

1 **Practical considerations for amino acid isotope analysis**

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14

15 **ABSTRACT**

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

37

38 *Keywords:* Amino acids, compound-specific isotope analysis, position-specific isotope analysis,
39 acid hydrolysis, derivatization, separation, isotope ratio mass spectrometry, OrbitrapTM, nuclear
40 magnetic resonance

41

42 *Abbreviations:*

43 *Amino acids:* ala, alanine; arg, arginine; asn, asparagine; asp, aspartic acid; asx, asparagine +
44 aspartic acid; cys, cysteine; his, histidine; gln, glutamine; glu, glutamic acid; glx, glutamine +
45 glutamic acid; gly, glycine; ile, isoleucine; leu, leucine; lys, lysine; met, methionine; phe,
46 phenylalanine; pro, proline; ser, serine; thr, threonine; trp, tryptophan; tyr, tyrosine; val, valine

47 *Chemicals:* HCl, hydrochloric acid; HF, hydrofluoric acid; dichloromethane (DCM); TFAA,
48 trifluoroacetic anhydride; HFB, heptafluorobutyric anhydride; PFP, pentafluoropropionic
49 anhydride; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; MTBSTFA, methyltributylsilyl
50 tetrafluoroacetamide

51 *Derivatives:* NPME, *n*-pivaloyl methyl ester; NPNP, *n*-pivaloyl *n*-propyl ester; NPIP, *n*-pivaloyl
52 isopropyl ester; TFA-IP, trifluoroacetyl isopropyl ester; TFA-ME, trifluoroacetyl methyl ester;
53 PFP-IP, pentafluoropropionyl isopropyl ester; HFB-IB, heptafluorobutyryl isobutyl ester; NANP,
54 *n*-acetyl *n*-propylester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester; MOC
55 ME, methoxycarbonyl methyl ester; EOC EE, ethoxycarbonyl ethyl ester; TMS, trimethylsilyl; t-
56 BDMS, tert-butyldimethylsilyl

57 *Instruments and analytical techniques:* IRMS, isotope ratio mass spectrometry; GC-IRMS, gas
58 chromatography-IRMS; LC-IRMS, liquid chromatography-IRMS; HPLC, high performance
59 liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; IC, ion
60 chromatography; CE, capillary electrophoresis; PT-CF-IRMS, purge-and-trap continuous-flow

61 IRMS; FIA-NR-IRMS, flow injection analysis reaction with ninhydrin-IRMS; SWiM-IRMS,
62 spooling wire micro-combustion-IRMS; EA-IRMS, elemental analysis-IRMS; TCEA-IRMS,
63 thermal conversion-EA-IRMS; NMR, nuclear magnetic resonance
64 *Other*: KIE, kinetic isotope effect; VPDB, Vienna Pee Dee Belemnite; PES, polyethersulfone;
65 PVDF, polyvinylidenedifluoride; SD, standard deviation; USGS, United States Geological
66 Survey; IAEA, International Atomic Energy Agency

67

68 **1. Introduction**

69

70 Stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^2\text{H}/^1\text{H}$, and $^{34}\text{S}/^{32}\text{S}$) of amino acids record details
71 of biosynthesis, enabling interrogation of environmental and physiological processes. This
72 review covers common steps within the workflow for amino acid isotope analysis, including
73 protein hydrolysis, derivatization, chromatographic separation, and isotope ratio detection (Fig.
74 1). While these steps build on earlier studies quantifying amino acids (e.g., Homer, 1915;
75 Tristram, 1939; Rees, 1946), no reviews have covered amino acid isotope analysis in full –
76 instead providing overviews of C and N isotope analysis that emphasize data interpretation and
77 ecological applications (Ohkouchi et al., 2017; McMahon and Newsome, 2019; Whiteman et al.,
78 2019). Here we review published techniques encompassing sample preparation through isotope
79 ratio measurement for C, N, H, and S (Fig. 1). We highlight: (1) where nonquantitative reactions
80 and/or incomplete separations can fractionate isotopes, and (2) established and emerging
81 analytical options available to isotope geochemists that enable compound-specific and position-
82 specific isotope analysis of amino acids.

83

84 *1.1. Terminology*

85
86 Natural-abundance stable isotope compositions are typically reported in delta (δ) notation
87 (Urey, 1948; McKinney et al., 1950) to highlight small variations between samples. A δ value is
88 the relative difference in isotope ratio (R) between a sample and standard (Eqn. 1), commonly
89 expressed in parts per thousand (per mil, ‰). The heavy (i.e., rare) isotope is placed in the
90 numerator of R by convention.

91
$$\delta = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \quad (1)$$

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

101 Isotope effects result in measurable differences in isotopic composition between
102 products, pools, materials, etc., called “fractionations.” Isotope fractionations are commonly
103 expressed as fractionation factors (α), the ratio of isotope ratios between two pools of interest
104 (Hayes, 2002). For example, given the generic reaction $A \rightarrow B$, the isotopic fractionation would
105 be described as:

106
$$\alpha_{B-A} = \frac{R_B}{R_A} \quad (2)$$

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

$$111 \quad \varepsilon = (\alpha - 1) \times 10^3 \quad (3)$$

112

113 **2. Sample storage and preparation**

114

115 Sample storage conditions should be selected to minimize amino acid degradation and
116 contamination, ideally achieved by storing freeze-dried or frozen samples in clean plastic or
117 baked glassware with an anoxic headspace. Higher storage temperatures (> -20 °C) may
118 promote decomposition (Laegeler et al., 1974; Rutherford and Gilani, 2009; Whiteman et al.,
119 2019), while oxic conditions can degrade cysteine, methionine, and tryptophan (Hunt, 1985).
120 There is no consensus on whether plastic or glass containers are better for amino acid recovery.
121 Because dust, fingerprints, sweat, and reagents may introduce contaminant amino acids (Ozols,
122 1990; Henrichs, 1991; McCoy et al., 2019, and References therein), any glassware, foil, or glass
123 fiber filters that will contact samples should be baked (e.g., at 450 °C for 8 h; Molero et al.,
124 2011; Larsen et al., 2013; Unger and Holzgrabe, 2018; Whiteman et al., 2019). Most samples
125 should be homogenized – e.g., by mortar and pestle, bead beater, or cryogenic grinding – before
126 or after storage to increase efficiency of acid hydrolysis.

127 While general storage recommendations can be made, pretreatment is more sample-
128 dependent as geochemists process diverse sample matrices ranging from hard rocks to soft
129 tissues. The goal of pretreatment is to remove large, non-amino acid components that interfere

130 with measurement (e.g., through co-elutions or column overloading) and cannot be eliminated at
131 a later stage. However, as each additional step may lower recovery or fractionate isotopes, we
132 recommend minimizing pretreatments and monitoring procedural blanks and external standards.
133 Procedural blanks are controls that do not contain the sample matrix and are subjected to the
134 entire workflow, including pretreatment; external standards are well-characterized materials
135 (e.g., proteins like bovine serum albumin or amino acids with known isotope ratios) that are
136 processed alongside samples. Additional pretreatment steps are highly matrix-specific and
137 should be carefully assessed, but could include surface rinsing, mechanical abrasion, or deeper
138 cleaning (e.g., Hare et al., 1991; Johnson et al., 1998; Schiff et al., 2014); solvent extraction to
139 remove lipids from fatty tissues (Bligh and Dyer, 1959; Newsome et al., 2018; Whiteman et al.,
140 2019); demineralization with hydrofluoric acid (Cheng, 1975; Gelinas et al., 2001; Ingalls et al.,
141 2003; Nunn and Keil, 2006; Battmann et al., 2020); or decarbonation with hydrochloric acid
142 (e.g., Hare et al., 1991; Johnson et al., 1998; Schiff et al., 2014). Protein extraction prior to
143 hydrolysis, though tempting to avoid interfering components, is not recommended, as this
144 procedure is labor-intensive and introduces bias by preferentially removing hydrophilic or
145 hydrophobic peptides (Nguyen and Harvey, 1998; Wang et al., 2003; Nunn and Keil, 2006; Niu
146 et al., 2018).

147

148 **3. Acid hydrolysis**

149

150 The goal of acid hydrolysis is to liberate proteinogenic amino acids into their “free” (non-
151 peptide bound) forms (Fig. 2) with maximum recovery and minimal isotopic fractionation.
152 Studies have largely converged on heating samples in 6N HCl for 20–24 h at 110 °C under

153 anoxic conditions (e.g., flushed with N₂ gas; Moore and Stein, 1963; Fountoulakis and Lahm,
154 1998; Rutherford and Gilani, 2009; Fogel et al., 2016), hereafter referred to as “conventional
155 hydrolysis.” We recommend this method for most applications, as it results in high recoveries
156 and negligible fractionation of many amino acids with minimal additional chemical workups
157 (Fig. 3). Here we discuss this conventional hydrolysis method and its variations, including
158 different durations and additives that stabilize certain amino acid side-chains. We also discuss
159 fast hydrolysis alternatives, including microwave and vapor-phase methods. It is important to
160 emphasize that no single hydrolysis method recovers all amino acids (Fig. 4); in particular, no
161 current method prevents quantitative deamidation of asparagine and glutamine to aspartic acid
162 and glutamic acid (Supplementary Fig. S2; Supplementary Section 1.2; Hill, 1965; Wright, 1991,
163 Rutherford and Gilani, 2009). Yields and isotopic compositions are therefore commonly reported
164 as asx (asparagine + aspartic acid) and glx (glutamine + glutamic acid). Loss mechanisms are
165 influenced by amino acid side-chain chemistry (e.g., hydroxyl groups on serine and threonine;
166 see Supplementary Section 1), protein composition (e.g., the proportion of S-containing amino
167 acids), and hydrolysis method (e.g., duration, temperature). Sample matrix effects also likely
168 influence yields, but studies thus far have focused on loss and isotopic fractionations of pure
169 standards or synthetic polypeptides and cannot account for the complexity of geochemically
170 relevant samples.

171

172 *3.1. Conventional hydrolysis*

173

174 Conventional hydrolysis (6N HCl, 110 °C, 20–24 h, anoxic conditions) results in
175 consistently high yields for 13 of the 20 amino acids (Figs. 3 and 4; Supplementary Table S1),

176 including all aliphatic and basic amino acids. Lengthening or shortening hydrolysis duration
177 reduces the total number of stable residues but can maximize yields of particular amino acids
178 (Fig. 4). For example, extended hydrolyses (> 24 h) improve recoveries of most aliphatic amino
179 acids, especially valine, leucine, and isoleucine (Darragh and Moughan, 2005) at the expense of
180 other amino acids (Hirs et al., 1954; Smith and Stockell, 1954; Darragh et al., 1996). Meanwhile,
181 shortened hydrolysis times (< 20 h) optimize yields of serine and threonine (Rowan et al., 1992;
182 Albin et al., 2000), with maximum recovery between ~10 and 16 h (Gardner, 1981; Gehrke et
183 al., 1985; Rutherford, 2009). Although phenylalanine and tyrosine are stable regardless of
184 hydrolysis length, tryptophan has low and variable yields (11–55%; Fig. 3; Supplementary Table
185 1; Keutmann and Potts, 1969; Matsubara and Sasaki, 1969; Mondino and Bongiovanni, 1970;
186 Hunt, 1985; Manneberg et al., 1995, Rutherford and Gilani, 2009). Like tryptophan, sulfur-
187 containing and amidic amino acids are unstable regardless of hydrolysis length (Fig. 4; Hunt
188 1985).

189

190 *3.2. Isotopic fractionation*

191

192 Each amino acid experiences different mechanisms and magnitudes of loss during
193 conventional hydrolysis that can lead to isotopic fractionation. We review what is known about
194 isotopic fractionation during hydrolysis and peptide bond cleavage; mechanistic details
195 concerning amino acid losses are presented in Supplementary Section 1. Studies of pure amino
196 acid standards subjected to hydrolysis conditions revealed minimal C isotope changes
197 (Demmelmair and Schmidt, 1993; Metges and Daenzer, 2000), even for amino acids with
198 significant losses like serine and methionine. This is further supported by Jim et al. (2003), who

199 found no detectable C isotope fractionation upon hydrolysis of synthetic alanine, serine, glutamic
200 acid, phenylalanine, leucine, or proline polypeptides. Nitrogen isotopes may be more susceptible,
201 especially in aliphatic amino acids. Bada et al. (1989) observed ~20‰ ¹⁵N-enrichment of
202 residual, unhydrolyzed collagen, and Silfer et al. (1992) observed temperature-dependent normal
203 ¹⁵N-KIEs of 0.9960 to 0.9975 for residual diglycine. We also caution interpreting δ¹⁵N values of
204 asx and glx due to the loss of amide-N from asparagine and glutamine. Although few studies
205 have investigated H isotopes of amino acids, deuterated and tritiated hydrolysis experiments
206 suggest H isotope exchange with the aqueous medium is significant for tyrosine (C-3 atomic site;
207 Fig. 2), aspartic acid (C-3 site), and glutamic acid (C-4 site; Hill and Leach, 1964; Fogel et al.,
208 2016). Studies of S isotopes are also limited but indicate a ³⁴S-KIE of 0.985 associated with oxidative
209 degradation of cysteine during conventional hydrolysis (Phillips et al., 2021). Importantly, the
210 lack of geochemically relevant sample matrices in all the aforementioned studies limits the scope
211 of conclusions that can be drawn. Moving forward, a more comprehensive characterization of
212 isotope fractionations accompanying protein hydrolysis in complex matrices like sediments and
213 soils is needed.

214

215 3.3. *Alternative hydrolysis methods*

216

217 Variations on conventional hydrolysis offer some specific advantages, including: (1)
218 protection of certain amino acids through chemical additives and/or (2) much faster hydrolysis
219 times via microwave or vapor-phase methods. For additives, the use of β-mercaptoethanol
220 appears most promising, as it increases the number of stable residues from 13 to 17 (Fig. 4; Hunt,
221 1985; Ng et al., 1987). We especially recommend the use of β-mercaptoethanol for studies of

222 tryptophan, as this is the only method that can reproducibly recover this residue. Phenol is
223 another common additive as it mitigates halogenation of aromatic residues, but it does not
224 stabilize other amino acids so is not recommended for general use. Addition of the oxidizing
225 agent performic acid is one of the few hydrolysis methods that recovers cysteine, but it destroys
226 several aromatic and hydroxylic residues (Fig. 4; Hunt, 1985; Rutherford and Gilani, 2009).
227 Microwave-assisted and vapor-phase methods achieve complete hydrolysis in < 90 min and
228 generally recover the same amino acids as conventional hydrolysis (Fig. 4; Tsugita et al., 1987;
229 Chiou, 1989; Weiss et al., 1998; Yarnes and Herszage, 2017), although with slightly lower yields
230 (Enggrob et al., 2019). Vapor-phase methods have the added benefit of minimizing contact
231 between samples and liquids, reducing potential contamination. These high-temperature, short-
232 duration hydrolyses also limit amino acid racemization (Csapó et al., 1997) and potentially
233 minimize H isotope exchange with the aqueous medium.

234

235 **4. Analyte clean-up**

236

237 There are two main goals of clean-up: (1) removing large particles and (2) eliminating
238 extraneous compounds such as lipids, carbohydrates, and salts that are liberated during acid
239 hydrolysis. These components can interfere with derivatization and isotope analysis (e.g., by
240 consuming derivatizing reagents or damaging GC–IRMS combustion interfaces; Cheng et al.,
241 1975; Hedges and Stern, 1983; Takano et al., 2010). At a minimum, large particles must be
242 removed. While this can be accomplished via filtration with baked glass fiber filters (Amelung
243 and Zhang, 2001), quartz wool pipette columns (Enggrob et al., 2019), or non-protein binding
244 syringe filters (e.g., PES, PVDF; Larsen et al., 2013; Phillips et al., 2021), we recommend cation

245 exchange chromatography (Section 4.1) for most applications because it eliminates both salts
246 and particles. Less commonly, organic/aqueous solvent extraction can complement cation-
247 exchange chromatography to remove excess hydrophobic components (e.g., lipids from fatty
248 tissues). Solvent extraction is recommended for samples with > 2% lipid content (McMahon and
249 Newsome, 2019) and can be performed before or after hydrolysis. Some derivatizing reagents
250 (e.g., BSTFA) require moisture-free reaction conditions so samples must be carefully dried as a
251 final clean-up step. This can be achieved via rotary evaporation, addition of sodium sulfate
252 desiccants, or azeotropically with dichloromethane (DCM).

253

254 *4.1. Ion exchange*

255

256 Studies have converged on the Dowex 50WX8 hydrogen form resin (200–400 mesh) for
257 cation exchange. Most amino acids are recovered with > 90% yield, including from complex
258 matrices like clay minerals (Supplementary Table S2; Moore and Stein, 1951; Cheng et al.,
259 1975; Amelung and Takano, 2010). Cation exchange also preserves amino acid chirality and
260 introduces no background contaminants when resins are washed (Takano et al., 2010).
261 Additional rinses with 0.1N oxalic acid can be added to remove metal cations from soils, rocks,
262 and sediment samples (Amelung and Zhang, 2001). As cation exchange involves both binding of
263 the amine group and elution with ammonia, potential alteration of $\delta^{15}\text{N}$ values is a concern.
264 However, Takano et al. (2010) observed < 0.3‰ differences in $\delta^{15}\text{N}$ values for 12 amino acids
265 before versus after elution on the Dowex 50WX8 resin, despite losses of up to 17%
266 (Supplementary Table S2). Carbon is not involved in adsorption or elution and is not expected to

267 fractionate. Indeed, Abelson and Hoering (1961) found minimal C isotope fractionation (<
268 0.6‰) of alanine on the Dowex 50WX8 resin.

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

274

275 **5. Derivatization (for gas chromatography)**

276

277 Derivatization is required to make amino acids amenable to separation by gas
278 chromatography (GC). Polar functional (carboxyl, amine, hydroxyl, and thiol) groups are
279 modified via the addition of various organic moieties to make amino acids more volatile, with
280 the products termed “derivatives.” For H isotope analysis, derivatization serves the additional
281 purpose of removing exchangeable H atoms, such as on carboxyl and amine groups, that would
282 otherwise equilibrate with the aqueous medium and alter the original $\delta^2\text{H}$ value.

283 Derivatization strategies for amine and carboxyl groups can be chosen independently,
284 provided the reactions are compatible, leading to a variety of combinations (Tables 1 and 2).
285 Amine, hydroxyl, and thiol side-chains are typically derivatized by the same reagent used for the
286 amine group. No single derivatization strategy is optimized for all 20 amino acids (Tables 1 and
287 2). In particular, arginine and histidine are incompatible with many reactions (Table 1; Hušek
288 and Macek, 1975). Several factors govern the selection of GC derivatives, including reaction
289 time, ease of procedure, product volatility, derivative stability, the number of non-analyte atoms

290 added, reaction yield, byproduct formation, enantiomer preservation (i.e., lack of racemization),
291 combustion or pyrolysis efficiency, and chromatographic resolution. Tradeoffs abound: for
292 example, a less stable derivative may be preferable if the reaction is rapid, as samples can be
293 derivatized immediately before analysis.

294 Derivatizing reagents should be present in excess to enable reaction completion and avoid
295 isotopic fractionation of amino acids (Docherty et al., 2001). However, even under these
296 conditions, it appears that derivatization reactions are not always quantitative, as N isotope
297 fractionation accompanies formation of many derivative products (Table 1; Hofmann et al.,
298 2003; Walsh et al., 2014). All derivatizing agents add C and/or H atoms which alter the
299 molecular isotope ratio of derivatized amino acids (Fig. 5; Tables 1 and 2) and must be
300 subtracted. Large derivative groups are less suited for isotope analysis, as analytical uncertainty
301 scales with the number of atoms added by the derivative group(s) (Rieley, 1994). For details on
302 this data correction, error propagation, and associated isotope effects, see Supplementary Section
303 2.

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

313

314 *5.1. Amine group derivatives*

315

316 *5.1.1. Pivaloyl derivatives*

317

318 Pivaloyl esters are optimal for $\delta^{15}\text{N}$ analysis as they are stable, have excellent
319 chromatographic properties, and can be coupled to esterification of the carboxyl group to form a
320 variety of derivatives (Fig. 5, Tables 1 and 2; Metges et al., 1996; Chikaraishi et al., 2007; Corr
321 et al., 2007b; Tea and Tcherkez, 2017). Furthermore, enantiomers are preserved and can be
322 separated using chiral stationary phases (Abe et al., 2002; Takano et al., 2009). Pivaloyl esters
323 are not recommended for $\delta^{13}\text{C}$ or $\delta^2\text{H}$ analysis due to their many exogenous C and H atoms
324 (Tables 1 and 2; Supplementary Eqn. 6; Corr et al., 2007a). Pivaloylation is achieved with
325 pivaloyl chloride and targets amine, hydroxyl, and thiol groups (Table 2; Corr et al., 2007b). An
326 isotope effect is known for the carbonyl C of pivaloyl chloride during derivatization (Corr et al.,
327 2007b), and N isotope fractionation accompanying *n*-pivaloyl isopropyl ester formation has been
328 observed (Table 1; Hofmann et al., 2003).

329

330 *5.1.2. Fluorinated derivatives*

331

332 Trifluoroacetyl (TFA) esters (and less commonly, pentafluoropropionic (PFP) and
333 heptafluorobutyric (HFB) esters) are popular because they contain minimal exogenous atoms, are
334 resolved with short retention times on standard GC columns and can be synthesized in ~5–10
335 mins (Fig. 5; Tables 1 and 2; Silber et al., 1991; Veuger et al., 2005; Corr et al., 2007b;

336 Kayacelebi et al., 2015; Ohkouchi et al., 2017; Riekenberg et al., 2017; Tea and Tcherkez, 2017).
337 However, some TFA procedures are lengthier as they include several rounds of purification
338 (Hannides et al., 2009; McMahon et al., 2011). Despite their popularity, fluorinated derivatives
339 have notable limitations when analytes must be combusted or pyrolyzed (i.e., during GC–IRMS
340 analyses). During combustion, Cu and Ni oxides form fluorides, lowering the reactor’s oxidizing
341 capacity (Meier-Augenstein, 1999; Tea and Tcherkez, 2017) and potentially leading to
342 incomplete combustion of amino acids. This can compromise both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Dunn
343 et al., 2011; Ghashghaie and Tcherkez, 2013; Tea and Tcherkez, 2017). During pyrolysis, HF is
344 formed, which causes fractionation of H isotopes (Sauer et al., 2001; Renpenning et al., 2017)
345 and can potentially corrode metal and silica components downstream of the reactor (Meier-
346 Augenstein, 1999; Corr et al., 2007b; Dunn et al., 2011). TFA derivatives pose additional
347 analytical challenges, including their sensitivity to moisture and alcohol, and low stabilities both
348 in storage and during GC separation (Table 2; Darbre and Blau, 1965; Hušek and Macek, 1975;
349 Corr et al., 2007b).

350 While TFA derivatives should be avoided for combustion- and pyrolysis-based analyses,
351 their extensive fragmentation in electron impact ion sources (e.g., Jones, 2002) makes them
352 attractive targets for position-specific isotope analysis by high-resolution mass spectrometry
353 (e.g., Orbitrap; see Section 7.2). Moreover, enantiomers are preserved and can be separated on
354 chiral columns (Serban et al., 1988; Silber et al., 1991; Macko et al., 1997; McCarthy et al., 2004;
355 Yamaguchi and McCarthy, 2018), and fluoroacetylation reactions can be adapted for arginine,
356 which is not generally amenable to derivatization (Hušek and Macek, 1975; Amelung and
357 Zhang, 2001; Kayacelebi et al., 2015). TFA derivative $\delta^{13}\text{C}$ values must be corrected for the
358 isotope effect expressed at the TFA carbonyl C atom during derivatization (Table 1; Silber et al.,

359 1991; Corr et al., 2007b). N isotope fractionation during TFA isopropyl ester formation has been
360 observed (Table 1; Hofmann et al., 2003).

361

362 5.1.3. *Non-fluorinated acetyl derivatives*

363

364 Reaction with acetic anhydride targets amine, hydroxyl, and thiol groups to form *n*-
365 acetyl-based derivatives such as *n*-acetyl methyl esters (NACMEs; Fig. 5; Table 2; Corr et al.,
366 2007a,b). For C isotope analyses, these non-fluorinated analogues perform better than, or
367 similarly to, TFA derivatives on several metrics. Dunn et al. (2011) compared amino acid $\delta^{13}\text{C}$
368 values measured by LC-IRMS and EA-IRMS against GC-IRMS and found that the *n*-acetyl
369 derivatives consistently yielded better agreement among complementary measurements than the
370 TFA derivatives. *N*-acetyl derivatives are also more stable, introduce the same number of C
371 atoms, and can be separated on a variety of GC columns (Fig. 5; Tables 1 and 2; Adams, 1974;
372 Corr et al., 2007a,b; Enggrob et al., 2019). Acetylation can cause fractionation for C and N
373 isotopes, but it is easily correctable (Table 1; Hofmann et al., 2003; Corr et al., 2007a,b).

374

375 5.1.4. *Alkoxy carbonyl derivatives*

376

377 Methoxycarbonyl (MOC) methyl esters and ethoxycarbonyl (EOC) ethyl esters are
378 favorable for C, N, and H isotope analyses because their reactions are simple, rapid (≤ 5 mins),
379 and do not require heating (Hušek, 1991a,b), and can be carried out in aqueous conditions (e.g.,
380 0.1N HCl), allowing for easy isolation of the products via extraction with organic solvent, and
381 also introduce minimal non-analyte C and H (Tables 1 and 2). Carbamate derivatives do not

382 racemize (Zampolli et al., 2007) and can be baseline-separated on polar GC columns (Hušek,
383 1991b; Walsh et al., 2014). Alkyl chloroformate derivatizes amine groups (including the side-
384 chains of lysine and histidine), as well as the phenol group of tyrosine (Huang et al., 1993; Chen
385 et al., 2010). The side-chains of serine, threonine, and cysteine are either esterified, acylated, or
386 not derivatized, depending on the derivatizing reagents used (Huang et al., 1993; Zampolli et al.,
387 2007; Chen et al., 2010; Walsh et al., 2014). Carbon and nitrogen isotope fractionation
388 accompany MOC ester formation (Table 1; Sacks and Brenna, 2005; Walsh et al., 2014).

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

397

398 **5.2 Carboxyl group derivatives**

399

400 *5.2.1. Ester derivatives*

401

402 Methyl ester derivatives are attractive because they form rapidly and quantitatively with
403 few non-analyte C and H atoms (Tables 1 and 2). Ethyl, *n*-propyl, and isopropyl esters introduce
404 more exogenous atoms, but may be selected to improve GC separation. Esterification reactions

405 target carboxyl groups, but when coupled to derivatization with methyl chloroformate they can
406 additionally target the hydroxyl groups of serine and threonine (Table 2; Huang et al., 1993;
407 Zampolli et al., 2007; Chen et al., 2010; Walsh et al., 2014). Conditions for esterification are
408 usually acidic, but basic conditions have been used occasionally (e.g., Tuckey and Stevenson,
409 1979; Corr et al., 2007b). Acidic conditions are created with acetyl chloride, thionyl chloride, or
410 dilute HCl, although comparisons have found that acetyl chloride results in the highest reaction
411 yields of amino acids (Peláez et al., 2000; Corr et al., 2007b). Isopropylation and *n*-propylation
412 require heating and must be performed in moisture-free conditions (e.g., Silber et al., 1991;
413 Chikaraishi et al., 2007; Corr et al., 2007b), while requirements for methylation and ethylation
414 differ. When coupled to derivatization with alkyl chloroformates, reactions may be performed in
415 aqueous conditions at room temperature (Hušek, 1991a; Sacks and Brenna, 2005; Chen et al.,
416 2010; Walsh et al., 2014), but when combined with other amine group derivatization strategies
417 (e.g., acetylation or pivaloylation) anhydrous conditions and heating are required to avoid
418 expression of isotope effects at the carboxyl sites (Corr et al., 2007a,b). When excess reagent is
419 used and reactions are quantitative, negligible C isotope fractionations are expected, as C sites
420 within the alcohol reagents do not directly participate in bond breakage or formation (Silber et
421 al., 1991; Rieley, 1994; Corr et al., 2007a).

422

423 5.2.2. *Silyl derivatives*

424

425 Silylation is not recommended for amino acid isotope analysis despite being a popular
426 GC derivatization strategy for other organic compounds (e.g., Tea and Tcherkez, 2017), as it can
427 add a large number of non-analyte atoms (up to 18 C and 45 H; Table 1), products are moisture-

428 sensitive and degrade rapidly (Hušek and Macek, 1975; Colombini et al., 1998), and multiple
429 derivatives may add to amine or hydroxyl groups inconsistently (Colombini et al., 1998; Hušek
430 and Macek, 1975; Molnár-Perland and Katona, 2000; Zaikin and Halket, 2005; Tea and
431 Tcherkez, 2017). Carbon does not participate in silylation, precluding expression of C isotope
432 effects (Rieley, 1994). However, trimethylsilyl (TMS) derivatives may promote silicon carbide
433 formation in GC–IRMS combustion reactors, leading to C isotope fractionation via non-
434 quantitative conversion of analytes to CO₂ (Shinebarger et al., 2002; Tea and Tcherkez, 2017).
435 Nitrogen isotope fractionation can be significant due to non-quantitative derivatization of amino
436 acids and degradation of derivative products (Table 1; Hofmann et al., 1995, 2003).

437

438 **6. Separations**

439

440 Amino acids must be separated from complex mixtures prior to isotope analysis, without
441 inadvertently fractionating isotopes. Separation can be achieved using a variety of
442 chromatographic techniques, stationary phases, and mobile phases, but to date no combination
443 sufficiently separates all 20 proteinogenic amino acids. Separation is commonly achieved by gas
444 chromatography (GC) coupled directly to an IRMS (i.e., ‘online’ measurement) without
445 intermediate analyte collection, but requires derivatization and an associated correction for added
446 C or H atoms. Less commonly, LC is used for preparatory (i.e., ‘offline’) separation of
447 underivatized amino acids, which are recovered using a fraction collector, sometimes assessed
448 for purity, and then analyzed on a separate instrument (e.g., EA–IRMS). New LC–IRMS systems
449 enable LC separation online prior to isotope ratio measurement (Section 7.1.2). Considerations

450 for optimizing GC and LC separations are beyond the scope of this review, but the reader is
451 referred to Rood (2007) and Snyder et al. (2010) for excellent guides.

452 For many LC separations the lighter isotopologues elute from the column first
453 (McCullagh et al., 2006; Broek et al., 2013), whereas for GC separations on nonpolar columns
454 the heavier isotopologues typically elute first. Separation of isotopologues leads to isotopic
455 fractionation across the width of a chromatographic peak. For example, differences between the
456 front and tail halves of a peak separated using LC were 8.3‰ and 4.2‰ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$
457 values, respectively (Broek et al., 2013). Thus, peaks must be completely collected or integrated
458 to avoid altering the apparent isotope ratio of the sample (Meier-Augenstein, 1999; Sessions,
459 2006).

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

463

464 *6.1. Gas chromatography (GC)*

465

466 GC is currently the most popular choice for separating amino acids for isotope analysis,
467 yet there is no consensus on the best combination of stationary phase, derivative, and instrument
468 settings. Many published approaches separate at least 10–15 amino acids (Table 1) in an hour or
469 less. These separations commonly use 50 m or 60 m columns, carrier gas flow rates of 1–2
470 mL/min, and GC oven temperature programs from ~40 °C to 300 °C. While most separations are
471 on nonpolar or low-polarity stationary phases (e.g., Ultra-2, DB-5ms), high-polarity columns
472 (VF-23ms, ZB-FFAP, and ZB-WAX) substantially improve peak shapes of *n*-acetyl and

473 alkoxy carbonyl ester derivatives, have higher analyte capacities, and are compatible with other
474 common derivatives (Corr et al., 2007b; Walsh et al., 2014).

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

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

489

490 *6.2. Liquid chromatography (LC)*

491

492 High performance liquid chromatography (HPLC or, more generally, LC) is widely used
493 for offline separation and quantification of amino acids, but subsequent isotope analysis requires
494 specialized equipment (fraction collectors) or instrumentation (LC–IRMS). LC provides some
495 advantages over GC: derivatization is not needed for amino acids, and column capacity is

496 substantially higher. However, purifying and collecting fractions offline may necessitate larger
497 sample sizes, especially if an EA–IRMS is used for isotope analysis. Certain amino acids, like
498 isoleucine and leucine, are difficult to separate when underivatized.

499 Amino acid separation by LC typically uses nonpolar stationary phases and polar mobile
500 phases (e.g., water, acetonitrile, methanol). The Sielc Primesep A is the most popular column for
501 online $\delta^{13}\text{C}$ (McCullagh et al., 2006; Tripp et al., 2006; Smith et al., 2009; Dunn et al., 2011) and
502 offline $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ measurements (Broek et al., 2013; Broek and McCarthy, 2014; Sun et al.,
503 2020). Primesep A columns can separate 14 amino acids with run times of 105 mins (Broek et
504 al., 2013). An alternative is “hydrophilic interaction liquid chromatography” (HILIC). HILIC
505 initially employs a mobile phase with high organic and low aqueous content, allowing a small
506 water layer to form between the stationary and mobile phases, which provides good separation of
507 amino acids – particularly aspartic acid and serine which cannot be separated using other LC
508 stationary phases (Park et al., 2019). Finally, while not currently common instrumentation, the
509 LC–IRMS system (discussed further in Section 7.1.2) enables online C isotope analysis, has
510 similar sensitivity to GC–IRMS (Table 3), and may become more popular in future amino acid
511 isotope research.

512

513 *6.3. Ion chromatography (IC)*

514

515 Ion chromatography (IC) is primarily used to quantify amino acids, but has also been
516 used for online (Morrison et al., 2010) and offline (Zhang et al., 2021) IRMS analyses. IC
517 separation can be coupled to an IRMS for online C isotope analysis via an Isoprime Liquiface
518 system (Morrison et al., 2010). Abaye et al. (2011) used this system to measure the $\delta^{13}\text{C}$ values

519 of 11 amino acids, including arginine, lysine, and some aliphatic amino acids, which were
520 quickly resolved (70 mins) with adequate precisions ($SD < 1\%$; Abaye et al., 2011). IC has also
521 been used to separate 9 amino acids offline prior to N isotope analysis using a purge-and-trap
522 continuous-flow IRMS (Zhang et al., 2021). Advantages of separating amino acids by IC are that
523 pre- or post-column derivatization is not required and other matrix components (carbohydrates,
524 glycols, and sugar alcohols) can be simultaneously separated (Larson et al., 2002), minimizing
525 the sample workup steps needed (see Sections 2 and 4). A major disadvantage is lengthy run
526 times (180 mins or longer; Zhang et al., 2021).

527

528 *6.4. Capillary electrophoresis (CE)*

529

530 Capillary electrophoresis (CE) separates compounds based on mobility in an electric field
531 (Ewing et al., 1989) but has not yet been coupled to isotope measurements. Although CE
532 currently lacks selectivity compared to other separation methods, its speed, simplicity, and low
533 cost hold potential for future applications involving online amino acid isotope analysis. As with
534 LC, samples do not require derivatization or conversion to gases and CE can be coupled to
535 numerous detectors. Chiral buffers can be used to change the mobility of D- vs L-amino acids
536 and separate enantiomers (Hutt et al., 1999). Miniaturized versions of CE systems (microchip
537 electrophoresis) have been explored for inclusion on extraterrestrial sampling missions that
538 investigate amino acids to distinguish between biotic and abiotic sources (Hutt et al., 1999;
539 Creamer et al., 2017) and may prove useful when combined with sensitive techniques (e.g., high-
540 resolution mass spectrometry – Section 7.2).

541

542 6.5. Methods of assessing purity

543

544 Most compound-specific isotope analyses require purified samples. For example, isotope
545 ratio monitoring by nuclear magnetic resonance spectroscopy (NMR; Section 7.3) requires >
546 98% analyte. Other methods, such as EA–IRMS (Section 7.1.3), lack online separation so
547 geochemists must first purify amino acids offline for compound-specific applications and ensure
548 no contaminants are present. Numerous options exist for assessing sample purity. LC–MS or
549 GC–MS can be used to identify contaminants (Hare et al., 1991; Phillips et al., 2020), but some
550 contaminants may avoid detection if their mass falls outside of the analytical window selected.
551 Proton (^1H) NMR is an attractive option for purity verification as it is non-destructive, rapid (≤ 5
552 min), and commonly available at user facilities. Elemental composition determined using an EA
553 system can be used to indirectly assess purity because pure amino acids have a narrow range of
554 elemental ratios ($\text{C/N} = 1.5\text{--}9.0$, $\text{C/S} = 3.0\text{--}5.0$). We recommend verifying sample purity in
555 studies using offline separations that are decoupled from the final isotopic analyses.

556

557 7. Isotopic analysis

558

559 Potential goals of amino acid isotopic analysis include characterizing the stable isotope
560 ratios of one or more elements ($^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^{34}\text{S}/^{32}\text{S}$, and/or $^2\text{H}/^1\text{H}$), either averaged across
561 each amino acid molecule (“compound-specific” or “molecular-average”) or characterized at
562 individual atomic positions (“position-specific” or “intramolecular”). Established techniques for
563 characterizing isotopic compositions of amino acids from natural samples use isotope ratio mass
564 spectrometry (IRMS) paired with offline or online separation strategies (Section 6) and

565 combustion or pyrolysis of separated analytes. Additional techniques are in early stages of
566 development — especially for position-specific isotope analysis of amino acids — but have not
567 yet been applied to terrestrial materials. We describe the isotopic analysis techniques that are
568 currently useful to organic geochemists, as well as possible future advancements (e.g., high-
569 resolution mass spectrometry), summarizing figures of merit, required preparatory steps, and key
570 advantages and disadvantages. Methodological improvements will continue to combine different
571 online separation and isotope detection strategies in novel ways.

572

573 *7.1. Isotope ratio mass spectrometry (IRMS)*

574

575 IRMS achieves high levels of precision and accuracy (Table 3) via the simultaneous
576 comparison of two or more isotopes (i.e., the isotope ratio). Isotope ratios are further compared
577 between the sample and a standard of known composition on an international scale (e.g., VPDB)
578 and reported as δ values (Eqn. 1). Amino acids must be quantitatively converted to CO₂ (for
579 ¹³C/¹²C analysis), N₂ (¹⁵N/¹⁴N analysis), H₂ (²H/¹H analysis) or SO₂ (³⁴S/³²S analysis; Table 3)
580 for IRMS measurements. This goal was originally achieved by combusting pre-purified amino
581 acids in sealed tubes offline, then isolating the resulting CO₂ and N₂ for isotope analysis
582 (Abelson and Hoering, 1961; Macko et al., 1983; Tuross et al., 1988). Today, this is typically
583 achieved via online methods in which analytes are carried by an inert gas through a chemical
584 conversion interface on their way to the IRMS. The most common interfaces employ combustion
585 or pyrolysis, but other techniques such as chemical oxidation or high-temperature combustion-
586 desolvation have also been reported (Tea and Tcherkez, 2017). Here we cover GC–IRMS, LC–
587 IRMS, and EA–IRMS instrumentation. For more detailed reviews of IRMS principles, analytical

588 considerations, and historical context, see Brenna (1994), Brand (1996), Brenna et al. (1997),
589 Meier-Augenstein (1999), and Sessions (2006).

590

591 7.1.1. GC–IRMS

592

593 GC–IRMS is routinely used to measure compound-specific $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of
594 amino acids (Ohkouchi et al., 2017; Close, 2019), and, less commonly, $\delta^2\text{H}$ values (Fogel et al.,
595 2016; Newsome et al., 2020). The GC is typically coupled to the IRMS via a combustion (for
596 CO_2 or N_2) or pyrolysis (for H_2) interface. Amino acid samples must be derivatized for GC
597 separation and free of water, particles, elemental S, and salts to avoid damage to the GC column
598 and chemical conversion interface.

599 GC–IRMS can achieve instrumental precision of $\leq 0.1\text{‰}$ for C, $< 1\text{‰}$ for N, and 2‰ for
600 H in other common analytes (Table 3; Sessions, 2006). Amino acids have larger propagated
601 uncertainties of 0.6‰ to over 2‰ for C (e.g., Corr et al., 2007a; Smith et al., 2009; Dunn et al.,
602 2011) and up to $\sim 10\text{‰}$ for H (Table 3; Fogel et al., 2016) because the added C or H derivative
603 atoms increase the uncertainty on the final amino acid isotope ratio (see Section 5).

604 Measurements typically require ~ 0.1 to 10 's of nmol C or N, and one to two orders of magnitude
605 more H (Table 3). Specific advantages of GC–IRMS for amino acid isotope analysis are its high
606 sensitivity and high throughput. GC–IRMS is especially appropriate for small samples and for
607 researchers seeking isotopic data on as many amino acids as possible at the same time.

608 Disadvantages include difficulty of use, added sample workup steps, and larger uncertainties
609 associated with derivatization. Recent work couples narrow-diameter column (“fast”) GC to
610 IRMS (Sacks et al., 2007; Baczynski et al., 2018), paving the way for future amino acid isotope

611 analyses with sharper chromatographic peaks, faster run times, and enhanced sensitivity for $\delta^{13}\text{C}$
612 analyses.

613

614 7.1.2. LC-IRMS

615

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

626 A significant drawback to current LC-IRMS systems is that they must use acidic,
627 organic-free mobile phases, as any organic solvents would be oxidized to CO_2 along with the
628 analytes. Typical concentrations of organic solvents used in mobile phases for LC separations
629 would saturate the IRMS detector (Godin et al., 2005). Additionally, LC-IRMS is less sensitive
630 than GC-IRMS and can only measure C isotopes, not N or H. For information on LC-IRMS
631 technical challenges and solutions, we refer the reader to a review by Godin and McCullagh
632 (2011). Thus far, LC-IRMS systems have successfully measured amino acids from peptides and

633 archaeological samples (Godin et al., 2005; McCullagh et al., 2006), but methods are still in
634 development for complex materials like marine sediments (Close, 2019).

635

636 7.1.3. EA–IRMS

637

638 Elemental analyzer (EA)–IRMS instruments are most commonly used to measure the
639 bulk isotopic compositions of complex, solid samples, but can also be coupled with offline
640 preparatory techniques such as LC with fraction collection to provide isotopic measurements of
641 individual amino acids or proteins (e.g., Broek et al., 2013; Dong et al., 2017). Isolated amino
642 acids are packed into metal foil capsules, combusted to CO₂, N₂, H₂O, and/or SO₂, then dried and
643 separated using a short GC column for isotope ratio measurement.

644 Although three orders of magnitude less sensitive than GC–IRMS (requiring 1000s of
645 nmol C or N; Table 3) and requiring time-consuming offline separations, EA–IRMS systems
646 may be the ideal choice for certain specialized applications (e.g., $\delta^{34}\text{S}$ measurements of pre-
647 isolated cysteine or methionine or high-precision $\delta^{15}\text{N}$ measurements) or when sample size is not
648 limiting. Additionally, EA–IRMS instruments are more widely available, achieve better
649 precisions ($\sim 0.1\text{--}0.5\text{‰}$; Table 3), and are simpler to operate than GC–IRMS or LC–IRMS.
650 Perhaps most importantly, EA–IRMS offers the ability to simultaneously analyze the isotope
651 ratios of multiple elements in the same acquisition (e.g., C and N, with or without S; Fry, 2007;
652 Broek and McCarthy, 2014; Fourel et al., 2014; Brodie and Kracht, 2016; Sayle et al., 2019) —
653 although this strategy is not yet established for amino acids.

654 Recent optimizations have enhanced the sensitivity of EA–IRMS (Table 3). The nano-
655 EA–IRMS system (Polissar et al., 2009) improved sensitivities by 100- to 500-fold for $\delta^{13}\text{C}$ and

656 $\delta^{15}\text{N}$ measurements of pre-purified amino acids, while achieving similar precisions (Broek and
657 McCarthy, 2014; Swalethorp et al., 2020). Optimizations for S isotope analysis by EA–IRMS
658 (Fry, 2007; Fourel et al., 2014) enabled the first compound-specific $\delta^{34}\text{S}$ measurements of
659 cysteine and methionine (Phillips et al., 2020). Furthermore, H isotope analysis by pyrolysis EA
660 (also known as thermal conversion EA, or TCEA), has been demonstrated for ~ 0.3 mmol analyte
661 (Table 3; Gehre et al., 2015) but has only been applied thus far to pure amino acid standards
662 (Fogel et al., 2016; Newsome et al., 2020).

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

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

679

680 *7.1.4. IRMS configurations for position-specific isotope analysis*

681

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

702

703 *7.2. High-resolution mass spectrometry*

704

705 Directly converting amino acids to CO₂, N₂, and H₂ destroys information recorded in the
706 position-specific distributions of isotopes. For example, ¹³C-enrichment at the C-1 versus C-2
707 position in alanine has different implications for its origins (Chimiak et al., 2021), but cannot be
708 discerned from the ratio of ¹³C/¹²C in CO₂ produced by whole-molecule combustion (e.g., as in
709 GC–IRMS). High-resolution mass spectrometry of intact analyte ions (as opposed to whole
710 combustion or pyrolysis products) provides an avenue for analyzing position-specific isotope
711 distributions in amino acids. This approach is still in development for natural materials relevant
712 to organic geochemists (e.g., plant biomass – Wilkes et al., 2019), but represents a promising
713 future direction.

714 Position-specific isotope ratios can be accessed by measuring fragments of an amino acid
715 molecule, which form spontaneously during ionization and/or collision in an ion trap.
716 Constraining position-specific isotopic differences is accomplished by measuring and comparing
717 isotope ratios of two or more fragments of an amino acid in a mass spectrometer. These
718 measurements require that the spectrometer can distinguish molecular fragments containing
719 different rare isotopes, e.g., ¹³C vs ²H or ¹⁵N (Supplementary Section 4). Several mass
720 spectrometers achieve mass resolutions that can distinguish these different rare isotope
721 substitutions, but only Orbitrap™-based instruments have been studied in detail (e.g., Eiler et al.,
722 2017; Hofmann et al., 2020; Neubauer et al., 2020; Hilkert et al., 2021). Orbitrap mass analyzers
723 may be coupled to GC or LC for online isolation of amino acids; thus, required sample
724 preparation (e.g., derivatization) and clean-up steps would reflect the choice of instrumentation.

725 In addition, Orbitrap instruments hold the potential to measure clumped-isotope compositions of
726 amino acids (i.e., containing two or more rare isotopes).

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

742

743 *7.3. Nuclear magnetic resonance spectroscopy (NMR)*

744

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

748 requirements, NMR is best suited for characterizing pure standards but provides a path for
749 establishing position-specific isotope standards anchored to the international scale that may be
750 used with a more sensitive technique (e.g., Orbitrap). A separate radio frequency signal is
751 produced by each chemically distinct ^{13}C or ^2H atom within a molecule. These NMR signals are
752 quantified through peak integration and used to calculate position-specific isotope distributions
753 by comparison with the average isotope ratio for the entire molecule (measured separately by
754 IRMS; reviewed by Jézéquel et al., 2017). However, ^{13}C NMR and ^2H NMR typically require
755 hundreds of milligrams (~1 mmol) of pure analyte to achieve precisions of ~1‰ and 5‰,
756 respectively (Table 3; Romek et al., 2017; Gilbert, 2021). Such sample size requirements are
757 prohibitive for applications to amino acids from natural samples and all published NMR analyses
758 of amino acids to date have targeted pure standards (Vallet et al., 1991; Romek et al., 2017;
759 Rasmussen and Hoffman, 2020). An alternative NMR approach for position-specific $^{13}\text{C}/^{12}\text{C}$
760 analysis was recently applied to amino acid standards using ^1H NMR (Rasmussen and Hoffman,
761 2020; Supplemental Section 5). This technique has at least two advantages over direct ^{13}C NMR,
762 while achieving similar precisions (~1‰; Table 3) and accuracies: (1) it uses more commonly
763 available equipment, and (2) it is an order of magnitude more sensitive (Table 3; Hoffman and
764 Rasmussen, 2019). One drawback of using ^1H NMR to study C isotopes is that not all C
765 positions in amino acids are visible.

766 All of the above NMR techniques are non-destructive and can be readily calibrated to
767 internationally recognized scales, yet NMR has significant drawbacks compared to other isotopic
768 measurements – notably, poor sensitivity and precision (requiring up to six orders of magnitude
769 more C than IRMS for 0.5–3.5‰ precision; Table 3). NMR requires analytes to be purified (>
770 98%) offline prior to measurement, followed by several hours of analysis (Remaud et al., 2018).

771 While analytical advances are ongoing (see Jézéquel et al., 2017 and Hoffman and Rasmussen,
772 2019), we anticipate NMR will remain most useful as a complementary technique for calibrating
773 standards rather than a primary tool for measuring isotope ratios in (bio)geochemical samples.

774

775 *7.4. Referencing strategies for isotopic analysis*

776

777 Making accurate and precise isotope ratio measurements are major challenges in the
778 stable isotope community. As compound-specific (and emerging position-specific) isotope
779 applications expand across fields, the need for inter-laboratory comparability of data becomes
780 more urgent. In addition to variable sample preparation strategies, protocols used across
781 laboratories to calibrate and quality-check isotope ratio measurements are inconsistent, which
782 hampers data reproducibility between studies (Carter and Fry, 2013; Yarnes and Herszage,
783 2017). Inter-laboratory comparisons of amino acid $\delta^{13}\text{C}$ values recovered from the same samples
784 are not commonly published, but when done so, often show disagreement (e.g., Arthur et al.,
785 2014; Ayayee et al., 2015); comparisons for N isotope analyses are even less common.
786 Following recommendations by Carter and Fry (2013) and Yarnes and Herszage (2017), we urge
787 widespread adoption of the following standardization practices: (1) calibration of data based on
788 internal standards (synthetic amino acids like norleucine that are co-injected with samples)
789 and/or multipoint amino acid isotope standards (i.e., spanning a range of isotopic compositions
790 outside those of samples) to account for scale compression effects by instruments (e.g., Yarnes
791 and Herszage, 2017; Riekenberg et al., 2020; Zhang et al., 2021), (2) use of quality assessment
792 materials, such as an external standard measured repeatedly throughout sample analysis (e.g.,

793 Styring et al., 2015), to verify measurement accuracy, and (3) increased comparisons of isotopic
794 data between laboratories and publication of results.

795 Several internationally recognized amino acid reference materials are available for
796 compound-specific isotope analysis: glycine, L-valine, and L-glutamate standards with known
797 values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ have been calibrated through interlaboratory ring tests and are
798 distributed by the USGS, IAEA, and Indiana University (Qi et al., 2003, 2016; Schimmelmann et
799 al., 2016). Comparability of isotope ratio measurements would be greatly improved by the
800 development of: (1) additional amino acid standards encompassing a wider range of $\delta^{13}\text{C}$ and
801 $\delta^{15}\text{N}$ values, (2) amino acid $\delta^2\text{H}$ and $\delta^{34}\text{S}$ reference materials (the former effort is currently
802 challenged by lack of a reliable method to correct for exchangeable hydrogen (Schimmelmann et
803 al., 2016), (3) internationally-recognized protein standards with calibrated amino acid isotopic
804 compositions (Yarnes and Herszage, 2017), and (4) position-specific reference materials
805 anchored to international scales.

806

807 **8. Conclusions and outlook**

808

809 We have synthesized the extensive literature on amino acid isotope analysis from
810 preparation to measurement, highlighting established techniques and emerging technologies that
811 may offer future benefits to geochemists. We emphasize that there is no “one size fits all”
812 method for amino acid isotopic analysis: researchers have multiple options and choices will be
813 guided by sample type, individual applications, and available resources. Over the next decade,
814 attention to standardizing referencing strategies and developing reference materials is needed for
815 data generated across laboratories to be reproducible. A second beneficial area of attention is

816 measuring H and S isotope ratios in amino acids, which would expand environmental and
817 ecological applications. For example, $^2\text{H}/^1\text{H}$ ratios could provide information on migration and
818 energy flow (e.g., Rubenstein and Hobson, 2004; Bowen et al., 2005; Fogel et al., 2016), and
819 $^{34}\text{S}/^{32}\text{S}$ ratios could track dietary protein sources (e.g., Richards et al., 2001). To date, the $\delta^2\text{H}$
820 and $\delta^{34}\text{S}$ values of amino acids have been characterized in relatively few published studies
821 (Fogel et al., 2016; Newsome et al., 2020; Phillips et al., 2020), so the full potential of these
822 measurements is only beginning to be explored.

823 Finally, we see three areas of amino acid isotope research where methodologic
824 innovation will have the most impact. First, there are numerous opportunities for method
825 automation. In addition to the substantial improvements stemming from online LC-IRMS and
826 GC-IRMS techniques, further coupling (e.g., combining protein hydrolysis with high pressure
827 ion-exchange clean-up) would increase throughput. A second area for innovation is simultaneous
828 analysis of multiple isotope systems on the same sample and instrument. Because preparing
829 amino acids for isotope measurement is tedious, measuring H, C, N, and S concurrently offers
830 greater reward and is increasingly possible with techniques like optimized EA-IRMS and high-
831 resolution mass spectrometry. Multi-element analysis of amino acids will be especially helpful
832 for ecological and forensics studies that reconstruct modern and paleo diets, food webs, animal
833 and human movement, and behaviors of ancient civilizations. Third, we see a renaissance in
834 position-specific isotope analysis via high-resolution mass spectrometry and/or pyrolysis-GC-
835 IRMS. Specific intramolecular information, such as C-S bond clumping in methionine or C-H
836 bond clumping in aliphatic residues, could inform targeted questions about synthesis. Further,
837 the ability to measure position-specific isotope ratios at natural abundance may complement or
838 replace isotope labelling methods in metabolomics studies. None of these advancements will be

839 possible without the analytical expertise of isotope geochemists and ecologists, whose
840 experiments and observations will inform these promising frontiers.

841

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843

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856

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1670

1671 **Figure captions**

1672

1673 **Fig. 1.** A typical workflow for amino acid isotope analysis, highlighting the major preparatory
1674 and analytical steps examined in this review paper. We cover sample preparation, acid
1675 hydrolysis, clean-up, derivatization, separation, and isotopic analysis. Sample preparation
1676 includes drying and homogenization steps, and, less commonly, clean-up steps prior to
1677 hydrolysis. Peptide-bound amino acids can be released by acid hydrolysis and are typically
1678 followed by additional clean-up steps depending on the sample matrix. Amino acids must be
1679 derivatized to decrease their polarity and make them amenable to separation by gas
1680 chromatography. Samples can also be separated without derivatization via liquid
1681 chromatography methods. Finally, isotope measurements are achieved via a variety of
1682 established and emerging techniques including isotope ratio mass spectrometry (IRMS), high-
1683 resolution mass spectrometry (e.g., Orbitrap), or nuclear magnetic resonance (NMR). Insets

1684 show peptide bonds cleaved during acid hydrolysis, and example derivative groups added during
1685 derivatization.

1686

1687 **Fig. 2.** Structures of 20 common proteinogenic amino acids in zwitterion form at pH 7. Residues
1688 are grouped by side-chain chemistries. Numbering for amino acid C sites (e.g., C-1) used
1689 throughout this review is shown.

1690

1691 **Fig. 3.** Yields of mixtures of free amino acid standards (dark gray triangles) and of amino acids
1692 from proteins (light gray shapes) after conventional acid hydrolysis (6N HCl, 110 °C, 20–24 h,
1693 anoxic conditions). Corresponding yield data can be found in Supplementary Table S1.

1694 Abbreviations and references: AA STDs, amino acid standard 1 (Mondino et al., 1970) and
1695 amino acid standard 2 (Keutmann and Potts, 1969); RNASE, Ribonuclease (Keutmann and Potts,
1696 1969); CYT C, Cytochrome c (Matsubara and Sasaki, 1969); TMV, Tobacco mosaic virus
1697 (Matsubara and Sasaki, 1969); BSA, Bovine serum albumin (Manneberg et al., 1995); LYZ,
1698 Lysozyme (Manneberg et al., 1995); TRX, Thioredoxin (Manneberg et al., 1995); IFN A,
1699 Interferon A (Manneberg et al., 1995).

1700

1701 **Fig. 4.** Summary of stable and unstable residues during common acid hydrolysis procedures.
1702 Primary loss mechanisms are denoted by shapes. Conventional hydrolysis is defined as 20–24 h
1703 at 110 °C in 6N HCl under anoxic conditions. The recommended vapor phase hydrolysis uses 7N
1704 HCl, 10% trifluoroacetic acid, and 0.1% phenol for 22 min at 158 °C. The recommended
1705 microwave assisted hydrolysis uses 6N HCl, 0.02% phenol, 0.2% 3-(2-aminoethyl)-indole for 4
1706 min at 155 °C. See text for details and references.

1707

1708 **Fig. 5.** Derivatives commonly used for gas chromatography separations of amino acids.

1709 Abbreviations: t-BDMS, tert-butyldimethylsilyl; TMS, trimethylsilyl; MOC, methoxycarbonyl;

1710 EOC, ethoxycarbonyl; NPIP, *n*-pivaloyl isopropyl ester; NPNP, *n*-pivaloyl *n*-propyl ester;

1711 NPME, *n*-pivaloyl methyl ester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester;

1712 NANP, *n*-acetyl *n*-propyl ester; TFA-ME, trifluoroacetyl methyl ester; HFB-IB,

1713 heptafluorobutyryl isobutyl ester; TFA-IP, trifluoroacetyl isopropyl ester; PFP-IP,

1714 pentafluoropropionyl isopropyl ester.

1715

1716 **Fig. 6.** Summarized capabilities of common analytical techniques used for amino acid isotope

1717 analysis. Sensitivities given are lower limits. “Multi-element” refers to simultaneous

1718 measurement of different isotopic systems (i.e. $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$) in a single analysis. “Online

1719 separation” refers to the ability to measure multiple compounds from a mixture online. A

1720 “position-specific” measurement encompasses the ability to extract isotopic information from

1721 individual sites within a compound. We do not consider bulk or compound-specific isotope

1722 measurements on molecules containing single elements (e.g., N or S in amino acids), nor

1723 chemical approaches to site-specific measurements (e.g., decarboxylation reactions) as “position-

1724 specific” capabilities of the instrument. For more details and references, see Table 3 and Section

1725 7.

1726

1727

1728 **Table 1.** Common amino acid derivatives for GC separation.
1729

Derivative ^a	Added		Problematic amino acids ^b	Recommended columns	Co-elutions	Derivatization		References
	C	H				¹³ α ^c	¹⁵ α ^d	
NPME	6–11	12–21	arg, asn, gln	High polarity: ZB-WAX ZB-FFAP		0.938– 0.996		Corr et al. (2007b); Tea and Tcherkez (2017)
NPNP	8–13	16–25	arg, asn, gln	High polarity: ZB-WAX VF-23ms		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); Godin et al. (2007)
NANP	5–8	10–17	arg, asn, cys, gln, his, thr	High polarity: ZB-WAX ZB-FFAP VF-23ms	pro/thr (VF-23ms) 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-23ms	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-225ms VF-23ms DB-WAX	leu/ile pro/thr (VF-23ms)	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-23ms	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-225ms	ser/gln (DB-WAX) leu/ile			Hušek (1991a,b); Godin et al. (2007)

				VF-23ms DB-WAX	(DB-225ms)			
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)

1730

1731 ^aAbbreviations: NPME, *n*-pivaloyl methyl ester; NPNP, *n*-pivaloyl *n*-propyl ester; NPIP, *n*-
1732 pivaloyl isopropyl ester; TFA-IP, trifluoroacetyl isopropyl ester; TFA-ME, trifluoroacetyl methyl
1733 ester; PFP-IP, pentafluoropropionyl isopropyl ester; HFB-IB, heptafluorobutyryl isobutyl ester;
1734 NANP, *n*-acetyl *n*-propyl ester; NAIP, *n*-acetyl isopropyl ester; NACME, *n*-acetyl methyl ester;
1735 MOC ME, methoxycarbonyl methyl ester; EOC EE, ethoxycarbonyl ethyl ester; TMS,
1736 trimethylsilyl; t-BDMS, tert-butyldimethylsilyl.

1737 ^bAs reported in the specified literature.

1738 ^cCommonly referred to as kinetic isotope effects in the amino acid literature. Entries represent
1739 ranges of values reported in Corr et al. (2007b), except the range for MOC ME, which is
1740 calculated from data provided in Walsh et al. (2014) using Eqn. 2 in Corr et al. (2007b).

1741 ^dCalculated from data provided in Hofmann et al. (2003) using Eqn. 2 in Corr et al. (2007b).

1742 ^eCalculated assuming amine, hydroxyl, and thiol groups are each derivatized by only one t-
1743 BDMS group, which is not necessarily true (see discussion in main text).

1744

1745

1746

1747 **Table 2.** Summary of reaction information for six major derivatization strategies employed for
 1748 GC analysis of amino acids. Note that all derivatization methods for the amine group require
 1749 correction for C isotope fractionation.
 1750

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; $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values comprised by CuF_2 , NiF_2 , and CO generation during combustion; $\delta^2\text{H}$ 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); McMahan 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; products have low stabilities; 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; <i>n</i> -propanol and isopropanol add many H atoms	Hušek (1991a); Silber et al. (1991); Chikaraishi et al. (2007); Corr et al. (2007a,b)
	with ethanol		2	5					
	with <i>n</i> -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; $\delta^{13}\text{C}$ 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					

1751
 1752 ^aAbbreviations: TFAA, trifluoroacetic anhydride; HFB, heptafluorobutyric anhydride; PFP,
 1753 pentafluoropropionic anhydride; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; MTBSTFA,
 1754 methyltributylsilyl tetrafluoroacetamide
 1755 ^bThiol group of cysteine is derivatized when ethyl chloroformate is used, but not when methyl
 1756 chloroformate is used.
 1757 ^cHydroxyl groups not usually esterified, but serine and threonine can be methylated upon
 1758 derivatization to MOC methyl esters.
 1759

1760 **Table 3.** Summary of analytical techniques for isotopic analysis. Many of these methods have
 1761 yet to be applied to amino acids from the environment, although some have been used to
 1762 measure amino acid standards.
 1763

Analytical technique ^a		Isotopes	Measured species	Specificity	Typical precision (1 σ , ‰)	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	~100's	Wolyniak, (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 ₂ O	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	Sayle et al. (2019); Phillips et al. (2020) ^{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. (2020) ^{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 Hoffmann (2020) ^b

1764

1765 ^aAbbreviations: IRMS, isotope ratio mass spectrometry; GC-IRMS, gas chromatography-IRMS;
1766 LC-IRMS, liquid chromatography-IRMS; PT-CF-IRMS, purge-and-trap continuous-flow
1767 IRMS; FIA-NR-IRMS, flow injection analysis reaction with ninhydrin-IRMS; SWiM-IRMS,
1768 spooling wire micro-combustion-IRMS; EA-IRMS, elemental analysis-IRMS; TCEA-IRMS,
1769 thermal conversion-EA-IRMS; NMR, nuclear magnetic resonance.

1770 ^bMethod applied to pure amino acid standards.

1771 ^cMethod applied to amino acids in natural (terrestrial or extraterrestrial) materials.

1772

1773

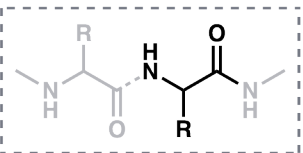
1774

1775



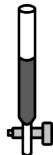
SAMPLE PREPARATION

freeze-drying, storage,
homogenization, etc.



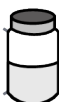
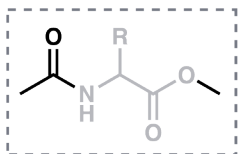
ACID HYDROLYSIS

6N hydrochloric acid, 110 °C,
20-24 h, anoxic, ± additives



CLEAN UP

cation exchange, filtration,
solvent extraction, etc.



DERIVATIZATION

esterification, acetylation,
alkoxycarbonylation, etc.



SEPARATION

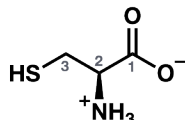
gas chromatography, liquid
chromatography, etc.



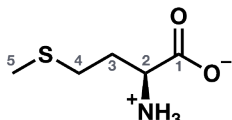
ANALYSIS

isotope ratio mass spectrometry,
high-resolution mass spectrometry, etc.

S-Containing

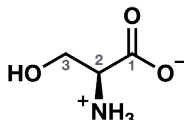


cysteine (cys)

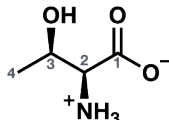


methionine (met)

Hydroxylic

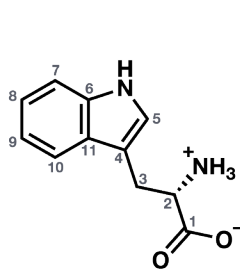


serine (ser)

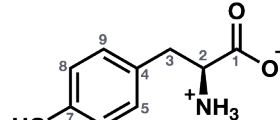


threonine (thr)

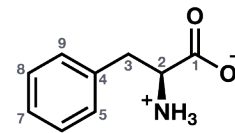
Aromatic



tryptophan (trp)

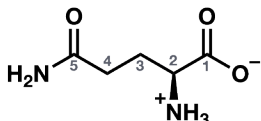


tyrosine (tyr)

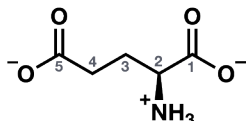


phenylalanine (phe)

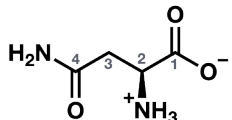
Acidic and Amidic



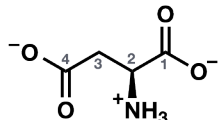
glutamine (gln)



glutamic acid (glu)

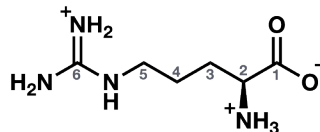


asparagine (asn)

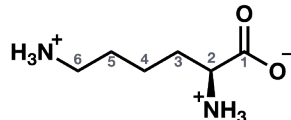


aspartic acid (asp)

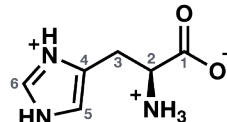
Basic



arginine (arg)

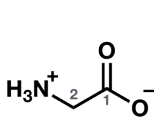


lysine (lys)

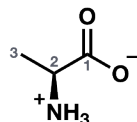


histidine (his)

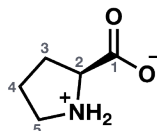
Aliphatic



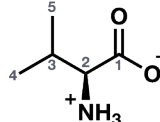
glycine (gly)



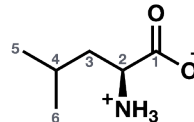
alanine (ala)



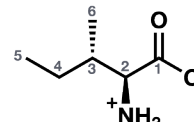
proline (pro)



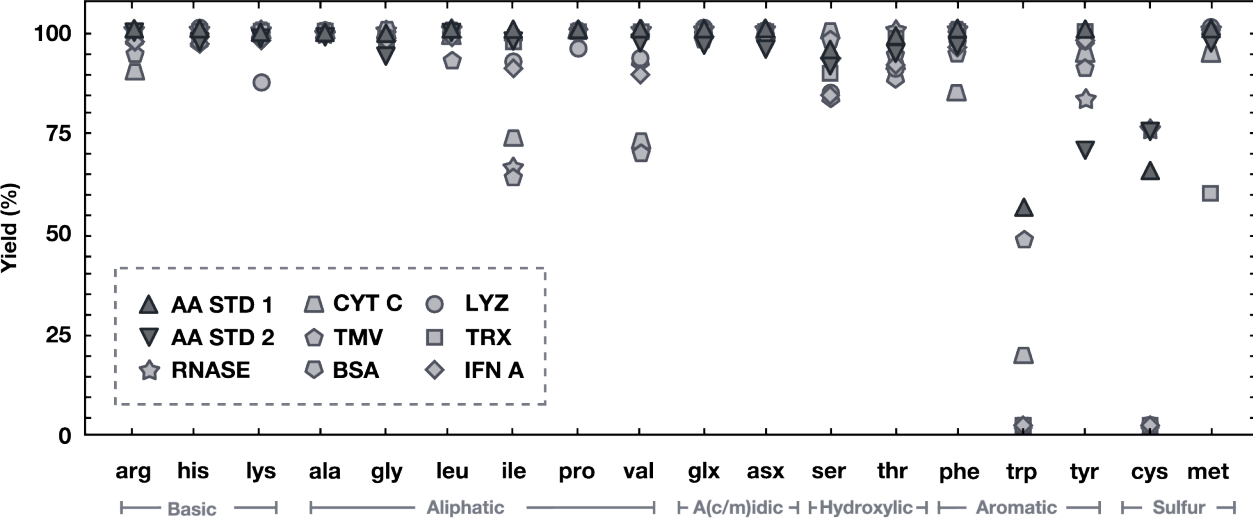
valine (val)

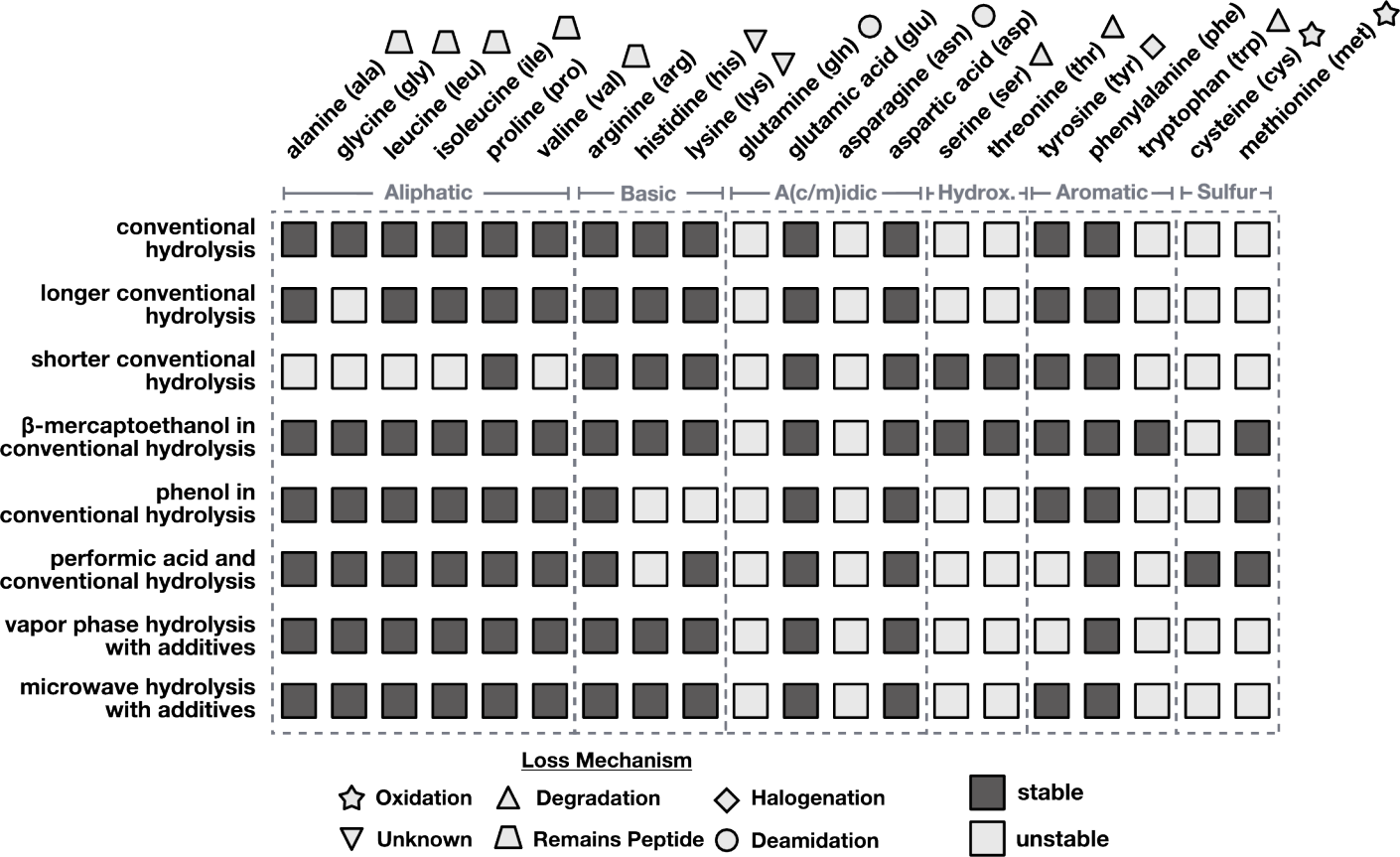


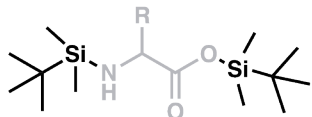
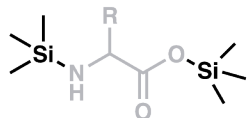
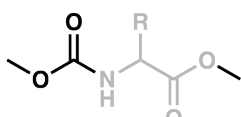
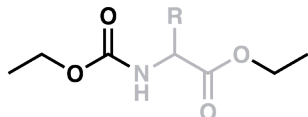
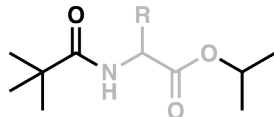
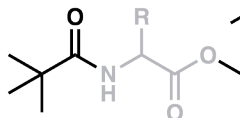
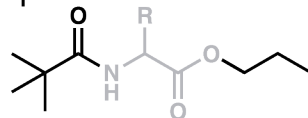
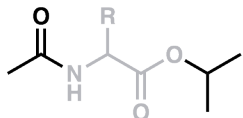
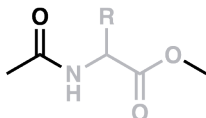
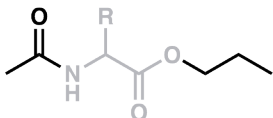
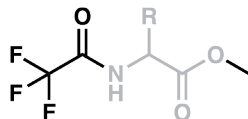
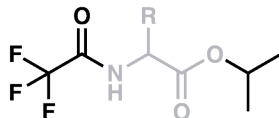
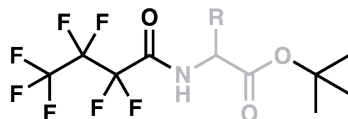
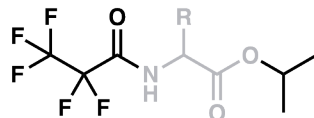
leucine (leu)



isoleucine (ile)





Silyl Derivatives**t-BDMS****TMS****Carbamates****MOC****EOC****Pivalamides****NPIP****NPME****NPNP****Acetamides****NAIP****NACME****NANP****Trifluoroacetamides****TFA-ME****TFA-IP****Perfluoroalkyl Amides****HFB-IB****PFP-IP**

