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Quantitative Analysis of Glycine Oligomerization by Ion-Pair Chromatography

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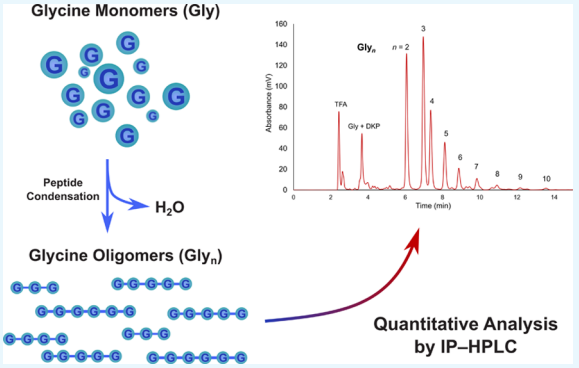
Supporting Information

ABSTRACT: This paper describes a method for the quantitative analysis of mixtures of glycine and its oligomers by ion-pair high-performance liquid chromatography (IP-HPLC), with a particular focus on applications in origins-of-life research. We demonstrate the identification of glycine oligomers (Gly_n) up to 14 residues long—the approximate detectable limit of their solubility in water—and measurement of the concentration of these species in the product mixture of an oligomerization reaction. The molar response factors for higher oligomers of glycine—which are impractical to obtain as pure samples—are extrapolated from direct analysis of pure standards of n = 3−6, which established a clear linear trend. We compare and contrast our method to those in previous reports with respect to accuracy and practicality. While the data reported here are specific to the analysis of oligomers of glycine, the approach should be applicable to the design of methods for the analysis of oligomerization of other amino acids.



INTRODUCTION

The investigation of systems for the efficient synthesis of polypeptides in prebiotically relevant conditions is a major focus of origins-of-life research. Enlivened by Miller's seminal experiment showing the synthesis of amino acids from a simulated Hadean atmosphere, researchers have studied the condensation of amino acids to form peptides in a variety of prospective prebiotic conditions.[1−3] Most proposed prebiotic syntheses of oligopeptides from amino acids can be classified into one or more of the following approaches: (i) catalysis by salts or surfaces at low water activity,[4−22] (ii) coupling mediated by stoichiometric reagents,[23−27] (iii) strand elongation via acyl-transfer reactions,[28−31] (iv) dry-downs and thermal/environmental cycling,[10,28,32−34] and (v) reactions driven by high-energy sources, like UV light or simulated asteroid impacts (see Figure 1).[35−39] Glycine is the most popular substrate for these studies because of its presumed prebiotic abundance and its simplicity—it lacks a side chain and is achiral; thus, no branched isomers or stereoisomers will form as products.[40,41]

Justification. Despite the popularity of amino-acid polymerizations—particularly of glycine—there is no standard approach to the analysis of their product mixtures. Often, the various methods employed are described in minimal experimental detail, and their underlying assumptions are overlooked. In this paper, we contrast the performance and limitations of our method versus those previously reported, with the goal of allowing future investigators to make informed decisions about how to analyze mixtures of oligopeptides and report yields in a manner that allows the meaningful comparison of results across the field.

Background. Prebiotic synthesis focuses on the conversion of simple feedstocks available on the early Earth into more complex, functional molecules and systems. For the condensation of amino acids, it is generally assumed to be advantageous to generate longer oligomers in higher yields with higher conversion of monomer into products. The motivating ideas are that (i) higher yields and conversions correspond to more efficient processes and (ii) longer peptides will have a greater chance of exhibiting the functional properties of proteins observed in biochemistry. The specific preference of a synthesis for one product over others may also be advantageous, though arguments can be made for the value of variety in polydisperse mixtures. Cyclic dipeptides (2,5-diketopiperazines, or DKPs) are generally regarded as undesirable "dead-ends" in these syntheses, as their lack of free amino and carboxyl groups limits their reactivity and, for many polymerizations, essentially removes amino acids from the reactive pool of building blocks.[42−44] An important aspect of evaluating and comparing proposed prebiotic syntheses of polypeptides is the characterization of the product mixtures. The general methods most commonly reported for these analyses are NMR spectroscopy, mass spectrometry (MS), and chromatography.

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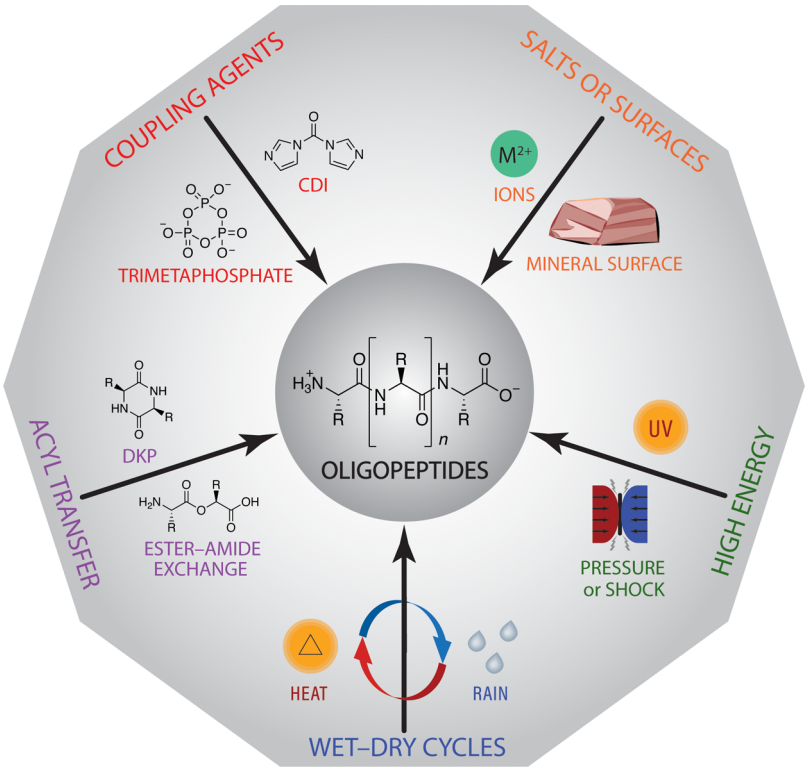

Figure 1. Prebiotically feasible methods for the condensation of amino acids into peptides. Five general approaches to the synthesis of oligopeptides include the use of catalytic salts or surfaces in the dry state, the use of stoichiometric coupling agents, elongation by acyl-transfer reactions, condensation through dry-downs or wet−dry environmental cycling, and reactions driven by sources of high energy, like UV light or shock impacts.

NMR spectroscopy can be used qualitatively to monitor the formation of amide bonds during amino-acid condensations.[34,45] We and others have used NMR spectroscopy quantitatively for applications like analyzing the hydrolysis of dipeptides of glycine and alanine,[46] but the technique becomes increasingly unsuitable for longer oligomers. Even the relatively short and simple oligomers Gly_{1-6} have overlapping signals that prevent accurate quantitative analysis of their mixtures (see Figure S17 in the Supporting Information).

Various MS techniques have been used to characterize the products of oligomerization reactions. MS methods are generally regarded as qualitative means of establishing the presence of various oligomers; the intensities of peaks are unreliable for measuring molecular concentrations, as peak intensities will also depend on each molecule's propensity to ionize and fragment. Parker et al. used ultraperformance liquid chromatography paired with quadrupole-traveling wave ion-mobility spectrometry and time-of-flight MS (UPLC-Q-TWIMS-TOF-MS) to analyze the dipeptides produced in Miller−Urey experiments.[47] Rodriguez-Garcia et al. used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS to identify the presence of oligomers up to Gly_{20}.[34] Forsythe et al. recently used liquid chromatography−ion-mobility spectrometry−tandem MS (LC−IM−MS/MS) to identify mixed oligomers of Gly, Ala, Leu, and glycolic acid up to $n = 8$.[48] Advances are being made in improving MS methods to provide reliable quantitative data,[49] but ionization and fragmentation are not the only barriers to the application of MS to the analysis of oligopeptides. Many prebiotic syntheses employ dry-down conditions with relatively high concentrations of salts as simulations of evaporating pools of "prebiotic soups".[21,41,50,51] The charged/zwitterionic nature of amino acids and peptides makes them difficult to separate from other ions (i.e., to de-salt them), and high salt is known to adversely affect a number of MS methods—limiting spectral quality, reducing quantitative accuracy, and potentially damaging the detector components of spectrometers.[52]

Chromatographic methods—sometimes used in conjunction with MS—have been the most common methods for the analysis of mixtures of peptide oligomers. Sugahara and Mimura as well as Fujimoto et al. used gas chromatography−MS (GC−MS) for the analysis of product mixtures from pressure-induced oligomerization of Ala.[35,36] The analytes were derivatized with isopropanol and trifluoroacetic anhydride to produce *N*-trifluoroacetic acylated-$(Ala)_n$-*O*-isopropyl esters. Only small oligomers of Ala_n were formed, and the authors note that this method is limited to Ala_n oligomers of $n \leq 3$, possibly because longer oligomers are insufficiently volatile for the analysis by GC.

Derivatization involves the functionalization of analytes with moieties that aid their detection. These procedures operate on the assumption that the oligomers present will react with the detection label with quantitative yield and that each tagged oligomer will have the same molar absorptivity (unless individual standards are tagged and analyzed as well). In-line tagging methods require LC systems with greater sophistication than may be accessible to some groups. Kitadai and coworkers used the method of Torres et al. for the quantitative analysis of oligomers of Gly_n up to $n = 6$ using HPLC on a cation-exchange column with post-column derivatization by *o*-phthalaldehyde and fluorescence detection.[4−6,53] Fuchida and

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co-workers used the same method for the analysis of their product mixtures with oligomers up to Gly_3.[19]

Ion-pair high-performance liquid chromatography (IP-HPLC) has proven in many studies to be an effective method for the separation of various oligomers of glycine.[7,11−14,16,20,21,34,38,39] In most of these studies, the Gly_n product mixtures were limited to $n \leq 6$; thus, analytical standards were commercially available. Ohara et al. employed the method of Rode and co-workers[21,54] in using HPLC with hexanesulfonate as an ion-pair reagent to measure oligomers up to Gly_6 by UV detection and then used LC with a perfluorinated surfactant to observe Gly_{7-10} with detection by MS.[39]

Dalai et al. produced oligomers up to Gly_6 in their condensations and used IP-HPLC for the primary analysis of the products.[13] To quantify the short oligomers of Gly_n ($n \leq 3$), they compared the UV response of the products to analytical standards. For the longer oligomers ($n \geq 4$), the authors derivatized the oligomers with 2,4-dinitrophenyl groups using Sanger's reagent and analyzed the products by reverse-phase HPLC.

Rodriguez-Garcia et al. extended an IP-HPLC method reported by Rode and co-workers to quantify total combined yields of oligomers up to Gly_{14}.[21,34] They calculated the total yield of an oligomerization reaction as the proportion of Gly converted into $Gly_{\geq 2}$ (excluding DKP) based on "the mean absorbance per glycine unit in the larger standards, which was observed to become approximately constant >3-mer".

Yu et al. used hydrophilic interaction liquid chromatography for the quantification of depsipeptides and short oligomers of glycine.[55] They analyzed standards of Gly_n where $n = 1-3$ and hydroxyacid-*N*-capped oligomers of glycine and operated under the assumption that the trend in extinction coefficients extends to higher oligomers.

RESULTS

Measurement/Extrapolation of Molar Response Factors (f_n) for Each Gly_n. To determine whether there was a relationship between oligomer length and molar UV response (f_n) that could be used to extrapolate values of f_n for higher oligomers, we obtained commercial samples of Gly_{1-6} and a custom order of Gly_8. We also ordered Gly_{10}, but the vendor was unable to synthesize a pure sample. IP-HPLC analysis of the Gly_{1-6} samples showed them to be free of detectable impurities, whereas the Gly_8 sample had detectable quantities of other Gly_n oligomers (see Figure S1). As such, we relied on samples of Gly_{1-6}—and not Gly_8—in our extrapolation of molar response factors for higher oligomers.

The IP-HPLC chromatograms plot absorbance (in units of mV, from detector signal) versus retention time as analytes elute from the column. We measured the UV response (A_n, peak area) for each Gly_n standard at varying concentrations to calculate the molar response factors (f_n) of each oligomer (Figure 2). Equation 1 relates the UV response to the number of moles of standard (m_n) injected per analyte

$$A_n = f_n \cdot m_n \quad (1)$$

We plotted the values of f_n versus oligomer length for the Gly_{1-6} standards and generated least-squares regression lines through the first three points, last four points, and all six points (Figure 3). All three lines had similar slopes and intercepts, which surprised us for the shortest oligomers, which we

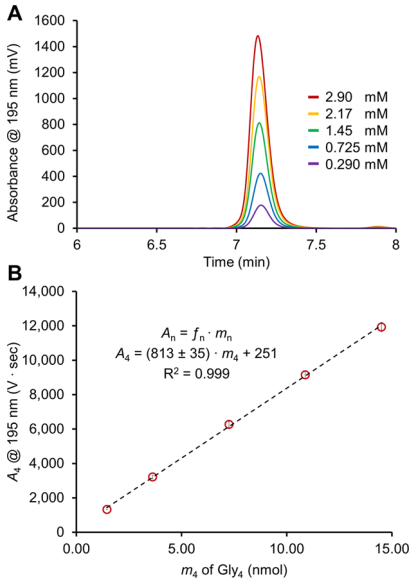

Figure 2. (A) Overlaid chromatograms of standard solutions of Gly_4 of varying concentrations, with injections of 5 μL and detection at 195 nm. (B) Plot of the UV response vs the number of molecules of Gly_4 injected. The slope of the line equals the molar response factor (f_n) for Gly_4, in accordance with eq 1. Each point represents the mean average of three standard samples of the corresponding concentration, and the errors bars represent 95% confidence intervals.

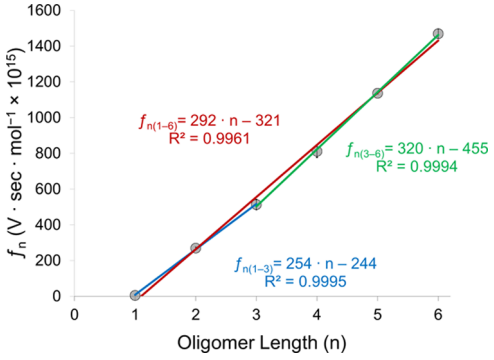

Figure 3. Plot of molar response factor (f_n) at 195 nm vs oligomer length for standards of oligomers of glycine (Gly_n, $n = 1-6$), with least-squares linear regression curves plotted through $n = 1-6$ (red), $n = 1-3$ (blue), and $n = 3-6$ (green). The error bars represent 95% CIs for the response factors, based on three measurements obtained independently from the slopes of linear calibration curves constructed for each oligomer, as for Gly_4 in Figure 2B. Note the small differences in the least-squares linear regression curves; the green curve ($n = 3-6$) most accurately predicts the measured values of the longest oligomers within the experimental error of the measurements, and as such, it was used to extrapolate the values of f_n for $Gly_{>7}$ employed to determine yields in subsequent experiments (Table 1).

hypothesized could have very different absorbances. Of these three curves, the line fit to the Gly_{3-6} data most accurately models the measured values of the longest standards within the experimental error of the measurements (Figure 3, Table 1).

From this regression curve for Gly_{3-6}, we extrapolated the molar response factor (f_n) values for Gly_{7-14} (Table 1). With these values in hand, eq 1 is used to determine the concentration of each Gly_n oligomer in a mixture separated by IP-HPLC. We validated the method by using it to verify the concentration of oligomers in a mixed standard to demonstrate

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Table 1. Molar Response Factors (f_n) for Oligomers of Glycine (Gly_n) from n = 1 to 14[a]

oligomer Gly_n	response factor ($V \cdot s \cdot mol^{-1} \times 10^{15}$) measured f_n	calculated $f_{n(1-3)}$	calculated $f_{n(3-6)}$	calculated $f_{n(1-6)}$
1	**5.8** ± 0.2	9	−136	−29
2	**270** ± 6	263	184	263
3	**513** ± 29	516	504	555
4	**813** ± 35	770	823	847
5	**1137** ± 4	1024	1143	1139
6	**1470** ± 27	1277	1462	1431
7		1531	**1782**	1723
8		1784	**2101**	2015
9		2038	**2421**	2307
10		2292	**2741**	2599
11		2545	**3060**	2891
12		2799	**3380**	3183
13		3053	**3699**	3475
14		3306	**4019**	3768
15		3560	**4338**	4060

[a]Values measured from commercial standards appear in the second column, while values calculated from the three linear regression curves shown in Figure 3 appear in the rightmost columns. The bolded values of f_n are those used in our determination of yields of oligomers in Gly_n syntheses.

that the oligomers did not interfere with each other's measurement (see Figure S10 in the Supporting Information). We also measured a molar response factor for Gly_8 from the impure commercial sample by correcting f_8 to account for the measurable Gly_6, Gly_7, and Gly_9 impurities in the sample (details in the Supplemental Experimental section of the Supporting Information). This corrected value for f_8 was simply used as a "check standard" for the extrapolation model described above and was not used to extrapolate higher values of f_n.

Characterization of Prebiotic Oligomerizations. As a final demonstration of the utility of the method, we applied it to the analysis of an actual condensation reaction that produced a mixture of oligoglycines under conditions reported by Cronin and co-workers.[34] We prepared three identical 4.0 mL aqueous solutions of 87.5 mM Gly, 250 mM NaCl, and 25 mM NaOH. The vials were placed on a hot plate maintained at 130 °C for 12 h and left open to evaporate to dryness during the heating period. The product was allowed to cool and subsequently dissolved in 4.0 mL of 0.1% trifluoroacetic acid (TFA) in water at room temperature for analysis by the IP-HPLC method described above. We determined the combined yield of all of the linear oligomers of glycine ($Gly_{\geq 2}$) to be 49.5 ± 2.0% (95% confidence interval, based on the measurement of three samples, see Figure 4 and Table S3 in the Supporting Information). Cronin reported a yield of "ca. 45%" in an identical experiment.[34]

DISCUSSION

We began from the IP-HPLC method for the analysis of oligoglycine originally reported by Rode and used by both Ohara and Cronin.[21,34,39] The technique appears to be limited by the solubility of Gly_n oligomers in the running buffer. When we analyzed a commercial sample of poly(glycine) with molecular weight cutoff of approximately 5000 g/mol, the chromatogram comprised peaks that we felt comfortable

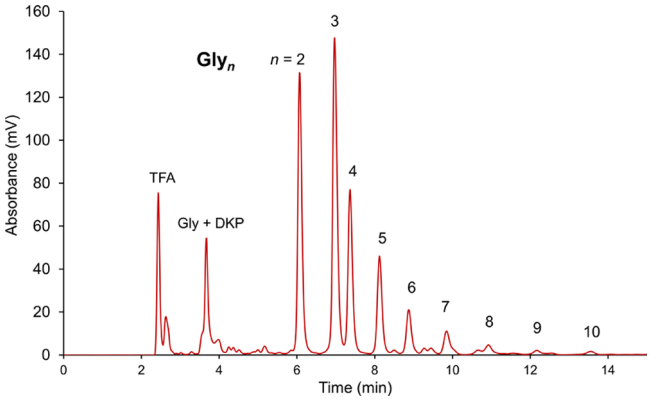

Figure 4. IP-HPLC chromatogram of the product mixture from the oligomerization of glycine described in the Results section. The total yield of linear oligomers (based on initial glycine, excluding DKP as an undesirable product) was determined to be 49.5 ± 2.0% Total yield = $(\sum_{n=2}^{n} n \cdot m_n)_{final} / (m_1)_{initial}$ = 49.5%.

integrating up to Gly_{14} (details available in the Supporting Information, Figure S19). The higher peaks faded into the baseline and broke in the pattern of having progressively longer differences in retention time from the previous oligomer. In the preparation of the polyglycine sample, some of the solid did not dissolve—likely higher oligomers that are insoluble or poorly/partially soluble. Cronin and co-workers noted similar behavior in their samples from the condensation of Gly.[34] Reanalysis of the solid by IP-HPLC found oligomers that were presumably saturated in the first sample. They also analyzed the undissolved solid by MALDI MS and identified oligomers up to Gly_{20}. As noted previously, MS methods are useful for the qualitative determination of the presence of oligomers, but samples with high salt can be difficult to analyze quantitatively.

We attempted to modify the IP-HPLC method to enable quantitative analysis of longer oligomers by addition of water-miscible organic solvents, like acetonitrile, to the running buffer to improve the solubility of the longer Gly_n species. However, in all attempts, the cosolvent drastically and unfavorably altered the retention times and resolution of the oligomers. We also attempted to optimize the separation by testing alkylsulfonates of different lengths (with C_6, C_8, and C_{10} alkyl chains) but returned to hexanesulfonate as the best option.

When optimizing conditions for the separation of oligoglycines, we paid particular focus to the separation of Gly_3 and Gly_4. For unknown reasons, these peaks run closer together than other oligomers. Their resolution vastly improved upon switching to a column with a lower particle size (3 vs 5 μm) and increasing the flow rate (up to 1.0 mL/min). Following these modifications, the method reported here can give near-baseline resolution of the linear Gly_n oligomers ($n \geq 2$), which compares favorably to previous separations (Figure 4).[34,55] Glycine and DKP coelute, but their mutual interference is not necessarily a significant limitation to the evaluation of prebiotic systems (see the section on Guidance for Reporting Yields, below).

Method Validation. The concentration of each Gly_n oligomer in a sample is measured by integrating its peak in the chromatogram and dividing by the corresponding molar response factor measured or calculated from standards (Table 1). We rely on external standards because the chromatogram is

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already crowded with Gly_n peaks, and the inclusion of an extra analyte would interfere with measurement of the oligomers.

To verify the accuracy of the method, we compared the total concentration of Gly (in all forms of Gly_n) in a standard sample of mixed Gly_n standards (n = 1−6) measured by both quantitative NMR spectroscopy and the IP-HPLC method described above. The concentrations measured by the two methods differed by just 3.2% (see the Supporting Information, Figure S17 and Table S2).

Guidance for Reporting Yields. Quantitative analysis provides useful information with respect to evaluating prebiotic syntheses, and in the context of oligoglycine synthesis, there have been a variety of approaches. Conversions and yields are the standard metrics of synthetic reactions.[56] In theory, the simplest approach to analyze the efficiency of oligomerization would be to measure the remaining Gly and calculate its conversion—the percentage of it consumed in the reaction, assuming that all of the consumed glycine was converted to higher oligomers. However, in practice, Gly often coelutes with other byproducts or—in the case of nonchromatographic techniques—interferes with the signal for other analytes, which prevents a quantitative measure of Gly alone. Additionally, such an approach would not penalize side reactions like pyrolysis of glycine or the formation of prebiotically undesirable byproducts, such as diketopiperizine (DKP).[57]

Yields of the individual Gly_n oligomers—calculated from their UV responses—are more robust metrics for the evaluation of peptide syntheses than single values for the conversion of glycine. The molar response factors (f_n) measured by our method permit the determination of the yield of each oligomer with a distinct peak. It is clear from comparison of the chromatogram for authentic low-molecular-weight (LMW) polyglycine and that of the prebiotic oligomerization reaction that there are products of the reaction that are not Gly_n oligomers. We have not attempted to characterize these side products, but MS would be helpful in this regard. It is possible that unknown side products are coeluting with some of the Gly_n oligomers, possibly inflating the calculated yields of one or more of the individual oligomers. Based on the characteristic stability of amino acids and peptides, as well as the sharpness of the peaks in our chromatograms, we believe the presence of these side products is small relative to the peptide oligomers expected as products of the reaction.

Assumptions and Limitations. Our method relies on the assumption that the trend in the molar UV response observed in our Gly_n standards continues linearly through Gly_{14}. An advantage of using isocratic LC is that the oligomers all elute in the same solvent. By analyzing the standards in the same solvent, we can ensure that there are no variations based on changes to the molar absorptivities of the analytes across a gradient of solvents. Reports in the literature disagree as to whether these effects are significant or not.[58,59] Furthermore, we operate under the assumption that any potential secondary structures that may form in longer oligomers do not significantly impact the UV absorption of those analytes. We also assume that no byproducts of the reaction are coeluting with the oligomers of glycine. And as with other methods that use external standards, our method functions on the assumption that the instrument is precise and reproducible in delivering consistent injection volumes, which is reasonable, given the reproducibility of the data obtained on our standards.

The biggest limitation of the method is the reliable quantification of products that are not soluble in water. The solubility of oligomers of glycine appears to decrease as the number of residues increases. If a sample of Gly_n is completely soluble in the running buffer, the IP-HPLC method will permit separation and accurate quantitative analysis. A significant limitation is that if there is any remaining solid that does not dissolve, the quantitative analysis will be flawed, as the solid sample will contain an unknown amount of Gly_n oligomers. Attempts to dissolve the remaining solid and subject it to a second analytical run will be frustrated for the least soluble, higher oligomers. Fortunately, the vast majority of glycine oligomerizations reported in the literature produce mixtures of Gly_n suitable for quantitative analysis by IP-HPLC. Only a few reports generate high enough yields of longer oligomers to preclude their complete analysis. Researchers should be careful to note whether their samples dissolve completely when reporting Gly_n products and their yields.

In the future, as syntheses become progressively better in terms of yielding longer oligomers, it will be increasingly important for the field to develop a technique for the convenient quantitative analysis of these compounds. In the absence of such a method, researchers could draw incorrect conclusions when comparing multiple systems on the basis of yield. For example, if a significant portion of the starting material is converted into insoluble products that are not included in the chromatographic analysis, then the total yield of oligomers will be artificially undercalculated. A system with a lower overall yield, but no insoluble products, might erroneously appear to have a higher yield than a comparable system that did generate insoluble Gly_n oligomers. It appears that future techniques will require analyte derivatization and/or nonaqueous solvents to overcome the limitation of the solubility of $Gly_{>14}$ in water, but the use of these solvents poses a challenge for the effective separation of all of the analytes in the product mixtures.

Context. We wish to be clear about what we believe to be the main contributions of this paper. The idea of condensing glycine and other amino acids has a long history in origins-of-life research, but the vast majority of studies only report the synthesis of $Gly_{\leq 6}$. These studies determine yields by comparison to standard samples of these oligomers, which can be obtained commercially. Despite the long history of work in this field, amino-acid oligomerizations and polymerizations are still a very active area of research. Recently, several systems have "taken off" in terms of producing oligomers of n > 6. These successes have introduced the challenge of analyzing mixtures of $Gly_{>6}$, as these compounds are not easily purchased or synthesized in sufficient mass and purity to use as standards. The inaccessibility of these standards necessitated the development of a means of extrapolating information about higher oligomers in order to characterize mixtures.

We were skeptical that the molar UV response factors of higher oligomers could be accurately extrapolated from Gly_{1-4}. While the addition of each residue to longer oligomers (e.g., Gly_{12} to Gly_{13} to Gly_{14}) places each chromophore in roughly the same environment, we expected addition of an extra residue to shorter oligomers to have less predictable consequences. After all, in shorter oligomers, the central residues are closer to the polar/charged C- and N-termini. The termini are also closer to each other in shorter oligomers, and we thought that it was unsound to assume that these

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differences would have no effect on absorptivity of the amide bonds. Therefore, we made it a point in this study to carefully measure the molar response factors of as many oligomers as possible to determine a proper calibration curve to extrapolate values of f_n for longer oligomers. In a previous study of a different system, Codari et al. established that the response factors for oligo(lactic acid)—from hydrolysis of the parent polyester—are linear by measuring up to $n = 9$.[60] Similar to our approach described here, they used their data to extrapolate UV responses for higher oligomers. For the case of glycine oligomers, it appears that the extrapolation of f_n values for $Gly_{>7}$ is best obtained from a linear regression curve fitted to Gly_{3-6} (over Gly_{1-6} or Gly_{1-3}) because of the smaller deviation of that line through the longer standards.

We also wish to be clear that IP-HPLC has existed for a long time. Rode first employed it for the analysis of glycine oligomerizations.[21] Cronin used it recently to analyze $Gly_{\geq 6}$,[34] and in this paper, we report modifications to the method to improve resolution of peaks and ensure the quantitative rigor of the analysis. We are unaware of better separations of Gly_3 and Gly_4 by IP-HPLC.

CONCLUSIONS

IP-HPLC offers a simple, robust, and convenient method for the quantitative analysis of condensation reactions of glycine. These reactions are of particular interest to the study of the origins of life. Over the years, research groups interested in the origins of life have employed a variety of methods to analyze condensation reactions of glycine and other amino acids. Yields in these systems are complicated metrics, as a wide mixture of products is produced. But having a simple, sensitive, and reliable method to quantify the products is vital when comparing multiple systems and studying experimental parameters. Although this method has limitations because of the solubility of products, it offers advantages compared to other methods with regard to improved resolution and robustness of the analysis of oligomers for which pure standards cannot be obtained. Oligomers higher than Gly_{15} have been synthesized in proposed prebiotic systems and detected by MS; thus, the development of methods that enable the quantitative analysis of these oligomers—which are practically insoluble in aqueous media—would be valuable for this field.

METHODS

Design. Our interest in studying amino-acid condensations, coupled with the variety of approaches to their analysis, motivated our search for a robust and reliable method to quantify glycine oligomerization reactions. We expect to produce higher oligomers of Gly_n for which standards can be challenging to synthesize in high yield and purity. The lack of standards presents a problem for estimating the concentration of longer products in calculations of yield. We found previously reported methods to be lacking in one or more of the following characteristics: (i) they did not attempt to quantify higher oligomers, (ii) they extrapolated a trend for molar absorptivity based on a few short oligomers, with $n < 4$, or (iii) they did not describe the quantification of higher oligomers in detail. We were also troubled by the resolution of many of the reported separations, where overlapping peaks would hinder accurate determination of the UV response of individual oligomers in a sample. The separation of Gly_3 and Gly_4 can be particularly difficult.[34]

Our general approach entailed verifying, optimizing, and extending the IP-HPLC method for the separation of glycine oligomers reported by Rode and co-workers that was later modified by Cronin and co-workers.[21,34] Once the method produced chromatograms with acceptable resolution of peaks on our instrument, we were able to determine concentrations of each oligomer based on their UV responses and then use the data from standards to characterize reaction mixtures of glycine condensations under prebiotic conditions.

Materials, Safety, and General Procedures. All reagents, including glycine, oligomers of glycine up to Gly_6, and LMW polyglycine were obtained from MilliporeSigma or VWR and their affiliate suppliers and were not purified further. TFA is a strong acid and care should be taken in its use. A standard of Gly_8 was ordered for custom synthesis from Fisher Scientific USA. The purity of this sample was checked by IP-HPLC and found to be contaminated by other oligomers of glycine, including Gly, Gly_6, Gly_7, and Gly_9 (Figure S1). As a result, we did not incorporate data collected on this sample of Gly_8 into our model for the extrapolation of molar response factors (f_n) of higher oligomers.

Standard solutions of each oligomer were prepared using a minimum of 10 mg of sample, and the solutions were prepared using a volumetric flask (of at least 50 mL) for accuracy. These solutions were diluted to five different concentrations using micropipettes, giving five individual standards for each oligomer (Figure S2). This procedure was repeated three different times for each oligomer of glycine, yielding three measurements for each oligomer at five different concentrations (Figures S3–S9). The three trials for each oligomer at each concentration were run with unique samples that were prepared individually for the determination of the molar response factor (f_n).

Experimental Parameters of the IP-HPLC Method for the Quantification of Gly_n. Oligomers of glycine were analyzed using IP-HPLC on a Shimadzu Prominence LC20-AR instrument equipped with a Phenomenex Luna C18 column (250 mm long × 4.6 mm diameter, 3 μm particle size). The mobile phase was an aqueous solution of 50 mM KH_2PO_4 and 7.2 mM sodium hexanesulfonate (SHS), adjusted to pH 2.5 by the addition of HPLC-grade 85% H_3PO_4. The mobile phase was used isocratically with a flow rate of 1.00 mL·min^{-1}. When preparing a column for its first use, the mobile phase was passed through the column at 0.50 mL·min^{-1} for 12 h to establish an equilibrium with respect to adsorption of the SHS ion-pair reagent on the C18 stationary phase, in essence, establishing a modified stationary phase suitable for IP-HPLC. The column temperature was maintained at 30 °C by a column oven, and samples were injected in 5.0 μL aliquots by an autosampler to a 100 μL injection loop. The instrument is equipped with a dual-wavelength UV–vis detector, which was set to record absorbance at 195 and 214 nm. Our analysis relies exclusively on the data collected at 195 nm, where the analytes have higher molar absorptivity. With these experimental parameters, we achieved separation of all of the linear oligomers of glycine up to $n = 14$ in a commercial sample of LMW polyglycine (Figure S19).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01492.

Material available: additional experimental details of the IP-HPLC method and practical notes for its application, NMR spectra of standards, calibration curves for each Gly_n standard, additional IP-HPLC chromatograms, determination of limits of quantitation, and additional references (PDF)

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The authors declare no competing financial interest.

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