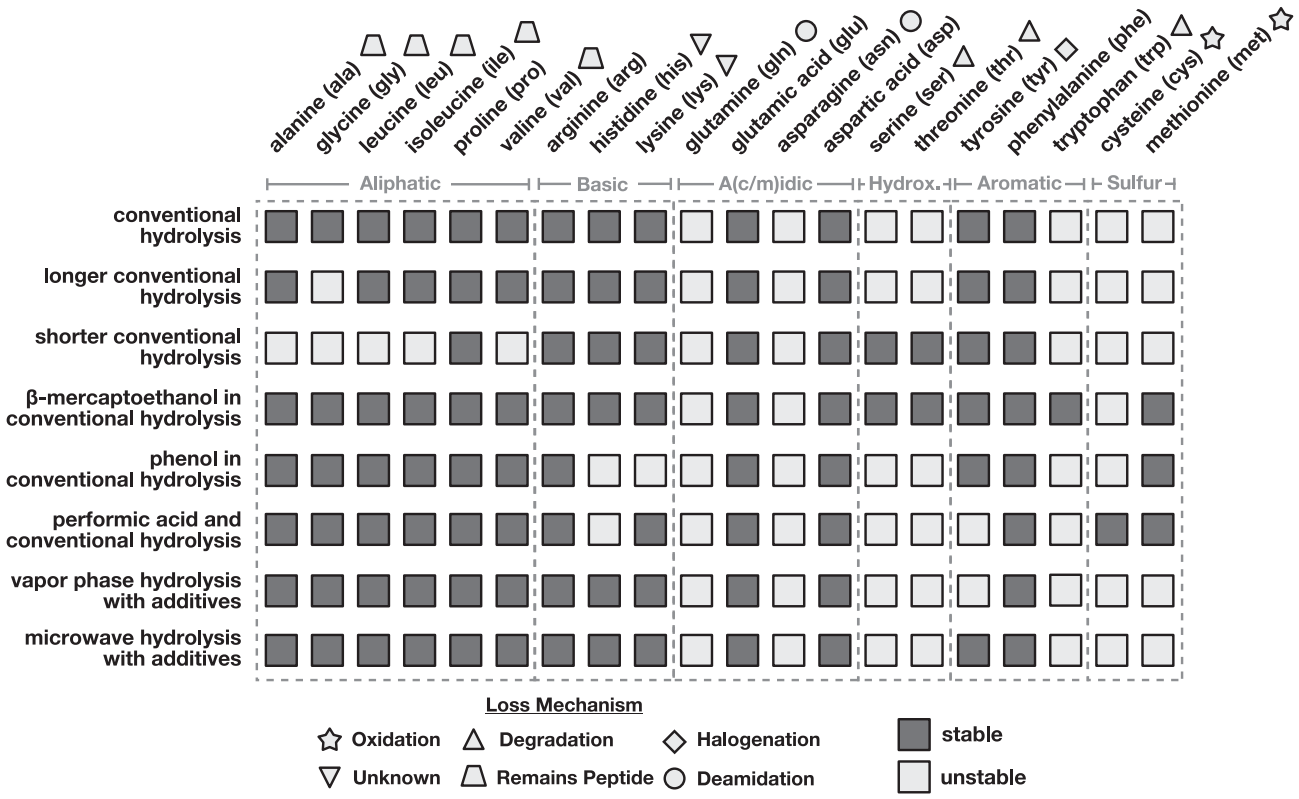

Fig. 4. Summary of stable and unstable residues during common acid hydrolysis procedures. Primary loss mechanisms are denoted by shapes. Conventional hydrolysis is defined as 20–24 h at 110 °C in 6 N HCl under anoxic conditions. The recommended vapor phase hydrolysis uses 7 N HCl, 10% trifluoroacetic acid, and 0.1% phenol for 22 min at 158 °C. The recommended microwave assisted hydrolysis uses 6 N HCl, 0.02% phenol, 0.2% 3-(2-aminoethyl)-indole for 4 min at 155 °C. See text for details and references.

complete hydrolysis in < 90 min and generally recover the same amino acids as conventional hydrolysis (Fig. 4; Tsugita et al., 1987; Chiou, 1989; Weiss et al., 1998; Yarnes and Herszage, 2017), although with slightly lower yields (Enggrob et al., 2019). Vapor-phase methods have the added benefit of minimizing contact between samples and liquids, reducing potential contamination. These high-temperature, short-duration hydrolyses also limit amino acid racemization (Csapó et al., 1997) and potentially minimize H isotope exchange with the aqueous medium.

4. Analyte clean-up

There are two main goals of clean-up: (1) removing large particles and (2) eliminating extraneous compounds such as lipids, carbohydrates, and salts that are liberated during acid hydrolysis. These components can interfere with derivatization and isotope analysis (e.g., by consuming derivatizing reagents or damaging the gas chromatography-isotope ratio mass spectrometer (GC–IRMS) combustion interfaces; Cheng et al., 1975; Hedges and Stern, 1983; Takano et al., 2010). At a minimum, large particles must be removed. While this can be accomplished via filtration with baked glass fiber filters (Amelung and Zhang, 2001), quartz wool pipette columns (Enggrob et al., 2019), or non-protein binding syringe filters (e.g., PES, PVDF; Larsen et al., 2013; Phillips et al., 2021), we recommend cation exchange chromatography (Section 4.1) for most applications because it eliminates both salts and particles. Less commonly, organic/aqueous solvent extraction can complement cation-exchange chromatography to remove excess hydrophobic components (e.g., lipids from fatty tissues). Solvent extraction is recommended for samples with > 2% lipid content (McMahon and Newsome, 2019) and can be performed before or after hydrolysis. Some derivatizing reagents (e.g., BSTFA) require moisture-free reaction conditions so samples must be carefully dried as a final clean-up step. This can be achieved via rotary evaporation, addition of sodium sulfate desiccants, or azeotropically with dichloromethane (DCM).

4.1. Ion exchange

Studies have converged on the Dowex 50WX8 hydrogen form resin (200–400 mesh) for cation exchange. Most amino acids are recovered with >90% yield, including from complex matrices like clay minerals (Supplementary Table S2; Moore and Stein, 1951; Cheng et al., 1975; Amelung and Zhang, 2001; Takano et al., 2010). Cation exchange also preserves amino acid chirality and introduces no background contaminants when resins are washed (Takano et al., 2010). Additional rinses with 0.1 N oxalic acid can be added to remove metal cations from soils, rocks, and sediment samples (Amelung and Zhang, 2001). As cation exchange involves both binding of the amine group and elution with ammonia, potential alteration of $\delta^{15}N$ values is a concern. However, Takano et al. (2010) observed < 0.3‰ differences in $\delta^{15}N$ values for 12 amino acids before versus after elution on the Dowex 50WX8 resin, despite losses of up to 17% (Supplementary Table S2). Carbon is not involved in adsorption or elution and is not expected to fractionate. Indeed, Abelson and Hoering (1961) found minimal C isotope fractionation (< 0.6‰) of alanine on the Dowex 50WX8 resin.

Anion exchange (Dowex 1X8) is another option for desalting (Cheng et al., 1975), but is far less common, perhaps due to (1) the fact that in geochemical samples, interfering anions (i.e., sugars and organic acids) are present in greater concentrations than cations, (2) evidence of C isotope fractionation during elution (Abelson and Hoering, 1961), and (3) the need to work with corrosive HF to condition some anion exchange resins (Abelson and Hoering, 1961).

5. Derivatization (for gas chromatography)

Derivatization is required to make amino acids amenable to

separation by GC. Polar functional (carboxyl, amine, hydroxyl, and thiol) groups are modified via the addition of various organic moieties to make amino acids more volatile, with the products termed "derivatives." For H isotope analysis, derivatization serves the additional purpose of removing exchangeable H atoms, such as on carboxyl and amine groups, that would otherwise equilibrate with the aqueous medium and alter the original δ^2H value.

Derivatization strategies for amine and carboxyl groups can be chosen independently, provided the reactions are compatible, leading to a variety of combinations (Tables 1 and 2). Amine, hydroxyl, and thiol side-chains are typically derivatized by the same reagent used for the amine group. No single derivatization strategy is optimized for all 20 amino acids (Tables 1 and 2). In particular, arginine and histidine are incompatible with many reactions (Table 1; Hušek and Macek, 1975). Several factors govern the selection of GC derivatives, including reaction time, ease of procedure, product volatility, derivative stability, the number of non-analyte atoms added, reaction yield, byproduct formation, enantiomer preservation (i.e., lack of racemization), combustion or pyrolysis efficiency, and chromatographic resolution. Tradeoffs abound: for example, a less stable derivative may be preferable if the reaction is rapid, as samples can be derivatized immediately before analysis.

Derivatizing reagents should be present in excess to enable reaction completion and avoid isotopic fractionation of amino acids (Docherty et al., 2001). However, even under these conditions, it appears that derivatization reactions are not always quantitative, as N isotope fractionation accompanies formation of many derivative products (Table 1; Hofmann et al., 2003; Walsh et al., 2014). All derivatizing agents add C and/or H atoms which alter the molecular isotope ratio of derivatized amino acids (Fig. 5; Tables 1 and 2) and must be subtracted. Large derivative groups are less suited for isotope analysis, as analytical uncertainty scales with the number of atoms added by the derivative group(s) (Rieley, 1994). For details on this data correction, error propagation, and associated isotope effects, see Supplementary Section 2.

Numerous derivatives are used in ecological and geochemical studies. For N isotope analysis, we recommend pivaloyl derivatives, while for C and H isotope analysis, *n*-acetyl methyl esters (NACMEs) or methoxycarbonyl (MOC) methyl esters are ideal, as they introduce few exogenous atoms (Ohkouchi et al., 2017). Fluorinated derivatives, though popular, are best reserved for applications that do not rely on combustion or pyrolysis of analytes. We discuss these and other common amino acid derivatives for GC analysis below, with sections separated by targeted functional group (amine versus carboxyl). Reagent toxicity is not individually discussed, but it should be noted that many are acutely toxic (particularly pivaloyl chloride and methyl/ethyl chloroformate; Walsh et al., 2014; Ohkouchi et al., 2017).

5.1. Amine group derivatives

5.1.1. Pivaloyl derivatives

Pivaloyl esters are optimal for δ^{15}N analysis as they are stable, have excellent chromatographic properties, and can be coupled to esterification of the carboxyl group to form a variety of derivatives (Fig. 5, Tables 1 and 2; Metges et al., 1996; Chikaraishi et al., 2007; Corr et al., 2007b; Tea and Tcherkez, 2017). Furthermore, enantiomers are preserved and can be separated using chiral stationary phases (Abe et al., 2002; Takano et al., 2009). Pivaloyl esters are not recommended for δ^{13}C or δ^2H analysis due to their many exogenous C and H atoms (Tables 1 and 2; Supplementary Eq. (6); Corr et al., 2007a). Pivaloylation is achieved with pivaloyl chloride and targets amine, hydroxyl, and thiol groups (Table 2; Corr et al., 2007b). An isotope effect is known for the carbonyl C of pivaloyl chloride during derivatization (Corr et al., 2007b), and N isotope fractionation accompanying *n*-pivaloyl isopropyl ester formation has been observed (Table 1; Hofmann et al., 2003).

5.1.2. Fluorinated derivatives

Trifluoroacetyl (TFA) esters (and less commonly, pentafluoropropionic (PFP) and heptafluorobutyric (HFB) esters) are popular because they contain minimal exogenous atoms, are resolved with short retention times on standard GC columns and can be synthesized in ~5–10 mins (Fig. 5; Tables 1 and 2; Silfer et al., 1991; Veuger et al., 2005; Corr et al., 2007b; Kayacelebi et al., 2015; Ohkouchi et al., 2017; Riekenberg et al., 2017; Tea and Tcherkez, 2017). However, some TFA procedures are lengthier as they include several rounds of purification (Hannides et al., 2009; McMahon et al., 2011). Despite their popularity, fluorinated derivatives have notable limitations when analytes must be combusted or pyrolyzed (i.e., during GC–IRMS analyses). During combustion, Cu and Ni oxides form fluorides, lowering the reactor's oxidizing capacity (Meier-Augenstein, 1999; Tea and Tcherkez, 2017) and potentially leading to incomplete combustion of amino acids. This can compromise both δ^{13}C and δ^{15}N values (Dunn et al., 2011; Ghashghaie and Tcherkez, 2013; Tea and Tcherkez, 2017). During pyrolysis, HF is formed, which causes fractionation of H isotopes (Sauer et al., 2001; Renpenning et al., 2017) and can potentially corrode metal and silica components downstream of the reactor (Meier-Augenstein, 1999; Corr et al., 2007b; Dunn et al., 2011). TFA derivatives pose additional analytical challenges, including their sensitivity to moisture and alcohol, and low stabilities both in storage and during GC separation (Table 2; Darbre and Blau, 1965; Hušek and Macek, 1975; Corr et al., 2007b).

While TFA derivatives should be avoided for combustion- and pyrolysis-based analyses, their extensive fragmentation in electron impact ion sources (e.g., Jones, 2002) makes them attractive targets for position-specific isotope analysis by high-resolution mass spectrometry (e.g., Orbitrap; see Section 7.2). Moreover, enantiomers are preserved and can be separated on chiral columns (Serban et al., 1988; Silfer et al., 1991; Macko et al., 1997; McCarthy et al., 2004; Yamaguchi and McCarthy, 2018), and fluoroacetylation reactions can be adapted for arginine, which is not generally amenable to derivatization (Hušek and Macek, 1975; Amelung and Zhang, 2001; Kayacelebi et al., 2015). TFA derivative δ^{13}C values must be corrected for the isotope effect expressed at the TFA carbonyl C atom during derivatization (Table 1; Silfer et al., 1991; Corr et al., 2007b). N isotope fractionation during TFA isopropyl ester formation has been observed (Table 1; Hofmann et al., 2003).

5.1.3. Non-fluorinated acetyl derivatives

Reaction with acetic anhydride targets amine, hydroxyl, and thiol groups to form *n*-acetyl-based derivatives such as NACMEs (Fig. 5; Table 2; Corr et al., 2007a,b). For C isotope analyses, these non-fluorinated analogues perform better than, or similarly to, TFA derivatives on several metrics. Dunn et al. (2011) compared amino acid δ^{13}C values measured by liquid chromatography (LC)–IRMS and elemental analysis (EA)–IRMS against GC–IRMS and found that the *n*-acetyl derivatives consistently yielded better agreement among complementary measurements than the TFA derivatives. *N*-acetyl derivatives are also more stable, introduce the same number of C atoms, and can be separated on a variety of GC columns (Fig. 5; Tables 1 and 2; Adams, 1974; Corr et al., 2007a,b; Enggrob et al., 2019). Acetylation can cause fractionation for C and N isotopes, but data is easily correctable (Table 1; Hofmann et al., 2003; Corr et al., 2007a,b).

5.1.4. Alkoxycarbonyl derivatives

Methoxycarbonyl (MOC) methyl esters and ethoxycarbonyl (EOC) ethyl esters are favorable for C, N, and H isotope analyses because their reactions are simple and rapid ($\leq$ 5 mins), do not require heating (Hušek, 1991a,b), introduce minimal non-analyte C and H (Tables 1 and 2), and can be carried out in aqueous conditions (e.g., 0.1 N HCl), allowing for easy isolation of the products via extraction with organic solvent. Carbamate derivatives do not racemize (Zampolli et al., 2007) and can be baseline-separated on polar GC columns (Hušek, 1991b; Walsh et al., 2014). Alkyl chloroformate derivatizes amine groups (including the side-chains of lysine and histidine), as well as the phenol group of tyrosine (Huang et al., 1993; Chen et al., 2010). The side-chains

Table 1
Common amino acid derivatives for GC separation.

Derivative[a]	Added		Problematic amino acids[b]	Recommended columns	Co-elutions	KIE		References
	C	H				$^{13}\alpha$[c]	$^{15}\alpha$[d]	
NPNP	8–13	16–25	arg, asn, gln	High polarity: ZB-WAX VF-23 ms		0.891–0.982		Corr et al. (2007b); Tea and Tcherkez (2017)
NPIP	8–13	16–25	arg, asn, cys, gln, his, trp, val	Low to high polarity: Ultra-2 ZB-WAX ZB-FFAP		0.874–1.03	0.994–1.002	Metges et al. (1996); Metges and Daenzer (2000); Hofmann et al. (2003); Chikaraishi et al. (2007); Corr et al. (2007b); Tea and Tcherkez (2017); Ohkouchi et al. (2017)
TFA-IP	5–8	7–14	arg, asn, cys, gln, his, trp	Low polarity: ZB-1 Ultra-2		0.919–0.992	0.986–1.008	Hušek and Macek (1975); Silfer et al. (1991); Docherty et al. (2001); Hofmann et al. (2003); Corr et al. (2007b); Ohkouchi et al. (2017)
TFA-ME	3–5	3–6	arg, asn, gln, his, ser, thr, tyr	Low polarity: ZB-5				Darbre and Blau (1965); Islam and Darbre (1972); Hušek and Macek (1975); Jim et al. (2006)
PFP-IP	6–9	7–14	arg, cys, his	Low polarity: Ultra-2	lys/cys/ tyr			Frank et al. (1982); Amelung and Zhang (2001); Glaser and Amelung (2002); Kayacelebi et al. (2015); Tea and Tcherkez (2017)
HFB-IB	8–12	9–18	his, met	Low polarity: DB-5				Engel and Hare (1985); Golan and Wolfe (1979); MacKenzie and Tenaschuk (1979)
NANP	5–8	10–17	arg, asn, cys, gln, his, thr	High polarity: ZB-WAX ZB-FFAP VF-23 ms	pro/thr (VF-23 ms) phe/glx (ZB-WAX)	0.948–0.997		Demmelmair and Schmidt (1993); Metges and Daenzer (2000); Corr et al. (2007b)
NAIP	5–8	10–17	asn, gln	High polarity: VF-23 ms	ile/gly glu/met	0.946–0.978	0.997–1.002	Adams (1974); Hofmann et al. (2003); Corr et al. (2007b); Yarnes and Herszage (2017)
NACME	3–5	6–9	arg, asn, gln, gly, his, lys, met	Mid to high polarity: DB-225 ms VF-23 ms DB-WAX	leu/ile pro/thr (VF-23 ms)	0.933–0.981		Corr et al. (2007a,b); Dunn et al. (2011)
MOC ME	3–5	6–9	arg, ser, his	High polarity: VF-23 ms	leu/ile	0.978–1.060	0.978–1.002	Hušek (1991a,b); Walsh et al. (2014)
EOC EE	5–8	10–15	arg	Mid to high polarity: DB-225 ms VF-23 ms DB-WAX	ser/gln (DB-WAX) leu/ile (DB-225 ms)			Hušek (1991a,b); Godin et al. (2007)
TMS	3–9	9–27		Low polarity: DB-5				Molnár-Perl and Katona (2000); Sobolevsky et al. (2003); Zaikin and Halket (2005); Tea and Tcherkez (2017)
t-BDMS	6–18	15–45		Low polarity: DB-5			0.999–1.080[e]	Molnár-Perl and Katona (2000); Hofmann et al. (2003); Sobolevsky et al. (2003); Tea and Tcherkez (2017)

[a] Abbreviations: NPNP, *n*-pivaloyl *n*-propyl ester; NPIP, *n*-pivaloyl isopropyl ester; TFA-IP, trifluoroacetyl isopropyl ester; TFA-ME, trifluoroacetyl methyl ester; PFP-IP, pentafluoropropionyl isopropyl ester; HFB-IB, heptafluorobutyryl isobutyl ester; NANP, *n*-acetyl *n*-propyl ester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester; MOC ME, methoxycarbonyl methyl ester; EOC EE, ethoxycarbonyl ethyl ester; TMS, trimethylsilyl; t-BDMS, *tert*-butyldimethylsilyl.
[b] As reported in the specified literature (References column).
[c] Range of $^{13}\alpha$ values reported in Corr et al. (2007b), except that for MOC ME, which is calculated from data in Walsh et al. (2014) using Eq. (2) in Corr et al. (2007b).
[d] $^{15}\alpha$ calculated using Eq. (2) in Corr et al. (2007b) with data from Hofmann et al., 2003 (NPIP, TFA-IP, NAIP, and t-BDMS) and from Walsh et al., 2014 (MOC ME).
[e] Calculated assuming amine, hydroxyl, and thiol groups are each derivatized by only one t-BDMS group, which may not be true (see discussion in main text).

Table 2
Summary of reaction information for six major derivatization strategies employed for GC analysis of amino acids. Note that all derivatization methods for the amine group require correction for C isotope fractionation.

Derivatization strategy[a]		Groups targeted	Atoms per group		Reaction time and temp	Derivative stability	Pros	Cons	References
			C	H					
Pivaloylation	with pivaloyl chloride	Amine, hydroxyl, thiol	5	9	120 mins, 110 °C	Months at –18 °C	Highly stable; products have excellent chromatographic resolution; enantiomers preserved	Generates co-eluting byproducts; adds many C and H atoms; reagent toxic	Metges et al. (1996); Abe et al. (2002); Chikaraishi et al. (2007); Corr et al. (2007b); Tea and Tcherkez (2017)
Fluoroacetylation	with TFAA	Amine, hydroxyl, thiol	2	0	5 mins to 1 d, 100 °C	Days at –18 °C	Derivatization can be rapid and targets arginine; adds few C and no H atoms; enantiomers preserved; products elute quickly, are well-resolved, and fragment extensively in electron impact ion sources	Moisture- and alcohol-sensitive; $\delta^{13}C$ and $\delta^{15}N$ values comprised by CuF_2, NiF_2, and CO generation during combustion; δ^2H values comprised by HF generation during pyrolysis; products can degrade during elution through some GC columns	Darbre and Blau (1965); Hušek and Macek, (1975); Meier-Augenstein (1999); Jones (2002); McCarthy et al. (2004); Corr et al. (2007b); Hannides et al. (2009); Dunn et al. (2011); McMahon et al. (2011); Kayacelebi et al. (2015); Renpenning et al. (2017)
	with HFB		4	0					
	with PFP		3	0	10 mins, 110 °C				
Non-fluorinated acetylation	with acetic anhydride	Amine, hydroxyl, thiol	2	3	10 mins, 60 °C	Months at –5 °C	Products have good chromatographic resolution and are highly stable; adds few C and H atoms		Adams (1974); Corr et al. (2007a,b); Dunn et al. (2011); Tea and Tcherkez (2017); Enggrob et al. (2019)
Alkoxycarbonylation	with methyl chloroformate	Amine, hydroxyl, thiol[b]	2	3	<5 mins, 25 °C	Weeks at −20 °C	Derivatization rapid; reaction in aqueous conditions; enantiomers preserved; adds few C and H atoms	Reagent toxic; byproducts may form; certain side-chains not consistently derivatized; amino acids may not react quantitatively	Hušek (1991a,b); Huang et al. (1993); Peláez et al. (2000); Montigon et al. (2001); Meier-Augenstein (2004); Zampolli et al. (2007); Chen et al. (2010); Walsh et al. (2014)
	with ethyl chloroformate		3	5					
Esterification	with methanol	Carboxyl, hydroxyl[c]	1	3	5–60 mins, 25–70 °C	Months at –18 °C	Can be coupled with any derivatization strategy; no fractionation correction needed; reactions are rapid and quantitative; adds few C atoms	Can be moisture or alcohol sensitive; *n*-propanol and isopropanol add many H atoms	Hušek (1991a); Silfer et al. (1991); Chikaraishi et al. (2007); Corr et al. (2007a,b)
	with ethanol		2	5					
	with *n*-propanol		3	7	60 mins, 100 °C				
	with isopropanol		3	7					
Silylation	with BSTFA	Carboxyl, amine, hydroxyl, thiol	3	9	15–150 mins, 60–150 °C	Hours at 4 °C	No extraction required; products very volatile; derivatization is quantitative and occurs in a single step	Derivatization inconsistent; $\delta^{13}C$ values compromised by silicon carbide formation in combustion reactor; adds many C and H atoms; products are moisture-sensitive and unstable	Hušek and Macek (1975); Hofmann et al. (1995); Colombini et al. (1998); Molnár-Perl and Katona (2000); Shinebarger et al. (2002) Sobolevsky et al. (2003); Zaikin and Halket (2005); Tea and Tcherkez (2017)
	with MTBSTFA		6	15					

[a] Abbreviations: TFAA, trifluoroacetic anhydride; HFB, heptafluorobutyric anhydride; PFP, pentafluoropropionic anhydride; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; MTBSTFA, methyltributylsilyl tetrafluoroacetamide

[b] Thiol group of cysteine is derivatized when ethyl chloroformate is used, but not when methyl chloroformate is used.

[c] Hydroxyl groups not usually esterified, but serine and threonine can be methylated upon derivatization to MOC methyl esters.

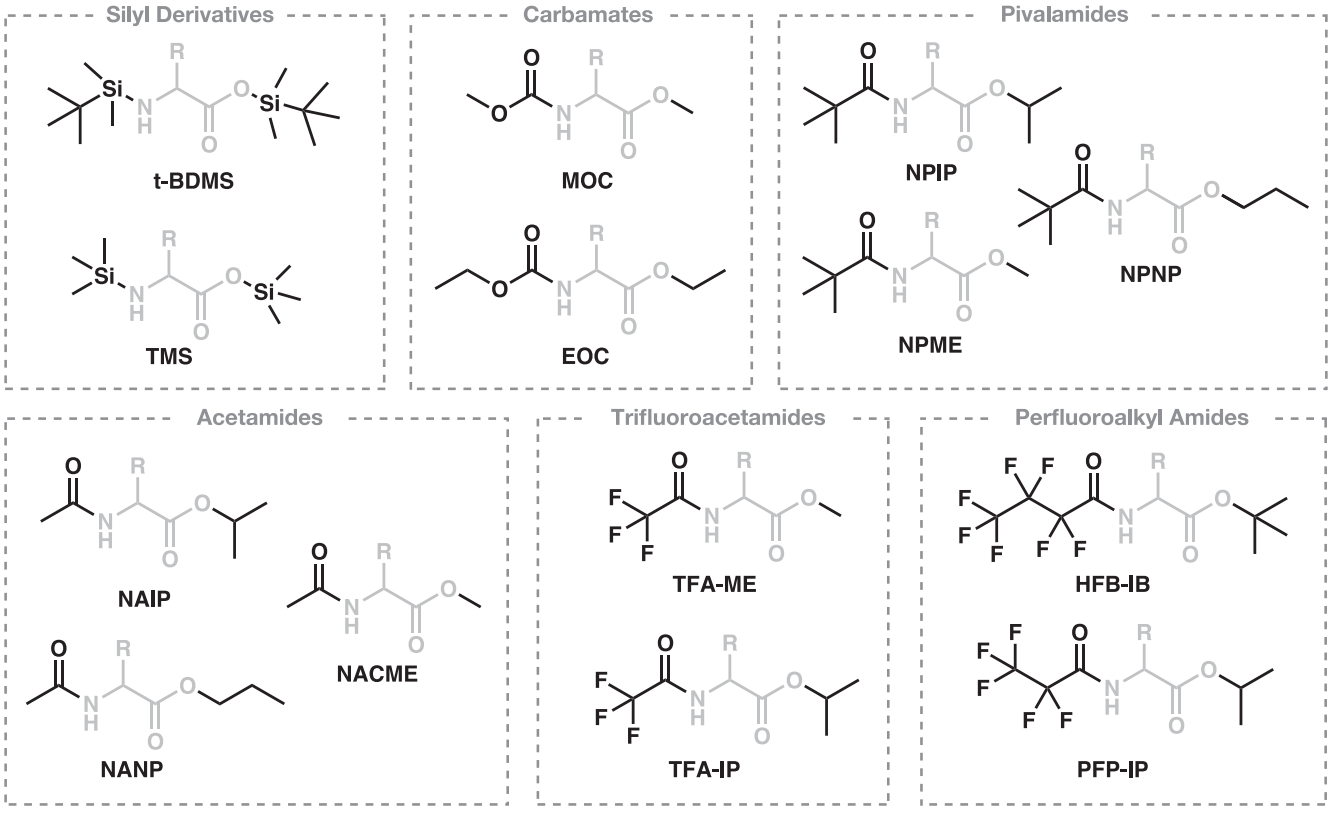

Fig. 5. Derivatives commonly used for gas chromatography separation of amino acids. Abbreviations: t-BDMS, *tert*-butyldimethylsilyl; TMS, trimethylsilyl; MOC, methoxycarbonyl; EOC, ethoxycarbonyl; NPIP, *n*-pivaloyl isopropyl ester; NPNP, *n*-pivaloyl *n*-propyl ester; NPME, *n*-pivaloyl methyl ester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester; NANP, *n*-acetyl *n*-propyl ester; TFA-ME, trifluoroacetyl methyl ester; TFA-IP, trifluoroacetyl isopropyl ester; HFB-IB, heptafluorobutyryl isobutyl ester; PFP-IP, pentafluoropropionyl isopropyl ester.

of serine, threonine, and cysteine are either esterified, acylated, or not derivatized, depending on the derivatizing reagents used (Huang et al., 1993; Zampolli et al., 2007; Chen et al., 2010; Walsh et al., 2014). Carbon and nitrogen isotope fractionation accompany MOC ester formation (Table 1; Sacks and Brenna, 2005; Walsh et al., 2014).

Byproduct formation is known to occur during derivatization with alkyl chloroformate, but these products are easily separated from amino acids on the GC column (Hušek, 1998; Peláez et al., 2000; Chen et al., 2010; Walsh et al., 2014). Additionally, a minor product can form in which the carboxyl group is esterified by the alkyl chloroformate rather than the alcohol (Peláez et al., 2000; Chen et al., 2010). Reaction conditions for chloroformate-based derivatization can cause glutamic acid to cyclize (Airaudo et al., 1987; Hušek, 1991b; Huang et al., 1993; Sacks and Brenna, 2005; Walsh et al., 2014), and acidic and amidic amino acid pairs to interconvert (Chen et al., 2010), although amidic residues deamidate anyways during hydrolysis.

5.2. Carboxyl group derivatives

5.2.1. Ester derivatives

Methyl ester derivatives are attractive because they form rapidly and quantitatively with few non-analyte C and H atoms (Tables 1 and 2). Ethyl, *n*-propyl, and isopropyl esters introduce more exogenous atoms, but may be selected to improve GC separation. Esterification reactions target carboxyl groups, but when coupled to derivatization with methyl chloroformate they can additionally target the hydroxyl groups of serine and threonine (Table 2; Huang et al., 1993; Zampolli et al., 2007; Chen et al., 2010; Walsh et al., 2014). Conditions for esterification are usually acidic, but basic conditions have been used occasionally (e.g., Tuckey and Stevenson, 1979; Corr et al., 2007b). Acidic conditions are created with acetyl chloride, thionyl chloride, or dilute HCl, although comparisons have found that acetyl chloride results in the highest yields of amino acids (Peláez et al., 2000; Corr et al., 2007b). Isopropylation and *n*-propylation require heating and must be performed in moisture-free conditions (e.g., Silfer et al., 1991; Chikaraishi et al., 2007; Corr et al., 2007b), while requirements for methylation and ethylation differ. When coupled to derivatization with alkyl chloroformates, reactions may be performed in aqueous conditions at room temperature (Hušek, 1991a; Sacks and Brenna, 2005; Chen et al., 2010; Walsh et al., 2014), but when combined with other amine group derivatization strategies (e.g., acetylation or pivaloylation) anhydrous conditions and heating are required to avoid expression of isotope effects at carboxyl sites (Corr et al., 2007a,b). When excess reagent is used and reactions are quantitative, negligible C isotope fractionation is expected, as C sites within the alcohol reagents do not directly participate in bond breakage or formation (Silfer et al., 1991; Rieley, 1994; Corr et al., 2007a).

5.2.2. Silyl derivatives

Silylation is not recommended for amino acid isotope analysis despite being a popular GC derivatization strategy for other organic compounds (e.g., Tea and Tcherkez, 2017), as it can add a large number of non-analyte atoms (up to 18 C and 45 H; Table 1), products are moisture-sensitive and degrade rapidly (Hušek and Macek, 1975; Colombini et al., 1998), and multiple derivatives may add to amine or hydroxyl groups inconsistently (Colombini et al., 1998; Hušek and Macek, 1975; Molnár-Perland and Katona, 2000; Zaikin and Halket, 2005; Tea and Tcherkez, 2017). Carbon does not participate in silylation, precluding expression of C isotope effects (Rieley, 1994). However, trimethylsilyl (TMS) derivatives may promote silicon carbide formation in GC–IRMS combustion reactors, leading to C isotope fractionation via non-quantitative conversion of analytes to CO_2 (Shinebarger et al., 2002; Tea and Tcherkez, 2017). Nitrogen isotope fractionation can be

significant due to non-quantitative derivatization of amino acids and degradation of derivative products (Table 1; Hofmann et al., 1995, 2003).

6. Separations

Amino acids must be separated from complex mixtures prior to isotope analysis, without inadvertently fractionating isotopes. Separation can be achieved using a variety of chromatographic techniques, stationary phases, and mobile phases, but to date no combination sufficiently separates all 20 proteinogenic amino acids. Separation is commonly achieved by GC coupled directly to an IRMS (i.e., 'online' measurement) without intermediate analyte collection. This method requires derivatization and an associated correction for added C or H atoms. Less commonly, LC is used for preparatory (i.e., 'offline') separation of underivatized amino acids, which are recovered using a fraction collector, sometimes assessed for purity, then analyzed on a separate instrument (e.g., EA–IRMS). New LC–IRMS systems enable LC separation online prior to isotope ratio measurement (Section 7.1.2). Considerations for optimizing GC and LC separations are beyond the scope of this review, but the reader is referred to Rood (2007) and Snyder et al. (2010) for excellent guides.

For many LC separations the lighter isotopologues elute from the column first (McCullagh et al., 2006; Broek et al., 2013), whereas for GC separations on nonpolar columns the heavier isotopologues typically elute first. Separation of isotopologues leads to isotopic fractionation across the width of a chromatographic peak. For example, differences between the front and tail halves of a peak separated using LC were 8.3‰ and 4.2‰ for $\delta^{15}N$ and $\delta^{13}C$ values, respectively (Broek et al., 2013). Thus, peaks must be completely collected or integrated to avoid altering the apparent isotope ratio of the sample (Meier-Augenstein, 1999; Sessions, 2006).

In the following sections, we compare GC and LC techniques for amino acid separation, examine the potential of IC and CE to be coupled to amino acid isotope analyses, and highlight several methods for determining sample purity.

6.1. Gas chromatography

GC is currently the most popular choice for separating amino acids for isotope analysis, yet there is no consensus on the best combination of stationary phase, derivative, and instrument settings. Many published approaches separate at least 10–15 amino acids (Table 1) in an hour or less. These separations commonly use 50 m or 60 m columns, carrier gas flow rates of 1–2 mL/min, and GC oven temperature programs from ~40 °C to 300 °C. While most separations are on nonpolar or low-polarity stationary phases (e.g., Ultra-2, DB-5 ms), high-polarity columns (VF-23 ms, ZB-FFAP, and ZB-WAX) substantially improve peak shapes of *n*-acetyl and alkoxycarbonyl ester derivatives, have higher analyte capacities, and are compatible with other common derivatives (Corr et al., 2007b; Walsh et al., 2014).

The main advantage of using GC to separate amino acids is the ability to couple directly to an IRMS, thus increasing analysis speed and sensitivity. The main drawbacks are: (1) low analyte capacity on GC columns – i.e., only a small amount of analyte can be introduced without degrading peak shape – especially for H and N isotope analyses where more sample is needed; (2) low temperature limits for polar columns (resulting in long runs and high background signals from degradation of the stationary phase); (3) poor suitability for four amino acids (arginine, histidine, asparagine, and glutamine are challenging to derivatize); and (4) mandatory derivatization (introducing exogenous atoms, additional sample workup, and possible isotopic fractionations — see Section 5).

Comprehensive GC × GC (Tobias et al., 2008, 2011) is a promising advancement that uses two columns with different stationary phases to improve separation of complex mixtures and reduce preparatory steps. This method has been successfully coupled to online isotope measurements of other organic compounds (Tobias et al., 2008, 2011) and may expand to amino acid isotope analysis.

6.2. Liquid chromatography

High performance liquid chromatography (HPLC or, more generally, LC) is widely used for offline separation and quantification of amino acids, but subsequent isotope analysis requires specialized equipment (fraction collectors) or instrumentation (LC–IRMS). LC provides some advantages over GC: derivatization is not needed for amino acids, and column capacity is substantially higher. However, purifying and collecting fractions offline may necessitate larger sample sizes, especially if an EA–IRMS is used for isotope analysis. Certain amino acids, like isoleucine and leucine, are difficult to separate when underivatized.

Amino acid separation by LC typically uses nonpolar stationary phases and polar mobile phases (e.g., water, acetonitrile, methanol). The Sielc Primesep A is the most popular column for online $\delta^{13}C$ (McCullagh et al., 2006; Tripp et al., 2006; Smith et al., 2009; Dunn et al., 2011) and offline $\delta^{15}N$ and $\delta^{13}C$ measurements (Broek et al., 2013; Broek and McCarthy, 2014; Sun et al., 2020). Primesep A columns can separate 14 amino acids with run times of 105 mins (Broek et al., 2013). An alternative is "hydrophilic interaction liquid chromatography" (HILIC). HILIC initially employs a mobile phase with high organic and low aqueous content, allowing a small water layer to form between the stationary and mobile phases, which provides good separation of amino acids – particularly aspartic acid and serine which cannot be separated using other LC stationary phases (Park et al., 2019). Finally, while not currently common instrumentation, the LC–IRMS system (discussed further in Section 7.1.2) enables online C isotope analysis, has similar sensitivity to some GC–IRMS applications (Table 3), and may become more popular in future amino acid isotope research.

6.3. Ion chromatography

Ion chromatography (IC) is primarily used to quantify amino acids, but has also been used for online (Morrison et al., 2010) and offline (Zhang et al., 2021) IRMS analyses. IC separation can be coupled to an IRMS for online C isotope analysis via an Isoprime Liquiface system (Morrison et al., 2010). Abaye et al. (2011) used this system to measure the $\delta^{13}C$ values of 11 amino acids, including arginine, lysine, and some aliphatic amino acids, which were quickly resolved (70 mins) with adequate precisions (SD < 1‰; Abaye et al., 2011). IC has also been used to separate 9 amino acids offline prior to N isotope analysis using a purge-and-trap continuous-flow IRMS (Zhang et al., 2021). Advantages of separating amino acids by IC are that neither pre- nor post-column derivatization is required, and other matrix components (carbohydrates, glycols, and sugar alcohols) can be simultaneously separated (Larson et al., 2002), minimizing the sample workup steps needed (see Sections 2 and 4). A major disadvantage is lengthy run times (180 mins or longer; Zhang et al., 2021).

6.4. Capillary electrophoresis

Capillary electrophoresis (CE) separates compounds based on mobility in an electric field (Ewing et al., 1989) but has not yet been coupled to isotope measurements. Although CE currently lacks selectivity compared to other separation methods, its speed, simplicity, and low cost hold potential for future applications involving online amino acid isotope analysis. As with LC, samples do not require derivatization or conversion to gases and CE can be coupled to numerous detectors. Chiral buffers can be used to change the mobility of D- vs L-amino acids to separate enantiomers (Hutt et al., 1999). Miniaturized versions of CE systems (microchip electrophoresis) have been explored for inclusion on extraterrestrial sampling missions that investigate amino acids to distinguish between biotic and abiotic sources (Hutt et al., 1999; Creamer et al., 2017) and may prove useful when combined with

Table 3
Summary of analytical techniques for isotopic analysis. Many of these methods have yet to be applied to amino acids from the environment, although some have been used to measure amino acid standards.

Analytical technique[a]		Isotopes	Measured species	Specificity	Typical precision (1σ, ‰)	Typical sensitivity (nmol)	References[b,c]
IRMS	Conventional GC–IRMS	C	CO_2	Compound-specific	~0.6–2.3	0.1–10	McCarthy et al. (2004)[c]; Sessions (2006); Corr et al. (2007b)[b]; Baczynski et al. (2018)
		N	N_2	Compound-specific	0.5–1	1–10	McClelland and Montoya (2002)[b,c]; Sessions (2006); Rieckenberg et al. (2020)[b,c]
		H	H_2	Compound-specific	~10	10–50	Sessions (2006); Fogel et al. (2016)[b,c]
	Optimized GC–IRMS	C	CO_2	Compound-specific	0.9–1.5	0.05–0.6	Baczynski et al. (2018)
	Pyrolysis-GC–IRMS	C	CO_2 (from pyrolytic fragments)	Position-specific	~1	~100s	Wolyniak et al. (2005)[b]; Gilbert et al. (2016a,b)
	LC–IRMS	C	CO_2	Compound-specific	0.1–1.4	7–55	Smith et al. (2009)[b,c]; Dunn et al. (2011)[b,c]
	PT-CF–IRMS	N	N_2O	Compound-specific	0.3–0.7	<15	Zhang et al. (2021)[b,c]
	FIA-NR–IRMS	C	CO_2 (from carboxyl group)	Position-specific	0.1	15	Fry et al. (2018)[b]; Fry and Carter (2019)[b,c]
	SWiM–IRMS	C	CO_2	Bulk	0.6	1–10	Sessions et al. (2005)[b]; Eek et al. (2007)
	Conventional EA–IRMS	C	CO_2	Bulk	0.1–0.5	2000–8500	Polissar et al. (2009); Ogawa et al. (2010)[b]; Sun et al. (2020)[b,c]
		N	N_2	Bulk	0.1–0.5	1500–3500	Ogawa et al. (2010)[b]; Broek et al. (2013)[b,c]; Rieckenberg et al. (2020)[b,c]
		S	SO_2	Bulk	0.3	500–3000	Giesemann et al. (1994)
	TCEA–IRMS	H	H_2	Bulk	0.3–3	300,000	Gehre et al. (2015)[b]; Fogel et al. (2016)[b]; Newsome et al. (2020)[b]
	Optimized EA–IRMS	C	CO_2	Bulk	0.2–0.5	40–60	Polissar et al. (2009)
		N	N_2	Bulk	0.1–0.5	10–25	Polissar et al. (2009); Ogawa et al. (2010)[b]; Broek and McCarthy (2014)[b,c]; Swalethorp et al. (2020)[b,c]
		S	SO_2	Bulk	0.1–0.3	50–150	Phillips et al. (2021); Sayle et al. (2019)[b,c]
High-resolution MS	Orbitrap	C, N, S, H	Molecular ion, fragment ions	Compound-specific, position-specific	≤1	~0.1–10	Eiler et al. (2017); Neubauer et al. (2018)[b]; Chimiak et al. (2021)[b,c]
NMR	^{13}C NMR	C	Molecule	Position-specific	~1	~1,000,000	Romek et al. (2017)[b]
	^{1}H NMR	C	Molecule	Position-specific	0.5–3.5	50,000–300,000	Rasmussen and Hoffman (2020)[b]

[a] Abbreviations: IRMS, isotope ratio mass spectrometry; GC–IRMS, gas chromatography–IRMS; LC–IRMS, liquid chromatography–IRMS; PT-CF–IRMS, purge-and-trap continuous-flow IRMS; FIA-NR–IRMS, flow injection analysis reaction with ninhydrin–IRMS; SWiM–IRMS, spooling wire micro-combustion–IRMS; EA–IRMS, elemental analysis–IRMS; TCEA–IRMS, thermal conversion-EA–IRMS; NMR, nuclear magnetic resonance.
[b] Method applied to pure amino acid standards.
[c] Method applied to amino acids in natural (terrestrial or extraterrestrial) materials.

sensitive techniques (e.g., high-resolution mass spectrometry – Section 7.2).

6.5. Methods of assessing purity

Most compound-specific isotope analyses require purified samples. For example, isotope ratio monitoring by nuclear magnetic resonance spectroscopy (NMR; Section 7.3) requires > 98% analyte. Other methods, such as EA–IRMS (Section 7.1.3), lack online separation so geochemists must first purify amino acids offline for compound-specific applications and ensure no contaminants are present. Numerous options exist for assessing sample purity. LC–MS or GC–MS can be used to identify contaminants (Hare et al., 1991; Phillips et al., 2021), but some contaminants may avoid detection if their mass falls outside of the analytical window selected. Proton (^{1}H) NMR is an attractive option for purity verification as it is non-destructive, rapid (≤5 min), and commonly available at user facilities. Elemental composition determined using an EA system can be used to indirectly assess purity because pure amino acids have a narrow range of elemental ratios (C/N = 1.5–9.0, C/S = 3.0–5.0). We recommend verifying sample purity in studies using offline separations that are decoupled from the final isotopic analyses.

7. Isotopic analysis

Potential goals of amino acid isotopic analysis include characterizing the stable isotope ratios of one or more elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, and/or $^{2}H/^{1}H$), either averaged across each amino acid molecule ("compound-specific" or "molecular-average") or characterized at individual atomic positions ("position-specific" or "intramolecular"). Established techniques for characterizing isotopic compositions of amino acids from natural samples use IRMS paired with offline or online separation strategies (Section 6) and combustion or pyrolysis of separated analytes. Additional techniques are in early stages of development — especially for position-specific isotope analysis of amino acids — but have not yet been applied to terrestrial materials. We describe the isotopic analysis techniques that are currently useful to organic geochemists, as well as possible future advancements (e.g., high-resolution

mass spectrometry), summarizing figures of merit, required preparatory steps, and key advantages and disadvantages. Methodological improvements will continue to combine different online separation and isotope detection strategies in novel ways.

7.1. Isotope ratio mass spectrometry

IRMS achieves high levels of precision and accuracy (Table 3) via simultaneous comparison of two or more isotopes (i.e., the isotope ratio). Isotope ratios are further compared between the sample and a standard of known composition on an international scale (e.g., VPDB) and reported as δ values (Eq. (1)). Amino acids must be quantitatively converted to CO_2 (for $^{13}C/^{12}C$ analysis), N_2 ($^{15}N/^{14}N$ analysis), H_2 ($^{2}H/^{1}H$ analysis) or SO_2 ($^{34}S/^{32}S$ analysis; Table 3) for IRMS measurements. This goal was originally achieved by combusting pre-purified amino acids in sealed tubes offline, then isolating the resulting CO_2 and N_2 for isotope analysis (Abelson and Hoering, 1961; Macko et al., 1983; Tuross et al., 1988). Today, this is typically achieved via online methods in which analytes are carried by an inert gas through a chemical conversion interface on their way to the IRMS. The most common interfaces employ combustion or pyrolysis, but other techniques such as chemical oxidation or high-temperature combustion-desolvation have also been reported (Tea and Tcherkez, 2017). Here we cover GC–IRMS, LC–IRMS, and EA–IRMS instrumentation, and summarize capabilities of these methods in Fig. 6. For more detailed reviews of IRMS principles, analytical considerations, and historical context, see Brenna (1994), Brand (1996), Brenna et al. (1997), Meier-Augenstein (1999), and Sessions (2006).

7.1.1. GC–IRMS

GC–IRMS is routinely used to measure compound-specific $\delta^{13}C$ and $\delta^{15}N$ values of amino acids (Ohkouchi et al., 2017; Close, 2019), and, less commonly, $\delta^{2}H$ values (Fogel et al., 2016; Newsome et al., 2020). The GC is typically coupled to the IRMS via a combustion (for CO_2 or N_2) or pyrolysis (for H_2) interface. Amino acid samples must be derivatized for GC separation and free of water, particles, elemental S, and salts to avoid damage to the GC column and chemical conversion interface.

GC–IRMS can achieve instrumental precision of ≤0.1‰ for C, <1‰ for N, and 2‰ for H in other common analytes (Table 3; Sessions, 2006). Amino acids have larger propagated uncertainties of 0.6‰ to over 2‰ for C (e.g., Corr et al., 2007a; Smith et al., 2009; Dunn et al., 2011) and up to ~10‰ for H (Table 3; Fogel et al., 2016) because the added C or H derivative atoms increase the uncertainty on the final amino acid isotope ratio (see Section 5). Measurements typically require ~0.1 to 10s of nmol C or N, and one to two orders of magnitude more H (Table 3). Specific advantages of GC–IRMS for amino acid isotope analysis are its high sensitivity and high throughput. GC–IRMS is especially appropriate for small samples and for researchers seeking concurrent isotopic data on as many amino acids as possible. Disadvantages include difficulty of use, added sample workup steps, and larger uncertainties associated with derivatization. Recent work couples narrow-diameter column ("fast") GC to IRMS (Sacks et al., 2007; Baczynski et al., 2018), paving the way for future amino acid isotope analyses with sharper chromatographic peaks, faster run times, and enhanced sensitivity for $\delta^{13}C$ analyses.

7.1.2. LC–IRMS

A recent development, the Finnigan LC IsoLink system (LC–IRMS), provides the ability to measure $^{13}C/^{12}C$ isotope ratios of underivatized amino acids separated online by liquid chromatography (McCullagh et al., 2006; Juchelka and Krummen, 2008). Samples are introduced in dissolved form, separated by LC, and chemically oxidized to CO_2 (at 100 °C) before introduction into the IRMS (Juchelka and Krummen, 2008; Godin and McCullagh, 2011). Several studies demonstrate that quantitative conversion to CO_2 can be achieved across environmentally relevant sample sizes. Leucine samples with concentrations of ~50–300 ng μL^{-1} had $\delta^{13}C$ precisions ≤0.15‰ (Juchelka and Krummen, 2008), although standard deviations were higher (0.35‰) for a mixture of four amino acids at concentrations of ~50–400 ng μL^{-1} (Juchelka and Krummen, 2008).

A significant drawback to current LC–IRMS systems is that they must use acidic, organic-free mobile phases, as any organic solvents would be oxidized to CO_2 along with the analytes. Typical concentrations of organic solvents used in mobile phases for LC separations would saturate the IRMS detector (Godin et al., 2005). Additionally, LC–IRMS is generally less sensitive than GC–IRMS and can only measure C isotopes, not N or H (Fig. 6). For information on LC–IRMS technical challenges and solutions, we refer the reader to a review by Godin and McCullagh (2011). Thus far, LC–IRMS systems have successfully measured amino acids from peptides and archaeological samples (Godin et al., 2005; McCullagh et al., 2006), but methods are still in development for complex materials like marine sediments (Close, 2019).

7.1.3. EA–IRMS

EA–IRMS instruments are most commonly used to measure the bulk isotopic compositions of complex, solid samples, but can also be coupled with offline preparatory techniques such as LC with fraction collection to provide isotopic measurements of individual amino acids or proteins (e.g., Broek et al., 2013; Dong et al., 2017). Isolated amino acids are packed into metal foil capsules, combusted to CO_2, N_2, H_2O, and/or SO_2, then dried and separated using a short GC column for isotope ratio measurement.

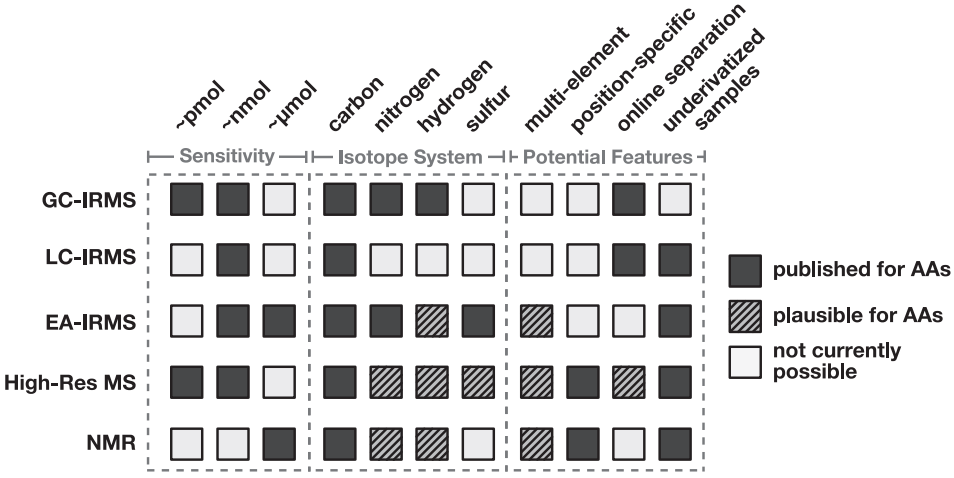

Fig. 6. Summarized capabilities of common analytical techniques used for amino acid isotope analysis. Sensitivities given are lower limits. "Multi-element" refers to simultaneous measurement of different isotopic systems (i.e. $^{13}C/^{12}C$ and $^{15}N/^{14}N$) in a single analysis. "Online separation" refers to the ability to measure multiple compounds from a mixture online. A "position-specific" measurement encompasses the ability to extract isotopic information from individual sites within a compound. We do not consider bulk or compound-specific isotope measurements on molecules containing single elements (e.g., N or S in amino acids), nor chemical approaches to site-specific measurements (e.g., decarboxylation reactions) as "position-specific" capabilities of the instrument. For more details and references, see Table 3 and Section 7.

Although three orders of magnitude less sensitive than GC–IRMS (requiring μmol of C or N; Fig. 6; Table 3) and requiring time-consuming offline separations, EA–IRMS systems may be the ideal choice for certain specialized applications (e.g., $\delta^{34}S$ measurements of pre-isolated cysteine or methionine or high-precision $\delta^{15}N$ measurements) or when sample size is not limiting. Additionally, EA–IRMS instruments are more widely available, achieve better precisions (~0.1–0.5‰; Table 3), and are simpler to operate than GC–IRMS or LC–IRMS. Perhaps most importantly, EA–IRMS offers the ability to simultaneously analyze the isotope ratios of multiple elements in the same acquisition (e.g., C and N, with or without S; Fry, 2007; Broek and McCarthy, 2014; Fourel et al., 2014; Brodie and Kracht, 2016; Sayle et al., 2019)—although this strategy is not yet established for amino acids (Fig. 6).

Recent optimizations have enhanced the sensitivity of EA–IRMS (Table 3). The nano-EA–IRMS system (Polissar et al., 2009) improved sensitivities by 100–500-fold for $\delta^{13}C$ and $\delta^{15}N$ measurements of pre-purified amino acids, while achieving similar precisions (Broek and McCarthy, 2014; Swalethorp et al., 2020). Optimizations for S isotope analysis by EA–IRMS (Fry, 2007; Fourel et al., 2014) enabled the first compound-specific $\delta^{34}S$ measurements of cysteine and methionine (Phillips et al., 2021). Furthermore, H isotope analysis by pyrolysis EA (also known as thermal conversion EA, or TCEA), has been demonstrated for ~0.3 mmol analyte (Table 3; Gehre et al., 2015) but has only been applied thus far to pure amino acid standards (Fogel et al., 2016; Newsome et al., 2020).

We see optimized EA–IRMS systems (in combination with automated, offline preparatory techniques) as an area of potential for amino acid isotope studies, especially when leveraged for analyzing multiple isotope systems simultaneously. We recommend optimized EA–IRMS configurations for measuring amino acid $\delta^{34}S$ values, as S cannot be measured by GC–IRMS or LC–IRMS systems, and we emphasize that EA–IRMS can achieve more precise and accurate $\delta^{15}N$ measurements than GC–IRMS (Table 3; Broek and McCarthy, 2014; Swalethorp et al., 2020). For individual applications, these advantages must be weighed against the need for offline sample separation and large sample sizes, and/or the availability of optimized instrumentation like the nano-EA–IRMS.

Finally, we note that a variety of other promising IRMS configurations have been applied to isotope analysis of individual amino acids or proteins by initially isolating analytes offline using preparatory techniques. These approaches include "spooling wire micro-combustion IRMS" (SWiM–IRMS; Sessions et al., 2005; Eek et al., 2007), and purge-and-trap continuous-flow IRMS (PT-CF–IRMS; Zhang et al., 2021). These configurations are not yet widely available but achieve sensitivities and precisions similar to GC–IRMS without requiring derivatization (see Supplementary Section 3).

7.1.4. IRMS configurations for position-specific isotope analysis

Other IRMS-coupled techniques are in development for position-specific isotope analysis. The approach is to introduce an initial chemical/thermal degradation step that isolates different atomic positions of the analyte prior to isotope ratio measurement. One example is "flow injection analysis reaction with ninhydrin IRMS" (FIA-NR–IRMS), which uses a chemical reaction to decarboxylate amino acids and measure the position-specific $\delta^{13}C$ value of the carboxyl-C position with ~0.3–0.5‰ precision (Table 3; Fry et al., 2018, Fry and Carter, 2019). Another is a method for the preparatory isolation and chemical work-up of free glutamine for position-specific $\delta^{15}N$ measurement by PT-CF–IRMS (Table 3; Lee et al., 2021). Glutamine is split into two fractions: (1) the amino-N is oxidized to nitrite, and (2) the amide-N is converted into ammonium by acid hydrolysis, then oxidized to nitrite. Both nitrite pools are reduced to N_2O and analyzed separately, recovering isotope ratios for the amino-N and amide-N, respectively. Finally, several variations of online-pyrolysis-GC–IRMS systems have been developed and applied to position-specific $^{13}C/^{12}C$ analysis of amino acid standards. Analytes are thermally converted (pyrolyzed) into fragments that encompass different carbon positions from the original amino acid molecule. These fragments are then separated by GC, individually combusted, and measured by IRMS to recover position-specific signatures (for reviews, see Gauchotte-Lindsay and Turnbull, 2016; Gilbert, 2021). Published precisions range from <0.2‰ for directly measured positions of alanine and phenylalanine standards to 0.9–6.5‰ for calculated position-specific $\delta^{13}C$ values due to error propagation (Table 3; Wolyniak, 2005). Application of these techniques to amino acids in geochemical samples has not yet been realized.

7.2. High-resolution mass spectrometry

Directly converting amino acids to CO_2, N_2, and H_2 destroys information recorded in the position-specific distributions of isotopes. For example, ^{13}C-enrichment at the C-1 versus C-2 position in alanine has different implications for its origins (Chimiak et al., 2021), but cannot be discerned from the ratio of $^{13}C/^{12}C$ in CO_2 produced by whole-molecule combustion (e.g., as in GC–IRMS). High-resolution mass spectrometry of intact analyte ions (as opposed to whole combustion or pyrolysis products) provides an avenue for analyzing position-specific isotope distributions in amino acids. This approach is still in development for natural materials relevant to organic geochemists (e.g., plant biomass – Wilkes et al., 2019), but represents a promising future direction.

Position-specific isotope ratios can be accessed by measuring fragments of an amino acid molecule, which form spontaneously during ionization and/or collision in an ion trap. Constraining position-specific isotopic differences is accomplished by measuring and comparing isotope ratios of two or more fragments of an amino acid in a mass spectrometer. These measurements require that the spectrometer can distinguish molecular fragments containing different rare isotopes, e.g., ^{13}C vs ^{2}H or ^{15}N (Supplementary Section 4). Several mass spectrometers achieve mass resolutions that can distinguish these different rare isotope substitutions, but only Orbitrap™-based instruments have been studied in detail (e.g., Eiler et al., 2017; Hofmann et al., 2020; Neubauer et al., 2020; Hilkert et al., 2021). Orbitrap mass analyzers may be coupled to GC or LC for online isolation of amino acids; thus, required sample preparation (e.g., derivatization) and clean-up steps would reflect the choice of instrumentation. In addition, Orbitrap instruments hold the potential to measure clumped-isotope compositions of amino acids (i.e., containing two or more rare isotopes).

Orbitrap analysis of pure amino acid standards indicates minimal sample sizes are required to obtain $\delta^{13}C$ precisions $\leq$1‰ for fragments of amino acids (Table 3; Eiler et al., 2017; Neubauer et al., 2018). An initial application to samples of the Murchison meteorite revealed substantial differences in $\delta^{13}C$ values between different atomic sites within meteoritic alanine (Chimiak et al., 2021), but had large propagated uncertainties for individual C positions. Limitations of this measurement approach for amino acids may include insufficient fragmentation or the lack of available position-specific isotopic reference materials for reporting results. For example, while Neubauer et al. (2018) calculated isotope ratios for most positions of pure methionine samples, not all amino acids fragment as easily (Piraud et al., 2003; Zhang et al., 2019). Accurately identifying the atomic positions from the original amino acid that ends up in each fragment is crucial but may present a bottleneck, as isotope labeling experiments may be required to resolve ambiguities. Further, standardization requires a separate working standard for each amino acid analyzed, which must then be characterized by a different position-specific isotopic technique (e.g., NMR) to anchor results to an international reference frame (e.g., VPDB).

7.3. Nuclear magnetic resonance spectroscopy (NMR)

Isotopic NMR (or SNIF-NMR®, hereafter simply NMR) provides stable C and H isotope ratios for individual atomic positions within an amino acid by ^{13}C NMR or ^{2}H NMR, respectively (Vallet et al., 1991; Romek et al., 2017). Because of its large sample size requirements

(Table 3), NMR is best suited for characterizing pure standards but provides a path for establishing position-specific isotope standards anchored to the international scale that may be used with a more sensitive technique (e.g., Orbitrap). A separate radio frequency signal is produced by each chemically distinct ^{13}C or ^{2}H atom within a molecule. These NMR signals are quantified through peak integration and used to calculate position-specific isotope distributions by comparison with the average isotope ratio for the entire molecule (measured separately by IRMS; reviewed by Jézéquel et al., 2017). However, ^{13}C NMR and ^{2}H NMR typically require hundreds of milligrams (~1 mmol) of pure analyte to achieve precisions of ~1‰ and 5‰, respectively (Table 3; Romek et al., 2017; Gilbert, 2021). Such sample size requirements are prohibitive for applications to amino acids from natural samples and all published NMR analyses of amino acids to date have targeted pure standards (Vallet et al., 1991; Romek et al., 2017; Rasmussen and Hoffman, 2020). An alternative NMR approach for position-specific $^{13}C/^{12}C$ analysis was recently applied to amino acid standards using ^{1}H NMR (Rasmussen and Hoffman, 2020; Supplementary Section 5). This technique has at least two advantages over direct ^{13}C NMR, while achieving similar precisions (~1‰; Table 3) and accuracies: (1) it uses more commonly available equipment, and (2) it is an order of magnitude more sensitive (Table 3; Hoffman and Rasmussen, 2019). One challenge of using ^{1}H NMR to study C isotopes is that not all C positions in amino acids are visible.

All of the above NMR techniques are non-destructive and can be readily calibrated to internationally recognized scales, yet NMR has significant drawbacks compared to other isotopic measurements – notably, poor sensitivity and precision (requiring up to six orders of magnitude more C than IRMS for 0.5–3.5‰ precision; Table 3). NMR requires analytes to be purified (>98%) offline prior to measurement, followed by several hours of analysis (Remaud et al., 2018). While analytical advances are ongoing (see Jézéquel et al., 2017; Hoffman and Rasmussen, 2019), we anticipate NMR will remain most useful as a complementary technique for calibrating standards rather than a primary tool for measuring isotope ratios in (bio)geochemical samples.

7.4. *Referencing strategies for isotopic analysis*

Making accurate and precise isotope ratio measurements are major challenges in the stable isotope community. As compound-specific (and emerging position-specific) isotope applications expand across fields, the need for inter-laboratory comparability of data becomes more urgent. In addition to variable sample preparation strategies, protocols used across laboratories to calibrate and quality-check isotope ratio measurements are inconsistent, which hampers data reproducibility between studies (Carter and Fry, 2013; Yarnes and Herszage, 2017). Inter-laboratory comparisons of amino acid $\delta^{13}C$ values recovered from the same samples are not commonly published, but when done so, often show disagreement (e.g., Arthur et al., 2014; Ayayee et al., 2015); comparisons for N isotope analyses are even less common. Following recommendations by Carter and Fry (2013) and Yarnes and Herszage (2017), we urge widespread adoption of the following standardization practices: (1) calibration of data based on internal standards (synthetic amino acids like norleucine that are co-injected with samples) and/or multipoint amino acid isotope standards (i.e., spanning a range of isotopic compositions outside those of samples) to account for scale compression effects by instruments (e.g., Yarnes and Herszage, 2017; Riekenberg et al., 2020; Zhang et al., 2021), (2) use of quality assessment materials, such as an external standard measured repeatedly throughout sample analysis (e.g., Styring et al., 2015), to verify measurement accuracy, and (3) increased comparisons of isotopic data between laboratories and publication of results.

Several internationally recognized amino acid reference materials are available for compound-specific isotope analysis: glycine, L-valine, and L-glutamate standards with known values of $\delta^{13}C$ and $\delta^{15}N$ have been calibrated through interlaboratory ring tests and are distributed by the USGS, IAEA, and Indiana University (Qi et al., 2003, 2016; Schimmelmann et al., 2016). Comparability of isotope ratio measurements would be greatly improved by the development of: (1) additional amino acid standards encompassing a wider range of $\delta^{13}C$ and $\delta^{15}N$ values, (2) amino acid $\delta^{2}H$ and $\delta^{34}S$ reference materials (the former effort is currently challenged by lack of a reliable method to correct for exchangeable hydrogen (Schimmelmann et al., 2016), (3) internationally-recognized protein standards with calibrated amino acid isotopic compositions (Yarnes and Herszage, 2017), and (4) position-specific reference materials anchored to international scales.

8. Conclusions and outlook

We have synthesized the extensive literature on amino acid isotope analysis from preparation to measurement, highlighting established techniques and emerging technologies that may offer future benefits to geochemists. We emphasize that there is no "one size fits all" method for amino acid isotopic analysis: researchers have multiple options and choices will be guided by sample type, individual applications, and available resources. Over the next decade, attention to standardizing referencing strategies and developing reference materials is needed for data generated across laboratories to be reproducible. A second beneficial area of attention is measuring H and S isotope ratios in amino acids, which would expand environmental and ecological applications. For example, $^{2}H/^{1}H$ ratios could provide information on migration and energy flow (e.g., Rubenstein and Hobson, 2004; Bowen et al., 2005; Fogel et al., 2016), and $^{34}S/^{32}S$ ratios could track dietary protein sources (e.g., Richards et al., 2001). To date, the $\delta^{2}H$ and $\delta^{34}S$ values of amino acids have been characterized in relatively few published studies (Fogel et al., 2016; Newsome et al., 2020; Phillips et al., 2021), so the full potential of these measurements is only beginning to be explored.

Finally, we see three areas of amino acid isotope research where methodologic innovation will have the most impact. First, there are numerous opportunities for method automation. In addition to the substantial improvements stemming from online LC–IRMS and GC–IRMS techniques, further coupling (e.g., combining protein hydrolysis with high pressure ion-exchange clean-up) would increase throughput. A second area for innovation is simultaneous analysis of multiple isotope systems on the same sample and instrument. Because preparing amino acids for isotope measurement is tedious, measuring H, C, N, and S concurrently offers greater reward and is increasingly possible with techniques like optimized EA–IRMS and high-resolution mass spectrometry. Multi-element analysis of amino acids will be especially helpful for ecological and forensics studies that reconstruct modern and paleo diets, food webs, animal and human movement, and behaviors of ancient civilizations. Third, we see a renaissance in position-specific isotope analysis via high-resolution mass spectrometry and/or pyrolysis-GC–IRMS. Specific intramolecular information, such as C–S bond clumping in methionine or C–H bond clumping in aliphatic residues, could inform targeted questions about synthesis. Further, the ability to measure position-specific isotope ratios at natural abundance may complement or replace isotope labelling methods in metabolomics studies. None of these advancements will be possible without the analytical expertise of isotope geochemists and ecologists, whose experiments and observations will inform these promising frontiers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Kate Freeman, Sarah Zeichner, and Phillip M. Riekenberg for their helpful feedback on early versions of this review. The authors also acknowledge Karli Holman for her expertise in

reviewing proposed acid hydrolysis reactions and thank members of the Sessions and Eiler labs at Caltech and of the Freeman lab at Penn State for their informative discussions with authors, as well as the two anonymous reviewers and the Associate Editor for their constructive comments. This work was supported in part by grants from the NSF GRFP (to SNS, Fellow ID 2018259065), NASA Astrobiology Institute (grant number 80NSSC18M094 to ALS and JME), NSF Geobiology (grant number EAR-1921330 to ALS), the Agouron Institute (grant number AI-F-GB54.19.2 to EBW) and the Simons Foundation (to JME, award number 626103) and DOE (to JME, award number DE-SC0016561). Any opinions, findings, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding agencies.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2021.104345.

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