Applied Chemistry Today **20(77)** (2025) 69-72

Semnan University

Applied Chemistry Today

Journal homepage: https://chemistry.semnan.ac.ir/

ISSN: 2981-2437

Research Article

Enhanced Quantification of Co-Eluting Amino Acids Using TLC based Image Analysis and Multivariate Calibration

Mehrdokht Hosseini Haghighi[a], Maryam Rajabi[a,*], Maryam Khoshkam [b,c*], Toktam Mohammadi Moghaddam[d]

[a]*Department of Chemistry, Faculty of Basic Sciences, Semnan University, Semnan, Iran*
[b]*Department of Chemistry, Faculty of Sciences, University of Mohaghegh Ardabili,56199-11367, Ardabil, Iran*
[c]*Department of Environmental Science, Faculty of Science, University of Zanjan, 45371-38791, Zanjan, Iran*
[d]*Workplace Health Research Center, Neyshabour University of Medical Sciences, Neyshabour, Iran*

PAPER INFO

Article history:
Received: 28/Apr/2025
Revised: 12/Aug/2025
Accepted: 19/Aug/2025

Keywords:
TLC based image analysis, Multivariate calibration, Co-eluting components, Amino acids

ABSTRACT

L-Glutamine (Gln) and Glycine (Gly) are two amino acids with overlapping retention times in thin-layer chromatography (TLC). This overlap presents a significant analytical challenge, particularly when both compounds are present in the same mixture, as their spots on the TLC plate may not be fully resolved. Here, we used smartphone-based image analysis combined with PLS regression for semi-separated Gln/Gly quantification in TLC. A smartphone captured TLC plates images under consistent lighting conditions, and custom software processed them to generate chromatographic profiles. Calibration curves showed linear responses, with detection limits of 0.007 M and 0.008 M for Gln and Gly, respectively. Despite mobile-phase optimization, complete spot resolution was unachievable. To address this, partial least squares (PLS) regression deconvoluted the merged signals, enabling accurate quantification. The method was validated using a pharmaceutical product, yielding recovery rates of 100.4 ± 1.4% for Gln and 100.6 ± 1.0% for Gly, demonstrating its reliability for complex samples.

DOI: https://doi.org/10.22075/chem.2025.37549.2365

[*]**. corresponding author:** Professor of Analytical Chemistry. E-mail addresses: mrajabi@semnan.ac.ir

How to cite this article: Hosseini Haghighi, M., Rajabi, M., Khoshkam, M. & Mohammadi Moghaddam, T. (2025). Enhanced Quantification of Co-Eluting Amino Acids Using TLC based Image Analysis and Multivariate Calibration. *Applied Chemistry Today*, **20(77)**, 69-72. (in Persian)

1. Introduction

The quantification of amino acids (AAs) is critically important across scientific and industrial fields. In biochemistry and molecular biology, precise AA measurements are essential for studying protein synthesis, metabolic pathways, and enzymatic functions.

In the food and pharmaceutical industries, AA profiling ensures product quality, nutritional value, and the development of supplements and therapeutics. Clinically, AA quantification aids in diagnosing metabolic disorders and nutritional deficiencies [1].

As the building blocks of proteins, AAs influence protein turnover, cellular biology, and regulatory mechanisms. Their concentrations in biological samples (e.g., blood, urine, cerebrospinal fluid) serve as key biochemical indicators [2]. Thus, accurate AA analysis is foundational for scientific progress, industrial applications, and human health.

several methods are employed for the quantitative analysis of AAs. High-performance liquid chromatography (HPLC) is widely used due to its precision and ability to separate complex mixtures of amino acids [1-3].

Gas chromatography (GC), often combined with mass spectrometry (GC-MS), is another powerful technique for analyzing volatile amino acid derivatives[4, 5]. Spectroscopy, including UV-Vis and fluorescence spectroscopy, is employed for rapid and sensitive detection, often following derivatization of amino acids to enhance their spectral properties[4, 6]. Nuclear magnetic resonance (NMR) spectroscopy provides detailed information on the structure and concentration of amino acids in solutions[7, 8]. Additionally, Capillary electrophoresis (CE) offers high-resolution separation and quantification with minimal sample requirements [1, 9].

Each method has its advantages, making it suitable for specific applications based on the required sensitivity, accuracy, and sample complexity. Chromatographic techniques provide accurate results but may be unsuitable for the analysis of large sets of samples because the separation step is time-consuming. However, these methods usually require pre-column or post-column derivatization, followed by detecting the derivatized analyte using LC-UV, LC-fluorescence, or LC-MS/MS [10]. Spectrophotometric analysis of multicomponent mixtures reveals that quantifying compounds remains a challenging problem when there is no spectral difference. Because most common AAs are not readily detected by spectroscopic techniques (UV–Visible spectrophotometry or fluorimetry), a chemical procedure for the derivatization of AAs is required [11].

Thin-layer chromatography can also be employed to detect AAs, which requires no expensive outfits and tedious sample pretreatments, and allows a parallel separation of many samples [12-14]. As a convenient and economical method of detection, TLC has been successfully applied in the detection of AAs from several samples [15]. Compared to its qualitative use, quantitative applications with TLC are limited. Nevertheless, with advances in image-based detection [16, 17], quantitative approaches have increased in recent years [18].

Up to date, many TLC methods, especially using high-performance TLC (HPLC) plates, have been suggested for the separation of AAs in complex mixtures. By changing in mobile phase composition and/or modification of the stationary phase, analytical chemists were able to separate a lot of AAs by TLC or HPTLC [19, 20].

L-Glutamine (Gln) and Glycine (Gly) are two types of the 20 different amino acids in the human body. Gln is a building block of protein. Proteins help with many functions; for example, they can repair body

tissues, keep the digestive system working, and help the immune system fight germs.

Gly helps build proteins needed for tissue and hormone maintenance. Additionally, Gly supports heart and liver health, improves sleep, reduces the risk of diabetes, and reduces muscle loss.

The behavior of Gln and Gly in different chromatographic systems is similar. Since very close retention times have been reported for these amino acids, their separation by various thin-layer chromatography (TLC) methods is difficult. Band overlapping is a common problem in many chromatographic methods, especially TLC. Therefore, successful chromatographic separation requires a significant amount of time and solvent to achieve optimal conditions. In such cases, instead of finding conditions for the physical separation of analytes, their signals are separated mathematically using chemometric methods.

In the present study, we report multivariate image analysis for the simultaneous determination of Gln and Gly. Using homemade software, the colored spots of the analytes were converted to chromatograms and analyzed by multivariate data analysis methods, enabling the simultaneous determination of the non-separated amino acids. Partial least squares (PLS) regression was implemented as the multivariate calibration method. Model complexity was optimized to achieve the least prediction error.

2. Materials and Methods

2.1. Reagents and Solutions

All experiments were performed with analytical reagent-grade chemicals. n-butanol, acetic acid, L-Glutamine, Glycine, and brilliant green were provided by Sigma-Aldrich. Ninhydrin was obtained from Riedel–Dehaen AG Company. TLC plates (silica gel 60 F254, 20 * 20 cm) were purchased from Merck Company.

2.2. Homemade System

The assembled homemade system consists of three primary components: a TLC development chamber, an image capture device, and PLS_Toolbox software (Version 7.8 - released Jun. 2014) installed on a personal computer.

The first component is a standard TLC chamber, comprising a TLC plate and a mobile phase solution. The imaging component is the most important part of the homemade system. An iPhone 12 smartphone, equipped with a 12-megapixel camera, is used to capture images of the TLC plates. To facilitate image acquisition, the TLC plates are secured to a white plastic and are set at a distance of 20 cm from the smartphone and captured in room light (fig. S1). The third component of the homemade system is a personal computer running a multivariate image analysis program. All calculations are performed in **MATLAB (R2010b)**. In the image processing protocol, the recorded TLC plate images (saved in JPEG format) are imported into MATLAB. The three output matrices—red (R), green (G), and blue (B)—are then extracted from the selected image. Each matrix has dimensions of 141 × 124 pixels, corresponding to the length (141 pixels) and width (124 pixels) of the image.

After exporting the R, G, and B data, 11 derived datasets were generated for analysis (Fig. S2-S6). A custom-written subroutine is capable of transforming image data into three-dimensional (color value versus length and width of the TLC plate) and two-dimensional (color value versus length of the TLC plate) chromatograms. The collected data is smoothed and subsequently fed into various multivariate calibration methods to establish a correlation between the recorded images and the analyte concentrations.

2.3 Theory

We performed data analysis using Partial Least Squares (PLS) regression, a first-order multivariate calibration method. PLS requires a row vector of

predictor variables (in this study, the color values) for each sample. To facilitate PLS analysis, the three-dimensional arrays of color values (sub-matrices) were transformed into one-dimensional row vectors through a 3D-to-1D conversion process. After applying this conversion to all sample mixtures, a new predictor matrix (X) was constructed by sequentially arranging each sample's row-vector data. This matrix served as the independent variable in PLS regression to estimate analyte concentrations in the mixtures.

We employed the PLS-1 algorithm, which requires building separate PLS models for each analyte. The optimal number of latent variables was determined through leave-one-out cross-validation on the calibration dataset. Finally, the optimized PLS model was used to predict analyte concentrations in the validation set mixtures.

2.4 Procedure

2.4.1 Mobile Phase Selection

Various solvent systems reported in the literature for TLC analysis of AAs were evaluated. The mobile phase of n-butanol: acetic acid: water in a 3:1:1 volumetric ratio yielded satisfactory results.

This solvent system has been employed for the simultaneous determination of analytes using a conventional univariate calibration method. However, this solvent mixture did not completely separate Gln and Gly spots. Therefore, multivariate calibration was implemented for the analysis. Brilliant Green was utilized as an internal standard.

2.4.2 Staining Reagent

Ninhydrin was used as a staining reagent. To this end, Ninhydrin was added to the mobile phase at a 2% w/w concentration. Subsequently, after TLC development, the plates were dried in an oven at 70-75°C for 10 minutes.

2.4.3 Univariate Calibration Curve

Similar to all multivariate calibration-based analytical methods, the initial step involved determining the linear dynamic range of each analyte, or in other words, establishing the relationship between color intensity and the concentration of the studied AAs [21, 22].

Different solutions of each analyte, ranging in concentration from 0.00039 to 0.20 M, were prepared, and a fixed amount of Brilliant Green was added as an internal standard. Subsequently, a fixed and small volume (approximately 1.0 µL) of the prepared solutions was spotted on the TLC plate using a 1 µL syringe.

Calibration plots were constructed by plotting the ratio of the analyte peak area to that of Brilliant Green as the analytical signal against concentration. Separate calibration curves were generated for each color value (R, G, B) and their combinations.

2.4.4 Simultaneous Determination of Gln and Gly

The procedure for the simultaneous determination of two analytes is similar to that described by Hematinejad et al [19, 20]. Model development was achieved using two sets of standard solutions: calibration and prediction sets. A total of 23 standard solutions were prepared according to a five-level mixture design. All of the standard solutions were tested individually as test sets, and the remaining solutions were used as calibration sets.

TLC development and imaging were conducted as described in the previous section for univariate calibration. The input variables for multivariate calibration modeling were the obtained two-dimensional chromatograms (i.e., plots of color values as a function of pixel number from the spotting line).

In this case, the response signal comprised the color values (R, G, B, or a combination of color values), and the instrument channels were the pixels. The chromatograms were corrected for peak shift and peak intensity using Brilliant Green as an internal standard. PLS-1 methods were adopted. The overall

predictive performance of the models was evaluated using separate test set samples [23].

3. Results and Discussion

An important consideration in TLC separation is the selection of the appropriate mobile phase. After selecting the mobile phase, a critical aspect in the analysis of amino acids (AA) by TLC is their visualization.

The conventional method involves spraying a ninhydrin solution onto the surface of the TLC plate. However, this approach is time-consuming and often results in a non-homogeneous colored surface, making it unsuitable for quantitative imaging. Furthermore, spraying requires a certain level of expertise. To overcome these limitations, we adopted the recent method proposed by Li et al. [24]. In this approach, ninhydrin is dissolved directly into the mobile phase, resembling a pre-derivatization process. Then, the colors are visualized by drying the plates in an oven at a temperature of 70–75 °C for 10 minutes. This eliminates the need for the spraying step and produces uniformly colored spots, which are more suitable for quantitative imaging.

In imaging-based quantitative analyses, it is crucial to determine which color coordinates are most suitable. We found that using the G (green) value for Gln and the B (blue) color value for Gly yields more appropriate chromatograms. Specifically, these color values exhibited a linear dependency on the concentration of the analytes. The chromatograms obtained using either the G (green), B (blue), or R (red) values change as the analyte concentration varies.

Before performing multivariate calibration, the analytical performance of the proposed TLC method was evaluated for individual analytes. The three- and two-dimensional chromatograms generated by the written software, along with the corresponding calibration graphs, are shown in Figures 1 and 2 for Gln and Gly, respectively.

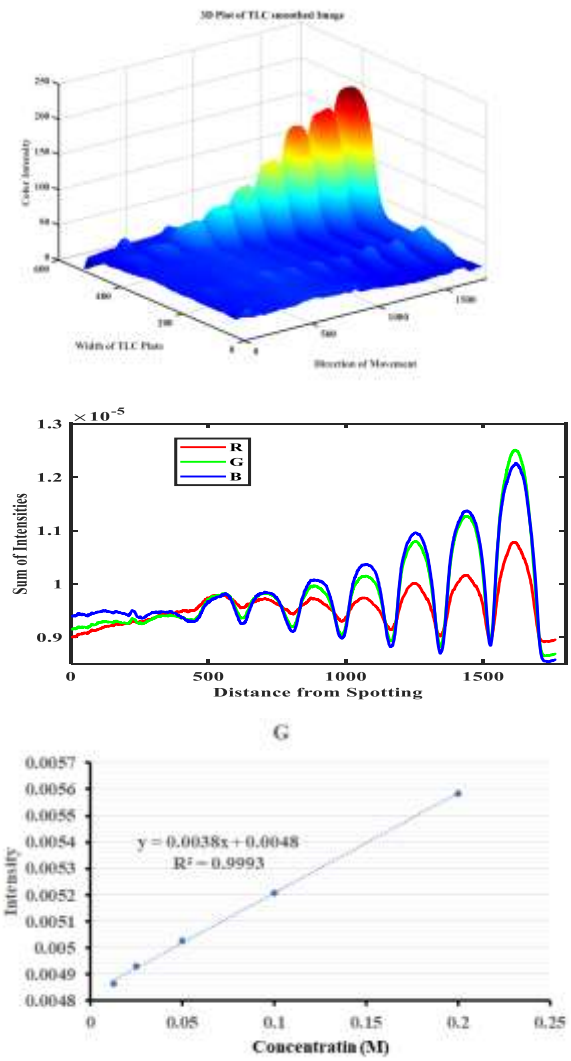
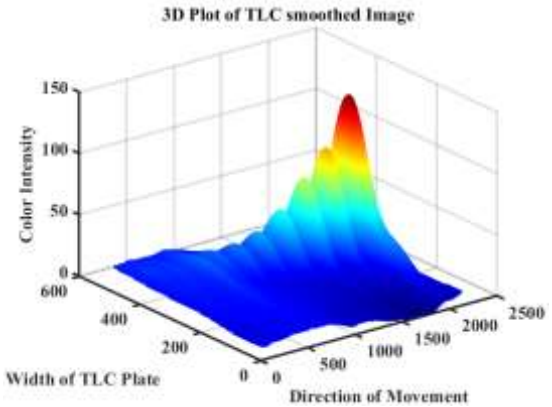


Fig.1. Individual calibration for Gln: **a** three-dimensional chromatogram, **b** two-dimensional chromatogram, and **c** resultant calibration curve

It should be noted that the data presented in these figures were obtained using the G (green) color index for Gln and the B (blue) color index for Gly. The analytical performance of the individual calibration curves is summarized in Table 1.



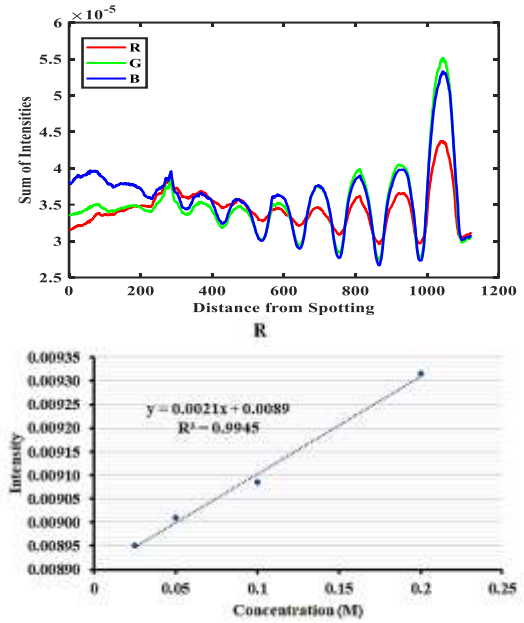


Fig.2. Individual calibration for Gly: **a** three-dimensional chromatogram, **b** two-dimensional chromatogram, and **c** resultant calibration curve

Well-defined linear relationships with excellent correlation coefficients were observed between the relative peak area (peak area of the analyte divided by the peak area of the internal standard) and the concentration of the analytes.

The lower limit of calibration was determined to be 0.0125 M for both Gln and Gly, respectively. At lower concentrations, the responses showed significant deviations from linearity.

Table 1. Analytical appraisals of the individual calibration curves of Gln and Gly

	Gln	Gly
Linear range (M)	0.0125-0.2	0.0125-0.2
Regression equation	y = 0.0038x + 0.0048	y = 0.0021x + 0.0089
R^2	0.9993	0.9945
Detection limit (M)	0.007	0.008

The detection limits, calculated as $3.3* \sigma_b$ (where σ_b is the standard deviation of the blank), were 0.007 M and 0.008 M for Gln and Gly, respectively.

The chromatograms of individual solutions of Gln and Gly, as well as their binary mixture, reveal significant overlap, making it impossible to simultaneously determine these amino acids using conventional calibration methods. Therefore, multivariate calibration was employed for the simultaneous determination of Gln and Gly.

In the next step, the R, G, and B data were exported for each sample, and 11 types of datasets were generated for further analysis using multivariate calibration methods. These datasets included:1) unfolded R, 2) unfolded B, 3) unfolded G, 4) Augmentation of unfolded R, G and B, 5) Sum of unfolded R, G, and B, 6) Row summation of R, 7) Row summation of B, 8) Row summation of G, 9) Column summation of R, 10) Column summation of B, and 11) Column summation of G was applied for further analysis with multivariate calibration method. PLS-1 was the best multivariate calibration method in this study.

PLS1 was applied to all **11 data types**. The results demonstrated that the **augmented unfolded R, G, and B data** (Fig. S4) provided the best performance for both **Gln and Gly**. Consequently, this data type was selected for further analysis.

The optimal number of **latent variables** for the PLS model was determined through **leave-one-out cross-validation** on the calibration dataset. The optimized PLS model was then used to predict analyte concentrations in the prediction set mixtures. The **statistical parameters** of the model are summarized in **Table 2**. To validate the predictive capability of the calibration models, they were applied to estimate analyte concentrations in the prediction samples. The results are presented graphically in **Figure 4**.

Table 2. Statistical parameters of the PLS-based multivariate calibration models

Analyte	LV	RMSCV	R^2_{CV}	RMS_P	R^2_P	REP (%)
Gln	4	0.7568	0.985	0.42	0.997	6.15
Gly	4	0.6272	0.984	0.40	0.981	8.01

As seen, the plot of predicted versus actual concentrations of both analytes represents a low degree of scattering around a line with squared correlation coefficients of 0.997 and 0.9812, for Gln and Gly, respectively.

To validate the multivariate model, we quantified Gln and Gly in pharmaceutical samples spiked with known concentrations of the analytes: Gln (0.10–0.12 M) and Gly (0.10–0.15 M). The results are presented in Table 3.

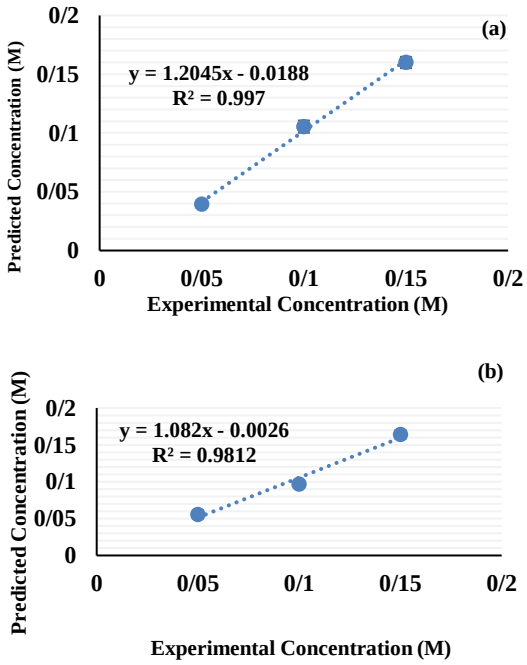


Fig. 4. Plot of predicted concentration versus experimental concentration for analysis of prediction: a Gln and b Gly (n=3)

Initial recovery values ranged from 103.3-108.3% for Gln and 106.6-110.0% for Gly, indicating a slight positive bias. After normalization, recoveries improved from 103.3–108.3% to 99.1%–101.7% (mean ± RSD: 100.400% ± 1.4) and for Gly from 106.6%-110.0% to 99.8%-101.3% (mean ± RSD: 100.550% ± 1.0), demonstrating the method's accuracy when accounting for matrix effects. Normalized recoveries showed no significant difference from 100% ($p>0.05$ by t-test), confirming the elimination of systematic bias.

The proposed model demonstrates effective simultaneous quantification of Gln and Gly in pharmaceutical samples.

Several advanced techniques have been reported for amino acid analysis, each with distinct advantages:

HPLC with UV detection [25] was used for glutamate, glycine, and alanine in human plasma after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). This method offers excellent linearity ($r > 0.999$) and precision (<10% RSD) but requires expensive instrumentation and derivatization steps.

In another study [26], capillary electrophoresis (CE) with LED-induced fluorescence achieves nanomolar (nM) sensitivity for Gly, Gln, and Glu in cerebrospinal fluid (CSF). However, this method requires specialized capillary conditioning but is highly suitable for clinical diagnostics.

A step-gradient HPLC with fluorescence detection enables rapid (<12 min) quantification of glutamate, glycine, and taurine in microdialysates and provides femtomole-level detection but is limited by instrument complexity [27].

Spectrophotometry with neural networks uses kinetic data for the simultaneous determination of Gly and Lys. This method is comparable to PLS in signal deconvolution but relies on spectrophotometric detection rather than TLC [28].

Enzymatic assays are highly specific for Gln quantification in CSF and plasma but are limited to single-analyte detection [29]. Compared to established techniques like HPLC and GC-MS , this TLC-based approach offers distinct advantages in operational simplicity, reduced equipment costs, and minimal training requirements.

However, three key limitations should be noted: (1) unlike HPLC/GC-MS, the method quantifies co-eluted analytes without physical separation, relying on multivariate signal deconvolution; (2) sensitivity remains lower than gold-standard methods (LODs of 0.007-0.008 M vs. µM-range for HPLC); and (3) precision (%RSD 1.0-1.4%), while acceptable for quality control, is slightly reduced compared to liquid chromatography techniques. These trade-offs position the method as particularly valuable for rapid screening applications where ultra-high sensitivity is not critical.

Table 3. Results of the analysis of Gln and Gly in pharmaceutical samples (n=3)

Sample	Spiked (M)		Raw Predicted (M)		Raw Recovery (%)		Normalized Recovery (%)		*%RE	
	Gln	Gly	Gln	Gly	Gln	Gly	Gln	Gly	Gln	Gly
1	0.100	0.100	0.103	0.110	103.300	110.000	99.100 ± 1.2	101.300 ± 0.9	0.900	-0.017
2	0.120	0.150	0.130	0.160	108.300	106.600	101.700 ± 1.5	99.800 ± 1.1	-1.300	0.130
Mean					105.800	108.300	100.400 ± 1.4	100.550 ± 1.0	-	-

*Relative Error (%) $= (\frac{True\ (Spiked)\ Concentration - Measured\ Concentration}{\text{True (Spiked) Concentration}}) \times 100$

4. Conclusion

We developed a simple thin-layer chromatography (TLC) method for the simultaneous determination of glutamine (Gln) and glycine (Gly) as representative amino acids. The approach combines multivariate analysis of chromatographic profiles with smartphone-based image processing, enabling quantification despite partial co-elution. Compared to existing methods for challenging amino acid pairs (e.g., leucine/isoleucine separation), this technique offers three key advantages: (1) reduced analysis time (<30 min), (2) lower operational costs (utilizing conventional TLC plates instead of HPTLC), and (3) simplified instrumentation (smartphone detection vs. HPLC/GC-MS). Validation using pharmaceutical samples yielded mean recovery rates of 100.40% and 100.55% for Gln and Gly, respectively, and demonstrated reliability for complex matrices. While optimized for Gln/Gly, the methodology shows potential for adaptation to other structurally similar amino acids like Leu/Ile, pending further validation of separation efficiency.

Acknowledgment

The authors would like to acknowledge the Chemistry Faculty at Semnan University for their support of this work.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this manuscript. In addition, the authors have entirely observed the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

References

[1] Teo, M., Khoo, L. W., & Chew, W. (2024). A simplified small-scale workflow for determination of complete protein-bound amino acids using pre-column derivatization HPLC method. *Journal of Food Composition and Analysis*, *135*, 106571.

[2] Zhang, J., Yu, X., Yang, R., Zheng, B., Zhang, Y., & Zhang, F. (2024). Quality evaluation of Lonicerae Japonicae Flos from different origins based on high-performance liquid chromatography (HPLC) fingerprinting and multicomponent quantitative analysis combined with chemical pattern recognition. *Phytochemical Analysis*, *35*(4), 647-663.

[3] de Sousa Fontes, V. M., Pimentel, T. C., da Silva, A. B. M., Madruga, M. S., Magnani, M., & dos Santos Lima, M. (2024). An improved method for determining free amino acids by RP-HPLC/DAD with o-phthalaldehyde derivatization: Method evaluation in beers and wines. *Food Chemistry*, *435*, 137591.

[4] Ichihara, K. I., Kohsaka, C., & Yamamoto, Y. (2021). Determination of proteinaceous free amino acids by gas chromatography. *Analytical Biochemistry*, *633*, 114423.

[5] Posma, R. A., Bakker, S. J., Nijsten, M. W., Touw, D. J., & Tsikas, D. (2024). Comprehensive GC-MS Measurement of Amino Acids, Metabolites, and Malondialdehyde in Metformin-Associated

Lactic Acidosis at Admission and during Renal Replacement Treatment. *Journal of Clinical Medicine*, *13*(13), 3692.

[6] Ferreira, C. P., Antunes, F. T. T., Rebelo, I. N., da Silva Junior, C. A., Vilanova, F. N., Corrêa, D. S., & de Souza, A. H. (2020). Application of the UV–vis spectrophotometry method for the determination of glutamate in the cerebrospinal fluid of rats. *Journal of Pharmaceutical and Biomedical Analysis*, *186*, 113290.

[7] Huang, C., & Kalodimos, C. G. (2017). Structures of large protein complexes determined by nuclear magnetic resonance spectroscopy. *Annual review of biophysics*, *46*, 317-336.

[8] Nord, L. I., Vaag, P., & Duus, J. Ø. (2004). Quantification of organic and amino acids in beer by 1H NMR spectroscopy. *Analytical chemistry*, *76*(16), 4790-4798.

[9] Soga, T., Kakazu, Y., Robert, M., Tomita, M., & Nishioka, T. (2004). Qualitative and quantitative analysis of amino acids by capillary electrophoresis-electrospray ionization-tandem mass spectrometry. *Electrophoresis*, *25*(13), 1964-1972.

[10] Wilson, S. F., James, C. A., Zhu, X., Davis, M. T., & Rose, M. J. (2011). Development of a method for the determination of glycine in human cerebrospinal fluid using pre-column derivatization and LC–MS/MS. *Journal of pharmaceutical and biomedical analysis*, *56*(2), 315-323.

[11] Hasani, M., Yaghoubi, L., & Abdollahi, H. (2007). A kinetic spectrophotometric method for simultaneous determination of glycine and lysine by artificial neural networks. *Analytical Biochemistry*, *365*(1), 74-81.

[12] Rajurkar, N. S., & Gaikwad, K. N. (2014). Identification and quantification of amino acids from medicinally important plants by using high-performance thin-layer chromatography. *Journal of Liquid Chromatography & Related Technologies*, *37*(15), 2197-2205.

[13] Hodisan, T., Culea, M., Cimpoiu, C., & Cot, A. (1998). Separation, identification and quantitative determination of free amino acids from plant extracts. *Journal of Pharmaceutical and Biomedical Analysis*, *18*(3), 319-323.

[14] Martens, J., & Bhushan, R. (1989). TLC enantiomeric separation of amino acids. *International Journal of Peptide and Protein Research*, *34*(6), 433-444.

[15] Qiu, T., Li, H., & Cao, Y. (2010). Pre-staining thin layer chromatography method for amino acid detection. *African Journal of Biotechnology*, *9*(50), 8679-8681.

[16] Sardesai, V. M., & Provido, H. S. (1970). The determination of glycine in biological fluids. *Clinica chimica acta*, *29*(1), 67-71.

[17] Botrè, F., Botrè, C., Lorenti, G., Mazzei, F., Porcelli, F., & Scibona, G. (1993). Determination of L-glutamate and L-glutamine in pharmaceutical formulations by amperometric L-glutamate oxidase based enzyme sensors. *Journal of pharmaceutical and biomedical analysis*, *11*(8), 679-686.

[18] Machado, T. R., & Fonseca, A. (2019). A new alternative method for quantitative analysis by thin layer chromatography using a lab-made multi-track device and image-based

chromatograms. *Microchemical Journal*, *147*, 127-132.

[19] Hemmateenejad, B., Farzam, S. F., & Mobaraki, N. (2014). Simultaneous measurement of leucine and isoleucine by multivariate image analysis-thin layer chromatography (MIA-TLC). *Journal of the Iranian Chemical Society*, *11*(6), 1609-1617.

[20] Hemmateenejad, B., Mobaraki, N., Shakerizadeh-Shirazi, F., & Miri, R. (2010). Multivariate image analysis-thin layer chromatography (MIA-TLC) for simultaneous determination of co-eluting components. *Analyst*, *135*(7), 1747-1758.

[21] Bedia, C., Sierra, A., & Tauler, R. (2021). Multimodal multisample spectroscopic imaging analysis of tumor tissues using multivariate curve resolution. *Chemometrics and Intelligent Laboratory Systems*, *215*, 104366.

[22] Dhanapal, L., & Erkinbaev, C. (2024). Portable hyperspectral imaging coupled with multivariate analysis for real-time prediction of plant-based meat analogues quality. *Journal of Food Composition and Analysis*, *126*, 105840.

[23] Brereton, R. G. (2003). *Chemometrics: data analysis for the laboratory and chemical plant*. John Wiley & Sons.

[24] Li, H., Qiu, T., Cao, Y., Yang, J., & Huang, Z. (2009). Pre-staining paper chromatography method for quantification of γ-aminobutyric acid. *Journal of chromatography A*, *1216*(25), 5057-5060.

[25] Li, Q. Z., Huang, Q. X., Li, S. C., Yang, M. Z., & Rao, B. (2012). Simultaneous determination of glutamate, glycine, and alanine in human plasma using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high-performance liquid chromatography. *The Korean journal of physiology & pharmacology*, *16*(5), 355-360.

[26] Lu, M. J., Chiu, T. C., Chang, P. L., Ho, H. T., & Chang, H. T. (2005). Determination of glycine, glutamine, glutamate, and γ-aminobutyric acid in cerebrospinal fluids by capillary electrophoresis with light-emitting diode-induced fluorescence detection. *Analytica chimica acta*, *538*(1-2), 143-150.

[27] Piepponen, T. P., & Skujins, A. (2001). Rapid and sensitive step gradient assays of glutamate, glycine, taurine and γ-aminobutyric acid by high-performance liquid chromatography–fluorescence detection with o-phthalaldehyde–mercaptoethanol derivatization with an emphasis on microdialysis samples. *Journal of Chromatography B: Biomedical Sciences and Applications*, *757*(2), 277-283.

[28] Hasani, M., Yaghoubi, L., & Abdollahi, H. (2007). A kinetic spectrophotometric method for simultaneous determination of glycine and lysine by artificial neural networks. *Analytical Biochemistry*, *365*(1), 74-81.

[29] Pye, I. F., Stonier, C., & McGale, E. H. (1978). Double-enzymic assay for determination of glutamine and glutamic acids in cerebrospinal fluid and plasma. *Analytical Chemistry*, *50*(7), 951-953.