Supporting Information for

Quantitative Analysis of Glycine Oligomerization
by Ion-Pair Chromatography

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Supplemental Experimental

General Considerations. All reagents, including glycine, oligomers of glycine up to Gly₆, and low-molecular-weight (LMW) polyglycine were obtained from MilliporeSigma or VWR and their affiliate suppliers and were not purified further. A standard of Gly₈ 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, Gly₆, Gly₇, and Gly₉ (Figure S1). As a result, we did not incorporate data collected on this sample of Gly₈ 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).

Quantitative Nuclear Magnetic Resonance (qNMR) Spectroscopy. The exact concentration of each standard was verified using quantitative ¹H NMR spectroscopy by comparing the signal from the analyte to that of an internal standard of 2.5 mM tert-butanol in D₂O (Figures S11–S16). All spectra were collected using a solvent-suppression, 1-D excitation sculpting pulse program (‘zgesgp’ on Bruker’s TopSpin 3.2 software). To ensure quantitative integration of the signals, the recycle delay (D1) was set to 15 seconds, which is approximately seven times the spin-lattice relaxation (T₁) for a glycine oligomer.¹² The low solubility of Gly₆ in
water resulted in an NMR spectrum of poor quality. Despite the poor quality of this spectrum, the concentration of the Gly₆ standard determined by qNMR spectroscopy closely matched the target concentration based on the mass of the analyte and the volume of the standard solution.

**UV–Vis Spectroscopy.** We collected a UV–vis spectrum of a Gly₄ standard in the IP–HPLC mobile phase solvent to help determine wavelengths for detection in the method. We selected 195 nm to match previous reports and because there was no obvious better alternative (see Figure S18). We also had the detector observe 214 nm, which is a commonly used wavelength for the analysis of peptides. The sensitivity at 214 nm is considerably lower than 195 nm and may not be suitable for analyzing higher oligomers that are likely to be present in low concentrations due to limited solubility and/or production in lower yields. Nonetheless, the calibration curves constructed from both sets of data followed similar trends.

**Estimation of Detectable Oligomers.** To estimate the maximum length of oligomers of glycine that we could reliably detect and analyze using this method, we collected an IP–HPLC chromatogram of a standard sample of low-molecular-weight (LMW) polyglycine (sold as molecular weight approx. 500–5,000 g/mol, but oligomers all the way down to free glycine were observed to be present). A saturated sample was prepared in 0.1% TFA in H₂O and filtered before analysis by IP–HPLC. The chromatogram revealed resolved signals for all oligomers of Glyₙ up to Gly₁₄ (Figure S19). While small peaks corresponding to Gly₁₅ and Gly₁₆ are also visible, we were not comfortable analyzing them due their small intensities and the start of a break in the pattern of retention times after Gly₁₄. As a result, we report the estimated molar response factors for oligomers up to Gly₁₄.

**Statistical Treatment of Uncertainty and Error.** Calibration curves for each of the six analytes for which we had standards (Gly₁ though Gly₆) appear in Figures S3–S8. The error bars
for each point represent 95% confidence intervals based on three replicate trials (degrees of freedom = 2, \( t^* \) critical value = 4.303). The slope of the least-squares regression line fitted to the plots of UV response vs. quantity of analyte is equal to the response factor, \( f_n \). The standard deviation of the slope, as determined by the \textsc{linest} function in Excel, was then used to calculate the 95% confidence interval for each response factor.\(^3\) These confidence intervals were determined using a \( t^* \) critical value corresponding to 95% confidence based on five points in each plot (degrees of freedom = 3, \( t^* \) critical value = 3.182).

**Analysis of the Gly\(_8\) Standard.** We commissioned the synthesis of Gly\(_8\) from a commercial peptide vendor, but the sample obtained was not pure, so we did not incorporate Gly\(_8\) into our model for the extrapolation of molar response factors (\( f_n \)) of higher oligomers. We did subject the sample to the same analysis as Gly\(_{1-6}\) and determined \( f_8 = 1940 \pm 15 \times 10^{15} \) V \cdot sec \cdot mol\(^{-1}\). We expect this value to be low, as some of the mass of sample used to prepare the standard solution was that of impurities (not Gly\(_8\)), and the molar response factor was based only on integrating the peak area for Gly\(_8\). In an attempt to correct the value of \( f_8 \), we integrated the peaks corresponding to the Gly\(_6\), Gly\(_7\), and Gly\(_9\) impurities in Figure S1, and used the values of \( f_n \) reported in Table 1 for these oligomers to determine the moles present of these analytes in the impure Gly\(_8\) standard solution. We then subtracted the mass of these impurities from the mass of Gly\(_8\) used to prepare the standard solution. The corrected value for \( f_8 \) was determined to be 2143 \( \times 10^{15} \) V \cdot sec \cdot mol\(^{-1}\), which compares favorably to 2101 \( \times 10^{15} \) V \cdot sec \cdot mol\(^{-1}\), the value for \( f_8 \) extrapolated using the regression line for Gly\(_{3-6}\). We do not report a confidence interval for \( f_8 \) due to the unclear uncertainty of our correction method.
Equations

**Equation S1.** Determination of response factors.

Variables:

\[
\text{Gly}_n = \text{peptide oligomer of n glycine residues}
\]

\[
m_n = \text{amount of injected } \text{Gly}_n \text{ analyte (units: mol)}
\]

\[
A_n = \text{UV response of injected } \text{Gly}_n \text{ analyte (units: mV \cdot sec)}
\]

\[
f_n = \text{molar response factor for } \text{Gly}_n \text{ (units: V \cdot sec \cdot mol}^{-1})
\]

UV response relations:

\[
A_n = f_n \times m_n \quad (S1)
\]

\[
f_n = A_n / m_n
\]

**Equation S2.** Determination of 95% confidence intervals for UV response values.

Variables:

\[
\bar{X} = \text{sample mean}
\]

\[
t^* = t\text{-distribution critical value}
\]

\[
s = \text{sample standard deviation}
\]

\[
n = \text{sample size}
\]

Confidence Interval:

\[
95\% \text{ CI} = \bar{X} \pm t^* \frac{s}{\sqrt{n}} \quad (S2)
\]
Practical Notes

Method Development. A mixed standard containing Gly2, Gly3, and Gly4 is a great check-sample for the optimization or development of an IP–HPLC separation method for the analysis of oligomers of glycine. Gly3 and Gly4 are the most difficult oligomers to separate—if they are separating well, the separation method will likely work for all of the oligomers. We observed the resolution of Gly3 and Gly4 to worsen as the column aged. For instance, this difference in performance is observable between Figures 4 and S10 (fresh column) and Figure S19 (column near the end of its life).

pH of Mobile Phase. It is important to ensure that the pH of the mobile phase is consistent from one batch to another. Not only will the pH affect the retention times of the analytes, it could also have an impact on their extinction coefficients, potentially reducing the accuracy of the measurements. Our mobile phase was adjusted to pH 2.5 with HPLC-grade H$_3$PO$_4$ in 2 L batches using a Jenway 3510 pH Meter.

Limits of Quantitation. We report limit of quantitation (LOQ) values for Gly2 through Gly6 in Table S4. These LOQ values were determined by taking the sample standard deviation of the three measured UV responses (peak areas in the chromatogram) for the lowest concentration of Gly2–6 used to prepare each of the calibration curves in Figures S4–S8, then multiplying by 10 and dividing by the slope of the calibration curve.

Control Experiment for Interference by DKP. Cyclic diglycine (2,5-diketopiperazine, DKP) hydrolyzes to form linear diglycine (Gly2). As we do not quantify DKP but do report yields of Gly2, we ran a control experiment to test whether the DKP present in a product mixture could hydrolyze during analysis to appear as Gly2 and thus inflate its yield. We prepared a 70 mM solution of DKP in the 0.1 % trifluoroacetic acid solution used to prepare samples for
analysis and let the sample stand for 5 hours to simulate the maximum time a sample would wait prior to injection on the HPLC instrument once prepared. After 5 hours, the peak corresponding to Gly$_2$ was below the limit of quantitation (LOQ) for Gly$_2$ reported in Table S4, suggesting the interference of DKP in our analysis is insignificant.
Figure S1. IP–HPLC chromatogram for the commercial sample of Gly₈. The inset chromatogram depicts a magnification of the parent chromatogram, with the peaks for Gly₈ aligned. Note the impurities present from other oligomers of glycine, including Gly₉. Due to the lack of purity of this sample, we did not incorporate data collected on Gly₈ into the extrapolation of the higher oligomers of Glyₙ.
Figure S2. Overlaid chromatograms of Gly₄ standards subjected to analysis by IP–HPLC. The peaks for each run were integrated to determine the UV responses, which were then plotted against the moles of analyte injected into the instrument to determine the molar response factor $f_4$ (see Figure S6). Identical experiments were conducted for each Glyₙ standard to determine values for $f_{1-6,8}$ (see Figures S3–S9). Concentrations of [Gly₄] = 0.290 mM (violet), 0.725 mM (blue), 1.45 mM (green), 2.17 mM (yellow), 2.90 mM (red).
Figure S3. The calibration curve of glycine. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_1$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI.

![Graph of Figure S3](image)

$$A_n = f_n \cdot m_n$$

$$A_1 = (5.79 \pm 0.23) \cdot m_1 + 10.44$$

$$R^2 = 0.999$$

Figure S4. The calibration curve of Gly2. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_2$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI.

![Graph of Figure S4](image)

$$A_n = f_n \cdot m_n$$

$$A_2 = (270 \pm 6) \cdot m_2 + 124$$

$$R^2 = 0.999$$
**Figure S5.** The calibration curve of Gly3. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_3$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI.

![Graph showing the calibration curve of Gly3.](image)

**Figure S6.** The calibration curve of Gly4. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_4$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI. An identical plot appears in the main paper as Figure 2b, where it represents a typical calibration curve.

![Graph showing the calibration curve of Gly4.](image)
**Figure S7.** The calibration curve of Gly5. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_5$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI.

![Graph of Figure S7](image)

The equation for the calibration curve is:

$$A_5 = f_5 \cdot m_5$$

$$A_5 = (1137 \pm 4) \cdot m_5 + 23$$

$$R^2 = 0.999$$

**Figure S8.** The calibration curve of Gly6. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor $f_6$ at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI.

![Graph of Figure S8](image)

The equation for the calibration curve is:

$$A_6 = f_6 \cdot m_6$$

$$A_6 = (1470 \pm 27) \cdot m_6 + 5$$

$$R^2 = 0.999$$
Figure S9. The calibration curve of Gly\textsubscript{8}. Error bars represent 95% confidence intervals based on three measurements at each concentration. The slope is equal to the molar response factor \( f_8 \) at 195 nm and is reported as a 95% confidence interval determined by the statistical analysis reported in the Supplemental Experimental section of the SI. This value for \( f_8 \) is uncorrected in the sense that this analysis did not account for the fact that the sample contained impurities of other Gly\textsubscript{n} compounds, up to Gly\textsubscript{9}. A corrected value of \( f_8 = 2143 \times 10^{15} \) V \( \cdot \) sec \( \cdot \) mol\(^{-1} \) was determined by a method described on page S5.

\[
A_n = f_n \cdot m_n
\]

\[
A_8 = (1940 \pm 15) \cdot m_8 + 11
\]

\[R^2 = 0.999\]
Figure S10. IP–HPLC chromatogram of a mixed standard of Gly1–6.

Table S1. Approximate retention times of standards of oligomers of glycine for the reported IP–HPLC method.

| Standard                               | Retention Time (min) |
|----------------------------------------|-----------------------|
| Glycine (Gly)                          | 3.8                   |
| Gly-Gly (Gly₂)                         | 5.9                   |
| Gly-Gly-Gly (Gly₃)                     | 6.7                   |
| Gly-Gly-Gly-Gly (Gly₄)                 | 7.1                   |
| Gly-Gly-Gly-Gly-Gly (Gly₅)             | 7.9                   |
| Gly-Gly-Gly-Gly-Gly-Gly (Gly₆)         | 8.6                   |
| Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly (Gly₈) | 10.9                  |
Figure S11. An $^1$H NMR spectrum of standard solution of glycine, including 2.5 mM tert-butanol to verify the concentration of the standard solution.

Figure S12. An $^1$H NMR spectrum of standard solution of Gly₂, including 2.5 mM tert-butanol to verify the concentration of the standard solution.
Figure S13. An $^1$H NMR spectrum of standard solution of Gly$_3$, including 2.5 mM tert-butanol to verify the concentration of the standard solution.

Figure S14. An $^1$H NMR spectrum of standard solution of Gly$_4$, including 2.5 mM tert-butanol to verify the concentration of the standard solution.
Figure S15. An $^1$H NMR spectrum of standard solution of Gly$_5$, including 2.5 mM tert-butanol to verify the concentration of the standard solution.

Figure S16. An $^1$H NMR spectrum of standard solution of Gly$_6$, including 2.5 mM tert-butanol to verify the concentration of the standard solution.
Figure S17. An $^1$H NMR spectrum of mixed standards of Gly$_{1-6}$, including 2.5 mM tert-butanol as an internal standard.
Figure S18. UV–vis spectra of the IP running buffer (blue curve, where a background spectrum of milli-Q has been subtracted) and a solution of Gly₄ standard (red curve, where a background spectrum of the IP running buffer has been subtracted).
Table S2. Measured UV responses (peak areas, in units of mV · sec) for each oligomer in a mixed standard of Gly\textsubscript{1–6} and the resulting concentration of total Gly\textsubscript{n}. Each column corresponds to the data collected from one of three trials. A sample chromatogram from one trial appears as Figure S10.

| Analyte | 1        | 2        | 3        |
|---------|----------|----------|----------|
| Glycine | 151232   | 151025   | 148850   |
| Gly\textsubscript{2} | 1296964 | 1298317  | 1298970  |
| Gly\textsubscript{3} | 2528292 | 2534156  | 2532483  |
| Gly\textsubscript{4} | 2186451 | 2187102  | 2185488  |
| Gly\textsubscript{5} | 532189  | 531497   | 530960   |
| Gly\textsubscript{6} | 81008   | 82908    | 80612    |
| Total Gly\textsubscript{n} (mM) | 12.79 | 12.79 | 12.71 |

Average ± 95% CI 12.76% ± 0.11%

Total Gly\textsubscript{n} by NMR (mM) 12.35

Percent Difference 3.2%

Table S3. Measured UV responses (peak areas, in units of mV · sec) of each Glyn oligomer in the product mixtures of three replicate trials of the oligomerization of glycine. A sample chromatogram from one trial appears as Figure 4 of the main paper.

| Analyte | 1        | 2        | 3        |
|---------|----------|----------|----------|
| Glycine | N/A      | N/A      | N/A      |
| Gly\textsubscript{2} | 10299513 | 10218977 | 10022841 |
| Gly\textsubscript{3} | 12161779 | 12545225 | 11981873 |
| Gly\textsubscript{4} | 6590787  | 6684185  | 6465976  |
| Gly\textsubscript{5} | 3946077  | 4157463  | 3936857  |
| Gly\textsubscript{6} | 2021211  | 2122018  | 2037391  |
| Gly\textsubscript{7} | 1415521  | 1423520  | 1432869  |
| Gly\textsubscript{8} | 674667   | 705527   | 696777   |
| Gly\textsubscript{9} | 360496   | 377628   | 361111   |
| Gly\textsubscript{10} | 438106  | 369598   | 425484   |
| Total Yield (%) | 49.52 | 50.30 | 48.70 |

Average ± 95% CI 49.5% ± 2.0%
Figure S19. An IP–HPLC chromatogram of an unmodified sample of low-molecular-weight polyglycine. The detection of up to Gly$_{14}$ establishes the upper limit for analysis of oligomers of glycine using this method.
Table S4. Measured limits of quantitation (LOQ) for Gly2 through Gly6 per injection for the described IP–HPLC method.

| Analyte | LOQ (nmol) |
|---------|------------|
| Gly2    | 0.203      |
| Gly3    | 0.134      |
| Gly4    | 0.053      |
| Gly5    | 0.048      |
| Gly6    | 0.031      |
Supplemental References

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