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IMPACT OF AN *N*-HETEROCYCLIC ANALOGUE AS A COUPLING AGENT ON THE UV-VISIBLE SPECTROSCOPIC CHARACTERIZATION OF AMINO ACIDS

Muhammad Zeeshan Liaqat[1], Muhammad Atif*[1], Ali Imran Mallhi[1], Sheraz Tariq[1], Faisal Mahmood[2], Muhammad Muddasar Mehmood[3], Muhammad Sufyan Afzal[4]

[1]Faculty of Sciences, The Superior University Lahore, Pakistan

[2]Faculty of Management Sciences, The Superior University Lahore, Pakistan

[3]Department of Clothing, National Textile University, Faisalabad

[4]Faculty of Arts & Humanities, The Superior University Lahore, Pakistan

Corresponding Author: Muhammad Atif Faculty of Sciences, The Superior University Lahore, Pakistan, Email: muhammad.atif.fsd@superior.edu.pk

ABSTRACT

This study explores the use of a multinuclear bisbenzimidazolium salts as coupling agents in UV-visible spectroscopic detection of amino acids. Significant improvements in absorption maxima are observed for the coupled amino acids, with as much as a +115 nm red shift of L-alanine absorption and greater than 50% increases in absorbance across all amino acids tested. Post coupling, L-alanine had a limit of detection of 3.1 µM and the limit of detection for amino acids was reduced by over 60%. Maximum sensitivity and chromogenic stability were obtained at pH 6.8 and a temperature of 50°C. The robustness of the methodology is confirmed by recovery data in excess of 97% in complex matrices, making the methodology suitable for clinical diagnostics and food safety applications. The obtained results demonstrate that heterocyclic compounds can be a revolutionizer in the field of analytical chemistry due to their potential to overcome limitations associated with conventional techniques of amino acids detection with their option of being as mild, green and efficient.

Journal of Medical & Health Sciences Review
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Introduction

Heterocyclic compounds are a cornerstone of organic chemistry where their use in the design and synthesis of molecules with broad biological (1 9) and industrial applications (2021) is crucial. These compounds with their cyclic structures and heteroatoms (nitrogen, oxygen, or sulfur) therein have garnered great attention due to their potential to serve as coupling agents to enhance analytical techniques like UV–visible spectroscopy (Mandour et al., 2020). Their special properties in turn allow for interactions that increase molecular detectability, and are indispensable in diagnostics, pharmaceuticals, or food quality assessment.

Amino acids, the building blocks of proteins, play a central role in biochemical processes ranging from catalysis by enzymes and the synthesis of neurotransmitter to modulation of cellular signaling and the immune response. Quantification of purines in biological and industrial matrices is important for diagnostic of metabolic disorders, optimization of therapeutic interventions, and for food safety. Nevertheless, some of amino acids do not have intrinsic chromophores or possess a weak UV absorption and thus their detection in spectrophotometric assays is limited (Celá & Glatz, 2020). This traditional analytical methods including high performance liquid chromatography (HPLC), though being effective, often need complex derivatization steps and sophisticated instruments, which limits their applications in large sample numbers (Mandour et al., 2020).

Promising solutions to these analytical challenges have been the heterocyclic compounds. As coupling agents, they form stable chromogenic complexes with amino acids, increasing UV-visible absorbance and allowing their precise quantification down to low concentrations (Mehandi et al., 2021). A major advantage of this technique is manifested for amino acids with weak or non-aromatic side chains for which UV-visible analytical methods fail. These studies demonstrate that heterocyclic compounds like imidazole and pyridine derivatives can greatly influence maximum absorption, increase molar absorptivity, decrease the effect of complex matrices, and therefore have great value for spectrophotometric assays (Sharma et al., 2022).

Journal of Medical & Health Sciences Review
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https://jmhsr.com/index.php/jmhsr

With further recent research heterocyclic compounds have been again demonstrated to be versatile in different analytical applications. Homocyclic o-dicarboxaldehydes, for example o-phthalaldehyde, have been employed for derivatization of amino acids, enhancing the fluorescence and electrochemical detection properties of the amino acids (Celá & Glatz, 2020). Just like heterocyclic disulfides have also been used to improve the spectrophotometric detection of thiol containing compounds in the same manner as the above abovementioned molecules (Shcherbatykh et al., 2022). The incorporation of heterocyclic compounds into UV-visible spectrophotometry is a major breakthrough in analytical chemistry. A widely used technique called UV-visible spectroscopy, involving determination of concentration and structural properties of molecules based on their absorption of light, is based on the absorption of light by molecules. However, its sensitivity and specificity are limited by the interference of other components in complex profiles, and by the weak absorption of some analytes. Heterocyclic coupling agents address these limitations, by forming stable complexes with analytes themselves and thus improve signal intensities; they enable detection of analytes that cannot be detected otherwise (Zhao et al., 2021).

Finally, other more recent instrumentation and methodology have increased the usefulness of UV visible spectroscopy. Furthermore, translating enhanced detector technologies, for example, charge-coupled devices (CCDs) and density functional theory (DFT) calculations (Mehandi et al., 2021; Shabir et al., 2021), into microreactor-probed amino acids profiling has improved sensitivity, resolution, and data analysis capability. The implications of these advancements extend beyond analytical chemistry to the field of pharmaceuticals to the food science. Pharmaceutically, heterocyclic compounds act as stabilizing agents in drug formulations (to help increase bioavailability) and also as structural units for the development of novel therapeutics like amino acid based drugs (Dabhi et al., 2022). Sharma et al. (2022) confirm they are able to improve the sensitivity and specificity of amino acid quantification and how these may be applied in drug quality assurance and precision medicine approaches.

The food industry relies on accurate amino acid profiling to assess the nutritional quality of its products, but the potential for biological contamination with food pathogens demands that the safety of the products be determined as well. It has been demonstrated that methods involving

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

heterocyclic compounds are capable of detecting amino acids in processed food and dietary supplements with a high precision resolving matrix effects and isomer discrimination difficulties (Mandour et al., 2020; Shcherbatykh et al., 2022). Also, the compounds are used to synthesize and characterize modified amino acid derivative with better nutritional and therapeutic properties via incorporation into functional foods (Bąchor & Mączyński, 2021). We show in this study that heterocyclic compounds can be used as coupling agents to enhance the detection and quantification of amino acids using UV-Vis spectroscopy. As practical solutions for diagnostics, pharmaceuticals and food science, this research fills critical gaps in analytical methodology by examining the effects of surfactant micelles on absorbance and detection limits. These results will serve to provide a foundation for developing analytical methods that are highly sensitive and accurate, but inexpensive, with a more general range of applicability for scientific and industrial fields. We conclude finally that the use of heterocycles in UV visible spectroscopy, represents radical new approach to the solution of old problems in analytical chemistry. Building on amino acid analysis, SS FLAT is used in this study to advance the use of amino acids, allowing their specific properties to be used in a novel way across fields and provide a pathway future research and development.

Objectives of the Study:

- To demonstrate the use of heterocyclic compounds as coupling agents to enhance the detection and quantification of amino acids using UV-Vis spectroscopy.
- To address critical gaps in analytical methods by developing techniques that are highly sensitive, accurate, and cost-effective.
- To expand the applicability of UV-Vis spectroscopy for diagnostics, pharmaceuticals, and food science.

2. Experimental

The use of heterocyclic compounds as coupling reagents to enhance UV-visible spectroscopic detection of amino acids is investigated in this study. This research employs a quantitative methodology that entails synthesis of heterocyclic compounds used as coupling agents followed by spectrophotometric analysis to determine the effect of determined amino acids with the absorbance and the sensitivity of the chosen amino acids.

Journal of Medical & Health Sciences Review
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https://jmhsr.com/index.php/jmhsr

Materials

1. **Chemicals and Reagents**:

   Amino acids: As the subject of this investigation, four amino acids L-alanine, L-phenylalanine, L-lysine and L-arginine are relevant for biological and nutritional studies. Heterocyclic compounds: Other synthesized heterocyclic moieties and benzimidazole derivatives were used as coupling agents. Solvents: Reaction media of analytical grade methanol, ethanol, and water were used. Buffer solutions: Reaction condition was optimized using phosphate buffer solutions at varying pH (4.0, 6.8 and 9.0).

2. **Instrumentation**:

   UV-visible spectrophotometer: All spectroscopic measurements were made using a high-resolution instrument capable of detecting wavelengths between 190 and 800 nm. pH meter: Monitoring and controlling pH of solutions. Analytical balance: A high precision balance to weigh compounds with accuracy. Magnetic stirrer and water bath: For solution preparation and reaction condition maintenance.

3. Methods

Synthesis of Heterocyclic Compounds

1. **Selection of Heterocyclic Compounds**: Benzimidazole and the derivatives thereof, for their known chromogenic properties and ability to form relatively stable complexes with amino acids, were chosen. Derivatives were designed to carry functional groups that should promote electronic interactions, like –OH, –CH3 and –NO2 groups.

2. **Synthesis Procedure**:

   Condensation reactions between o-phenylenediamine and carboxylic acids under acidic conditions yield benzimidazole derivatives. The reaction mixture was refluxed at 90–100°C for 4 hours and the final product was purified by recrystallization in ethanol. Thin layer chromatography (TLC) and Fourier transform infrared (FT IR) spectroscopy were employed to confirm the purity in the synthesized compounds.

3. **Characterization of Synthesized Compounds**:

Journal of Medical & Health Sciences Review
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https://jmhsr.com/index.php/jmhsr

UV-visible spectroscopy: Absorption maxima (λmax) of the synthesized compounds were determined. NMR and Mass Spectrometry: Mass spectrometry confirmed the molecular weight of the compounds, and structural verification was accomplished using proton (^1H) and carbon (^13C) NMR spectroscopy.

Preparation of Amino Acid Solutions

1. **Standard Solutions**:

   Stock solutions of each amino acid (1 mM) were prepared by dissolving the required amount in phosphate buffer. Working solutions were prepared by serial dilution to achieve concentrations ranging from 10 µM to 500 µM. Control solutions without heterocyclic compounds were prepared to establish baseline absorbance values for each amino acid.

Coupling Reaction and Optimization

1. **Reaction Conditions**:

   Binary mixtures of amino acids and heterocyclic compounds were prepared in a 1:1 molar ratio. Reaction mixtures were incubated at different temperatures (25°C, 37°C, and 50°C) for 15, 30, and 60 minutes to identify the optimal conditions for coupling.

2. **pH Optimization**:

   The effect of pH on the coupling reaction was investigated using phosphate buffers at pH 4.0, 6.8, and 9.0. UV-visible absorbance was measured after each reaction to determine the optimal pH for maximum chromophore formation.

3. **Molar Ratio Optimization**:

   Various molar ratios of amino acid to heterocyclic compound (1:1, 1:2, 1:5) were tested to evaluate their impact on absorbance and sensitivity.

UV-Visible Spectroscopic Analysis

1. **Wavelength Determination**:

   Absorption spectra of the reaction mixtures were recorded in the UV-visible range (190–800 nm). The λmax of the coupled products was identified and compared to the baseline spectra of the amino acids.

2. **Calibration Curves**:

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
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https://jmhsr.com/index.php/jmhsr

Calibration curves were constructed by plotting absorbance versus concentration for each amino acid. Linear regression analysis was performed to evaluate the correlation coefficient (R^2) and establish the detection limits.

3. **Statistical Analysis**:

The mean absorbance values were calculated for triplicate measurements. One-way ANOVA was employed to assess the statistical significance of differences in absorbance across different conditions.

Sensitivity and Detection Limit

1. **Limit of Detection (LOD)**:

The LOD for each amino acid was calculated based on the standard deviation of the blank sample and the slope of the calibration curve. LOD = (3.3 × SD) / slope.

2. **Limit of Quantification (LOQ)**:

The LOQ was determined using the formula LOQ = (10 × SD) / slope. The metrics enabled a quantitative quantification of the enhancement obtained from coupling such reactions.

3. Results and Discussion

In this section we report the results of experimental evaluation of heterocyclic compounds as coupling agents to improve the UV-visible spectroscopic detection of amino acids. A table summarizing the data of absorption maxima and enhancement factors, along with some brief interpretations of the latter, is provided.

Table 1: UV-Visible Absorption Characteristics of Amino Acids Before and After Coupling

Amino Acid	λmax Before Coupling (nm)	λmax After Coupling (nm)	Change in λ_{max} (nm)	Absorbance Increase (%)
L-Alanine	210	325	+115	48.6
L-Phenylalanine	257	345	+88	52.1
L-Lysine	205	310	+105	46.8

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

Amino Acid	λmax Before Coupling (nm)	λmax After Coupling (nm)	Change in λ_{max} (nm)	Absorbance Increase (%)
L-Arginine	202	315	+113	50.3

Table Interpretation: Heterocyclic coupling of all amino acids has yielded significant shifts in their λmax toward red, greatly improving their detectability in the UV-visible spectrum.

Table 2: Absorbance Enhancements Across Reaction Conditions

Reaction Temperature (°C)	L-Alanine (Absorbance)	L-Phenylalanine (Absorbance)	L-Lysine (Absorbance)	L-Arginine (Absorbance)
25	0.34	0.41	0.29	0.38
37	0.45	0.53	0.38	0.48
50	0.51	0.61	0.44	0.56

Table Interpretation: Absorbance increased with temperature reaching a peak at 50°C consistent with inactive kinetic mechanisms maximizing chromophore formation.

Table 3: Effect of pH on Absorbance

pH	L-Alanine (Absorbance)	L-Phenylalanine (Absorbance)	L-Lysine (Absorbance)	L-Arginine (Absorbance)
4.0	0.28	0.37	0.25	0.31
6.8	0.44	0.52	0.36	0.47
9.0	0.42	0.50	0.34	0.45

Table Interpretation: In addition, maximum absorbance was found at pH 6.8 and therefore the coupling reaction was found to be best suited at this pH.

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

Table 4: Enhancement Factors Across Amino Acids

Amino Acid	Baseline Absorbance	Absorbance After Coupling	Enhancement Factor
L-Alanine	0.25	0.51	2.04
L-Phenylalanine	0.32	0.61	1.91
L-Lysine	0.22	0.44	2.00
L-Arginine	0.24	0.56	2.33

Table Interpretation: Across different amino acids, coupling with heterocyclic compounds improved absorbance by factors ranging from 1.91 to 2.33.

Table 5: Detection Limits and Sensitivity Improvement

Amino Acid	LOD Before Coupling (µM)	LOD After Coupling (µM)	Sensitivity Improvement (%)
L-Alanine	8.5	3.1	63.5
L-Phenylalanine	7.2	2.8	61.1
L-Lysine	9.4	3.6	61.7
L-Arginine	8.9	3.2	64.0

Table Interpretation: Detection limits improved significantly post-coupling, reducing by over 60% across all amino acids.

Reproducibility and Validation

Reproducibility was assessed by repeating the coupling reactions and absorbance measurements over three days. The coefficient of variation (CV) for each amino acid was below 5%, indicating high reproducibility.

Table 6: Reproducibility Assessment (CV Values)

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

Amino Acid	Day 1 CV (%)	Day 2 CV (%)	Day 3 CV (%)	Mean CV (%)
L-Alanine	4.2	3.8	4.0	4.0
L-Phenylalanine	3.5	3.9	3.7	3.7
L-Lysine	4.4	4.1	4.3	4.3
L-Arginine	4.1	3.9	4.2	4.1

Table 7: Recovery Experiments in Complex Matrices

Amino Acid	Spiked Concentration (µM)	Recovered Concentration (µM)	Recovery Rate (%)
L-Alanine	50	48.6	97.2
L-Phenylalanine	50	49.1	98.2
L-Lysine	50	48.8	97.6
L-Arginine	50	49.3	98.6

Table Interpretation: High recovery rates (>97%) indicate the robustness of the method in complex matrices.

Discussion

This study shows that heterocyclic compounds have great potential for use as coupling agents to enhance the UV-visible spectroscopic detection of amino acids. The results are contextualized with respect to broader research in that field and discussed in terms of the implications of these results and areas for further exploration.

It has been found that the amino acids coupled with heterocyclic compounds exhibit great improvement in their UV-visible absorption characteristics. This is in line with previous studies which state that heterocyclic compounds similar to Schiff bases and azo dyes help stabilize electronic transitions leading to improvement of chromophoric properties. For example, the absorbed maxima and molar absorptivity are much better for Schiff base ligands complexed with

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

the nickel ion than reported in this study. In a similar way, a red shift in amino acid derivative absorption maxima after coupling also mimics the results reported by Abdelrehim (2020) which attributed a solvent dependent shift in the heterocyclic azo dye spectra to changes in electronic conjugation.

Mechanistic Insights into Coupling Reactions

The observed enhancement of spectroscopic sensitivity may be due to electron donating and withdrawing effects of substituents on heterocyclic compounds. As shown by Rautela et al. (2023), the amino acids are incorporated into heterocyclic frameworks similar to triazoles and oxadiazoles, leading to the formation of stable chromophores. This phenomenon is most important for UV inactive amino acids such as alanine and lysine, to which there is no intrinsic UV absorption. The results complement findings by Khalid et al. (2021), who pointed out heterocyclic compounds with larger π-conjugation system tend to have narrower HOMO-LUMO energy gaps resulting in better optical properties.

Furthermore, the implication of these findings are that they can be applied diagnostics and pharmaceuticals where precise detection of amino acids is required. Dabhi et al. (2022) have shown that quinoxaline −based heterocycles not only improve DNA binding, but also exhibit antimicrobial properties, which indicate double functionality in therapeutic and analytical applications. These observations are supported by the results of the current study, which show that via the use of heterocyclic coupling agents, the detection limits of biologically relevant compounds are enhanced. This study also demonstrates the potential of these methods for use in high throughput diagnostic assays through an over 60% reduction in the detection limits.

Matrix interference is one of the challenges presented in application of heterocyclic compounds in UV visible spectroscopy. To resolve this issue, Shcherbatykh et al. (2022) used heterocyclic disulfides as derivatizing agents which increased the specificity and lowered the hydrolysis susceptibility in complex biological samples. We demonstrate the robustness of the methodology through recovery rates better than 97% in these model systems, supporting its application in clinically and food safety relevant situations where analytical challenges due to matrix complexity are severe.

Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
Online ISSN: 3007-309X Print ISSN: 3007-3081
https://jmhsr.com/index.php/jmhsr

Future Directions

The promising outcomes still require further research to expand the phase of this methodology. The potential exists for additional heterocyclic frameworks, for example fused ring systems and heterocycles containing sulfur or oxygen, to provide compounds with still more dramatic chromophoric properties. The rational design of novel coupling agents for specific analytical applications could be assisted by the integration of computational techniques such as density functional theory (DFT) (also utilized by Zhao et al., 2021). This study demonstrates considerable implications for application in many different fields, having provided enhanced sensitivity and reduced detection limits. The ability to properly quantify amino acids in complex matrices would yield increased nutritional profiling and quality control in the field of food science. The method could be used for early detection of metabolic disorders and monitoring of therapeutic interventions in clinical diagnostics. In addition, these advancements can serve pharmaceutical industry to use these heterocyclic compounds in quality assurance of amino acid based formulations.

Conclusion

In brief, the investigation shows that heterocyclic compounds are effective at improving the UV-visible spectroscopic detection of amino acids. These experiments demonstrate significant improvements in sensitivity, reproducibility, and applicability, which indicate their potential to make them robust analytical tools. This research improves current methods by addressing existing challenges while also outlining new avenues to be addressed by future research to optimally apply the methods to a variety of scientific and industrial applications.

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Journal of Medical & Health Sciences Review
VOL-2, ISSUE-1, 2025
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