Underivatized Amino Acid Chromatographic Separation: Optimized Conditions for HPLC-UV Simultaneous Quantification of Isoleucine, Leucine, Lysine, Threonine, Histidine, Valine, Methionine, Phenylalanine, Tryptophan, and Tyrosine in Dietary Supplements

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ABSTRACT: Amino acids (AAs) are considered as the building blocks of life. Unlike nonessential AAs, the human body cannot synthesize essential AAs and should be supplied in food or dietary supplements. The aim of the work is simultaneous HPLC-UV determination of 10 structurally related AAs without pre- or postderivatization in powdered dietary supplements (PDSs). This was challenging, especially because PDS has no standardized procedures for its quality control. HPLC-UV chromatograms of the 10 AAs were recorded using a gradient elution of the mobile phase on a CLC-C18 column at room temperature. Good separation was achieved within a 25 min run time without pre- or postderivatization. The method was carefully validated according to the ICH guidelines over the linearity range of 100–200, 50–200, 20–150, 50–400, 20–250, 75–175, 50–250, 50–250, 50–300, and 5–100 μg/mL for l-lysine, l-threonine, l-histidine, l-valine, l-methionine, l-isoleucine, l-leucine, l-tyrosine, l-phenylalanine, and l-tryptophan, respectively, with mean recoveries ranging between 98.91 and 100.77. The method was found to be precise, and the relative standard deviation (RSD) was found to be between 0.28 and 1.92 with recoveries between 97.91 and 101.11. The method was found to be robust that resists deliberate changes in pH, flow rate, and mobile-phase percentages. It was successfully applied for the analysis of PDSs. The proposed method could be very useful for the quality control of the 10 structurally related AAs during their synthesis and for testing raw materials and pharmaceutical preparations.

1. INTRODUCTION

Amino acids (AAs) are considered as the building blocks of life. Proteins are made of 20 AAs, of which only 10 can be synthesized by humans. The others must be supplied in food or through dietary supplements in case of any food digestion problem that prevents its food intake. These essential AAs include leucine, isoleucine, histidine, lysine, valine, methionine, threonine, phenylalanine, and tryptophan.1

Amino acid analysis is a classic fundamental tool in life science that has long been used in different fields of application such as protein analysis, food chemistry, disease diagnosis, physiology, and pharmacology.2 AAs are polar, nonvolatile compounds, zwitterionic in nature, and exhibit a small UV absorbance.3 Traditionally, they have been analyzed using amino acid analyzers with postcolumn derivatization or liquid chromatography with pre- and/or postcolumn derivatization. Most of these methods are validated, interlaboratory studies showed that the derivatization step can represent a source of variability in the obtained results.15 Derivatization may introduce too many errors such as derivative instability, reagent interference, incomplete derivatization, side reactions, long analysis time (approximately 120 min), and additional peaks in chromatograms, more complicated if to be attempted in crude extracts.16 Consequently, the development of quantitative methods for
the determination of AAs in dietary supplements without the use of any derivatization reagents is warranted.

Few methods have been described for the chromatographic determination of underivatized AAs in different samples such as amino acids of plant extracts using liquid chromatography—electrospray ionization—tandem mass spectrometry (LC—MS/MS), amino acids in plasma, urine, and cerebrospinal fluid using LC—MS/MS, and amino acids in serum, plasma, cerebrospinal fluid, and tissue homogenates using ultra-high-performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). However, fewer methods have been reported for the quantification of AAs in nutritional supplements almost using capillary electrophoresis, HILIC-UV, and in parenteral nutritional solutions. There are two challenges in the quantitative determination of underivatized AAs: the first is the separation process as most AAs are very close in polarity and structure (Figure 1), all have weak UV absorbance, and basic, acidic, and neutral (zwitterionic) behaviors. The second limitation lies in the detection technology, but the detector development provides new opportunities in native AA analysis.

In the current work, an easy and relatively fast (30 min analysis time) method was described for the simultaneous quantification and separation of 10 underivatized essential AAs in one chromatographic run to include methionine, histidine, leucine, isoleucine, threonine, lysine, valine, tryptophan, phenylalanine, and tyrosine. They were directly quantified in a dietary supplement using HPLC-UV without the need for either pre- or postcolumn derivatization, which is considered an additional benefit compared to other traditional methods. The method was carefully validated and the obtained results were statistically compared to other published methods to highlight its advantages and limitations.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Solvents. AAs (L-leucine, L-isoleucine, L-histidine, L-lysine, L-methionine, L-valine, L-phenylalanine, L-tyrosine, L-threonine, and L-tryptophan) were purchased from (Sigma-Aldrich Co, Germany). Acetonitrile of HPLC grade was procured (Merck Co., Germany). Bidistilled water was used. Disodium hydrogen phosphate-2-hydrate and 0.817 g of sodium dihydrogen orthophosphate-1-hydrate were weighed and dissolved in 1000 mL of bidistilled water to get a phosphate buffer solution of a pH value of 7.4. A Shim-pack CLC-C18 column (particle size: 5 μm, L × I.D: 150 mm × 4.6 mm) was used for the separation of AAs. An IBM computer loaded with LC solution version 1.2 software (Shimadzu, Kyoto, Japan) was used for data processing.

2.2. Preparation of Standard Solutions. 0.025 g of each amino acid powder (L-leucine, L-isoleucine, L-histidine, L-methionine, L-lysine, L-valine, L-phenylalanine, L-threonine, L-tyrosine, and L-tryptophan) was weighed and placed in a 25 mL volumetric flask, separately. A phosphate buffer (pH 7.4) solution was then added to complete the volumes to obtain standard stock solutions of 1 mg/mL. 3.4 g of disodium hydrogen phosphate-2-hydrate and 0.817 g of sodium dihydrogen orthophosphate-1-hydrate were weighed and dissolved in 1000 mL of bidistilled water to get a phosphate buffer solution of a pH value of 7.4. L-Tryptophan required heating at 70 °C for less than 5 min until complete dissolution due to its low water solubility. Further dilutions were prepared for calibration and application.

2.3. HPLC Device. An HPLC instrument (Shimadzu, Kyoto, Japan) model LC-20AT series equipped with a degasser (DGU-20A), oven (CTO-20A), quaternary pumps (LC-20AD), autosampler (SIL-20A) with a 100 μL volume injection loop, and multiple wavelength detector (SPD-20A) was used. A Shim-pack CLC-C18 column (particle size: 5 μm, L × I.D: 150 mm × 4.6 mm) was used for the separation of AAs. An IBM computer loaded with LC solution version 1.2 software (Shimadzu, Kyoto, Japan) was used for data processing.

2.4. Optimum Chromatographic Conditions for AA Separation. The optimum separation conditions were obtained using a Shim-pack CLC-C18 column (particle size: 5 μm, L × I.D: 150 mm × 4.6 mm). All chromatograms of AAs were recorded using gradient elution of the mobile phase. The elution started with 100% of phosphate buffer (pH 7.4, 10 mM) for 10 min and then a linear increase of the concentration of acetonitrile to reach 50% for another 15 min; the mobile-phase flow was redirected to waste after 30 min and continued for 2 min of each run prior to column re-equilibration to its initial conditions for 5 min. The mobile-phase solution was filtered, degassed, and pumped at a flow rate adjusted to 1 mL/min, with the column maintained at 25 °C. The injection volume was 10 μL and the eluent was monitored using UV detection at 225 nm. Needle washes between each injection using an 80% water—20% acetonitrile...
mixture followed by 20% water–80% acetonitrile were carried out.

2.5. Validation of the HPLC Method. The chromatographic separation was validated following the ICH guidelines.

2.5.1. Construction of Calibration Curves, Linearity, LOD, and LOQ. The calibration profiles were constructed based on the concentrations observed in the dietary supplement under investigation. Different calibration curves were constructed using the standard solutions prepared before. Aliquots (0.05 and 2.5 mL) of each amino acid standard were separately placed into a series of 10 mL volumetric flasks and diluted to the volume using the mobile. Final five-point calibration standards at 100–200, 50–200, 20–150, 50–400, 20–250, 75–175, 50–250, 50–250, 50–300, and 5–100 μg/mL for L-lysine, L-threonine, L-histidine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, and L-tryptophan, respectively, were used. About 10 μL of each sample was measured using the proposed method following the optimum separation conditions.

Then, the obtained peak areas were plotted against the corresponding concentrations, and the regression equations were calculated. The limit of quantification (LOQ) and limit of detection (LOD) were calculated from the signal-to-noise ratio of 10 and 3, respectively.

2.5.2. Accuracy. Method accuracy was checked by employing the proposed method for the quantification of three blind concentrations prepared in the laboratory using the standard AAs purchased from Sigma-Aldrich Co, Germany. The blind concentrations were within the concentrations of the calibration range for each amino acid. The concentrations were calculated from the regression equations.

2.5.3. Precision. The precision method was determined by measuring method repeatability and intermediate precision. Repeatability (intraday precision) was assessed by analyzing three concentrations of each amino acid standard three times under the optimized HPLC conditions within the same day. The three concentrations were within the concentrations of the calibration range for each amino acid. The retention times and peak areas were determined, and the concentrations were calculated using the regression equations. Then, the relative standard deviation (RSD) for each amino acid was determined, while intermediate precision (interday precision) was determined by measuring the same three concentrations for each amino acid over a duration of 3 days. The retention times, peak areas, and RSD of each amino acid concentration were determined.

Figure 2. HPLC chromatogram of 10 AAs separated from the powdered Optaminess tablets. (a) Full chromatogram (30 min) that shows separation of the following AAs: (1) lysine, (2) threonine, (3) histidine, (4) valine, (5) methionine, (6) isoleucine, (7) leucine, (8) tyrosine, (9) phenylalanine, and (10) tryptophan. (b) Zoomed-in area (1) showing the baseline separation of AAs (1, 2, 3, and 4). (c) Zoomed-in area (2) that shows the baseline separation of AAs (6 and 7).
2.5.4. Robustness. Some of the experimental conditions were intentionally changed, and the resolution of amino acid standards was recorded. The flow rate was changed by 0.2 units to evaluate the effect of the flow rate on retention time. The acetonitrile percentage was changed by 0.2 units to evaluate the effect of organic substance strength on separated AA resolution.

2.5.5. Effect of Excipients. The tablet excipients reported in the investigated pharmaceutical preparation label such as maltodextrin, polyvinyl alcohol, starch, magnesium stearate, povidone, and sodium and magnesium salts were assessed at a concentration level of 0.02% (w/v). All these were mixed together and added to the amino acid standard mixture. The prepared solutions were filtered using a 0.45 μm nylon filter and analyzed using the optimum chromatographic conditions mentioned in the Optimum Chromatographic Conditions for AAS Separation section. This experiment was repeated three times.

2.6. Application to the Dietary Supplement “Optaminess”. Each tablet of Optaminess was labeled to contain 135 mg of l-valine, 45 mg of l-histidine, 90 mg of l-leucine, 60 mg of l-isoleucine, 90 mg of l-methionine, 65 mg of l-lysine, 70 mg of l-phenylalanine, 65 mg of l-threonine, 75 mg of l-tyrosine, and 25 mg of l-tryptophan. About 10 tablets were weighed and finely powdered. 0.055 g of the powder was weighed and dissolved in 10 mL of phosphate buffer of pH 7.4 and stirred for 15 min using a magnetic stirring device; then, it was filtered quantitatively into a 25 mL volumetric flask. The remaining residue was washed two times, each with 5 mL of the mobile phase and with volumes completed to the mark with the same solvent. The prepared dosage of the solution was analyzed using optimized conditions stated in Section 2.5 under linearity and with individual AA concentrations calculated using the regression equations of each standard.

3. RESULTS AND DISCUSSION

Up to now, few direct chromatographic separations have been validated for the quantification of AAs without pre- or postcolumn derivatization. Good separation was observed using the proposed HPLC-UV method for the 10 AAs in Optaminess tablets as shown in Figure 2 in a 30 min run time. The chromatographic separation was optimized, validated, and successfully applied for the quantification of l-threonine, l-lysine, l-histidine, l-valine, l-methionine, l-leucine, l-isoleucine, l-tyrosine, and l-tryptophan.

3.1. HPLC Method Optimization. The factors that may affect the simultaneous separation of AAs were determined with the method conditions optimized for the quantification of 10 AAs without the derivatization step. Good separation and resolution of AAs in the pharmaceutical dietary supplement were observed without pre- or post-derivatization steps as shown in system suitability parameters in Table 1. Several mobile phases, columns, and conditions were examined, aiming for method optimization. The chemical and physical characteristics of the column stationary phase (manufacturer, surface area, particle size, particle shape, and pore size) are known to influence the resolution and positions of the separated peaks.

The optimum stationary phase was examined using three columns including Shim-pack C8 (particle size: 5 μm, L × I.D: 250 mm × 4.6 mm, from Shimadzu, Japan), ACE5 C18 (particle size: 5 μm, L × I.D: 250 mm × 4.6 mm, from Advances Chromatography Technologies, Aberdeen, Scotland), and Shim-pack CLC-C18 (Particle size: 5 μm, L × I.D: 150 mm × 4.6 mm, from Shimadzu, Japan). Employing a C8 column, more AAs were retained on the column, although with longer separation time likely attributed to the higher polarity of the C8 column, especially for polar AAs such as histidine, lysine, threonine, and tyrosine. The obtained results in case of ACE 5 C18 columns were better, with most of the AAs well separated except in case of threonine and lysine showing coelution and no clear peak resolution. A satisfactory resolution and selectivity were obtained for all 10 AAs using a Shim-pack CLC-C18 column. There is no specific reason that Shim-pack C18 showed higher performance than ACE5 C18. Both were C18 columns, but the obtained resolution of AA separation and the number of theoretical plates is much greater in Shim-pack C18 than in the other column.

The elution time of AAs was found to be greatly influenced by the column temperature. For example, at higher temperatures (35 and 40 °C), the retention time values decreased, and critical pairs eluted together at nearly the same retention time, while at ambient room temperature best separation was achieved. The column temperature was accordingly set at 25 °C for optimum separation.

In general, AAs have to be derivatized when direct UV detection is selected, except in some reported methods where AAs were present at high levels, allowing for reasonable absorbance. Four wavelengths (200, 210, 225, and 240 nm) were investigated for selection of the optimum wavelength for the separation of AAs. The wavelength at 240 nm was found to be optimum for the separation of the highly conjugated AAs as tryptophan, histidine, methionine, and tyrosine, whereas the remaining AAs were not detected. Obviously, the peak response of the 10 AAs becomes stronger with a decrease in wavelength closer to 200 nm, while at wavelengths of 200 and 210, it was very difficult to obtain a stable baseline separation, especially by using gradient elution. Accordingly, UV detection was measured at 225 nm that revealed good baseline separation of AAs with a stable baseline. AAs are highly affected by pH as it can affect their ionization status. Basic AAs have an amine functional group in their side chain, while acidic AAs exhibit a carboxy group in their side chain with their water solubility being affected by a change in pH as it dissociates into different ionic forms in water: cations, anions, and zwitterions. Optimization of buffer pH is thus considered a key step in AA separation as it plays a fundamental role in achieving optimum resolution and separation, especially for critical pairs such as leucine, lysine, threonine, and tyrosine.
were prepared using different buffer compositions, and their baseline separation of leucine and isoleucine was observed except in the case of leucine, isoleucine, lysine, and threonine. The separation was capable of all AA separation with the acceptable resolution; hence, the effect on AA separation was assessed. Three pH values were employed. The separation of the 10 structurally related amino acids was challenging, and the addition of acetonitrile from the zero time of analysis caused coelution of AAs and bad resolution, so we have proposed a gradient system that warrants for the gradient addition of acetonitrile to be included. The optimum mobile phase for the elution of the 10 AAs isocratic for 10 min, followed by a gradual increase of acetonitrile to 50%, then 60%, and finally 80% for 15 min. These conditions were successfully used for eluting the AAs from the column using phosphate buffer, with only acetonitrile as a mobile phase succeeding to elute it and warranting for the gradient addition of acetonitrile to be employed. The separation of the 10 structurally related amino acids was challenging, and the addition of acetonitrile from the zero time of analysis caused coelution of AAs and bad resolution, so we have proposed a gradient system that depends on using buffer (pH 7.4) for the separation of nine amino acids, followed by the addition of acetonitrile for separation of one amino acid (tryptophan) after 28 min. The chromatographic separation method in which harmful solvents such as acetonitrile, methanol, and isopropanol are replaced with pure water used as the sole component of the mobile phase is green chromatography. Several publications have described that with high water content, the surface of silica becomes nonpolar. Siloxane groups contribute to the hydrophobic character of silica. There are many publications describing the possibility of separation of substances with a wide range of polarity, using pure water at ambient temperature as a mobile phase. The optimum mobile phase for the elution of the 10 AAs was thus a 100% solution of phosphate buffer (10 mM, pH 7.4) for 10 min, followed by a gradual increase of acetonitrile to 50% for 15 min. These conditions were successfully used for the separation of AAs in the following order: lysine, threonine, histidine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, and tryptophan.

### 3.2. Validation of the HPLC Method.

The proposed method for the analysis of AAs was validated for its application in routine analysis and quality control laboratories using the official ICH guidelines in terms of accuracy, linearity, range, precision (mean recovery percent ± RSD)\(^b\), and robustness\(^c\). The linearity for AAs was evaluated by three determinations for each amino acid. The linearity was validated by applying the regression method and calculating the correlation coefficient R\(^2\). The LOD and LOQ were calculated using the following equations:

\[ \text{LOD} = \frac{3.3 \times \text{RSD}}{S} \]

\[ \text{LOQ} = \frac{10 \times \text{RSD}}{S} \]

where \( S \) is the standard deviation of the regression equation.

### Table 2. List of AA Regression Parameters: Retention Times, Calibration Range, \( R^2\), LOD, and LOQ

| Amino acids | Retention times, min | Calibration range (\( \mu g/mL \)) | Regression equation | \( R^2 \) | RSD\(^b\) | LOD, \( \mu g/mL \) | LOQ, \( \mu g/mL \) |
|-------------|----------------------|----------------------------------|---------------------|--------|--------|-----------------|-----------------|
| L-lysine    | 2.63                 | 100–200                          | \( y = 58.28x + 15186 \) | 0.9898 | ±0.93 | 15              | 40              |
| L-threonine | 2.81                 | 50–200                           | \( y = 149.36x + 12038 \) | 0.9839 | ±0.89 | 25              | 26              |
| L-histidine | 3.27                 | 20–150                           | \( y = 13214x - 11991 \) | 0.9971 | ±1.07 | 10              | 17              |
| L-valine    | 3.81                 | 50–400                           | \( y = 81.67x + 10672 \) | 0.9934 | ±0.77 | 45              | 48              |
| L-methionine| 5.26                 | 20–250                           | \( y = 2677.8x + 9674.3 \) | 0.9949 | ±0.53 | 13              | 16              |
| L-isoleucine| 6.69                 | 75–175                           | \( y = 67.11x + 33.5 \)  | 0.9977 | ±0.28 | 7               | 22              |
| L-leucine   | 7.21                 | 50–250                           | \( y = 62.69x + 654.5 \)  | 0.9954 | ±0.54 | 20              | 37              |
| L-tyrosine  | 8.61                 | 50–250                           | \( y = 41830x + 49528 \) | 0.9982 | ±0.40 | 10              | 30              |
| L-phenylalanine | 22.97     | 50–300                           | \( y = 1975.1x - 3087 \) | 0.9996 | ±0.22 | 7               | 22              |
| L-tryptophan| 28.50                | 5–100                            | \( y = 111319x + 38461 \) | 0.9989 | ±1.64 | 4               | 5               |

\(^a\)y = Concentration of the corresponding AAs. \( x \) = Peak area. \(^b\)Average of three determinations for each amino acid. \(^c\)Mean of three determinations for each amino acid.

### Table 3. Chromatographic Method Validation Parameters

| Amino acids | Accuracy\(^d\) (mean ± RSD) | Precision (mean recovery percent ± RSD)\(^e\) | Robustness\(^d\) |
|-------------|-----------------------------|---------------------------------------------|-----------------|
|             | \( y = 58.28x + 15186 \)    | \( y = 149.36x + 12038 \)                  | \( y = 13214x - 11991 \) |
|             | \( y = 67.11x + 33.5 \)     | \( y = 62.69x + 654.5 \)                  | \( y = 41830x + 49528 \) |
|             | \( y = 111319x + 38461 \)   |                                             |                 |

\(^d\)Average of three determinations for each amino acid. \(^e\)Interday and intraday precision, each of \( n = 3 \) and average of three determination.

isoleucine, valine, threonine, tyrosine, and phenylalanine. Mobile phases with different pH values (4.6, 5.6, and 7.4) were prepared using different buffer compositions, and their effect on AA separation was assessed. Three pH values were capable of all AA separation with the acceptable resolution, except in the case of leucine, isoleucine, lysine, and threonine. Baseline separation of leucine and isoleucine was observed at pH values of 5.6 and 7.4, while lysine, threonine, and histidine showed coelution at the same retention time at pH values of 4.6 and 5.6. Adequate separation of all AAs with an acceptable resolution was achieved at a pH value of 7.4 (similar to physiological pH). At such pH, the carboxy group was unprotonated, concurrent with the amino group being protonated and in accordance with the reported pH values for optimized chromatographic separation of some AAs

Different mobile-phase buffers were assessed, with a high concentration of acetate buffer to achieve good separation, albeit to cause precipitation in the system and baseline instability in the chromatogram during gradient elution. Consequently, 20 mM phosphate buffer was tried; nevertheless, poor peak separation and bad resolution were observed. This may be due to an increase in buffer capacity at a high concentration of phosphate buffer. Optimum separation was observed using lower phosphate buffer capacity (10 mM).

It is worth mentioning that nine AAs can be well separated using an isocratic flow of 100% 10 mM phosphate buffer with a flow rate of 1 mL/min at 25 °C. These nine AAs were separated in the following order (lysine, threonine, histidine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine). Tryptophan was the only amino acid that failed to elute from the column using phosphate buffer, with only acetonitrile as a mobile phase succeeding to elute it and warranting for the gradient addition of acetonitrile to be employed.
Table 4. AA Content in Dietary Supplement “Optaminess Tablets” and the Effect of Excipients on the AA Recovery Percent

| Amino acids       | labeled “claimed” μg/mL | obtained results μg/mL | mean recovery % ± RSD | expected tablet excipients a |
|-------------------|-------------------------|------------------------|-----------------------|------------------------------|
| α-lysine          | 90                      | 89.09                  | 98.99 ± 0.88          | 101.31 ± 1.23               |
| α-threonine       | 120                     | 118.34                 | 98.62 ± 1.92          | 98.19 ± 0.89                |
| α-histidine       | 180                     | 177.21                 | 98.45 ± 1.11          | 99.03 ± 0.23                |
| α-valine          | 130                     | 129.95                 | 99.96 ± 0.93          | 100.90 ± 1.82               |
| α-methionine      | 180                     | 178.27                 | 99.04 ± 0.86          | 99.59 ± 0.26                |
| α-isoleucine      | 140                     | 139.47                 | 99.62 ± 0.33          | 99.78 ± 1.69                |
| α-tyrosine        | 130                     | 129.73                 | 99.79 ± 1.23          | 98.56 ± 0.98                |
| α-phenylalanine   | 150                     | 150.20                 | 100.01 ± 1.66         | 98.80 ± 0.63                |
| α-tryptophan      | 270                     | 268.90                 | 99.59 ± 0.63          | 99.50 ± 0.92                |

“Concentration dilution prepared using the labeled concentrations mentioned under "2.1" with the same ratio of the AAs as labeled. aCalculated using regression equations mentioned in Table 1. bAverage of three determinations. cCommon tablet excipients such as maltodextrin, polyvinyl alcohol, magnesium stearate, povidone, starch, sodium, and magnesium salts at a concentration level of 0.02% (w/v) were mixed with the AA standard mixture (number of samples = 3).

It showed the acceptable performance of the method in the presence of all the possible conditions or interactions. For example, the presence of different diluents and excipients may affect the method selectivity toward AA standard detection and separation. Consequently, the analysis of AAs in the presence of spiked excipients was essential to determine their effect on AA recovery. The tablet excipients including maltodextrin, polyvinyl alcohol, magnesium stearate, povidone, starch, sodium, and magnesium salts were used to examine all the possible conditions or interactions. Figure S1 shows the 10 AAs separated in the presence of the excipients. The obtained amino acid recoveries were not affected by the presence of these excipients as in Table 4. These results showed the acceptable performance of the method in the presence of tablet excipients.

3.3. Excipient Effect. The presence of different diluents and excipients may affect the method selectivity toward AA standard detection and separation. Consequently, the analysis of AAs in the presence of spiked excipients was essential to determine their effect on AA recovery. The tablet excipients including maltodextrin, polyvinyl alcohol, magnesium stearate, povidone, starch, sodium, and magnesium salts were used to examine all the possible conditions or interactions. Figure S1 shows the 10 AAs separated in the presence of the excipients. The obtained amino acid recoveries were not affected by the presence of these excipients as in Table 4. These results showed the acceptable performance of the method in the presence of tablet excipients.

3.4. Quantification of AAs in Commercial Dietary Supplements. The established validated method in this study was extended to detect the investigated 10 AAs in Optaminess tablets. These tables contain different concentrations of these AAs (135 mg of valine, 45 mg of histidine, 90 mg of leucine, 60 mg of isoleucine, 90 mg of methionine, 65 mg of lysine, 70 mg of phenylalanine, 65 mg of threonine, 75 mg of tyrosine, and 25 mg of tryptophan) for the treatment of damages due to faulty or deficient protein metabolism in chronic renal insufficiency in connection with limited protein in food. These AAs were found in a combination of being at diverged areas (and the regression equations were calculated. The mean peak concentration listed in Table 2. The LOQ and LOD were determined using the optimum chromatographic conditions, and the regression equations were calculated. The mean peak concentration listed in Table 2. The LOQ and LOD were calculated by the determination of the signal-to-noise ratio (S/N) of 10 and 3, respectively. The obtained results indicate the high sensitivity and precision, robustness, LOQ, and LOD, as shown in Tables 2 and 3.

The calibration plot of each standard amino acid was determined using the optimum chromatographic conditions, and the regression equations were calculated. The mean peak areas (n = 3) were plotted against the corresponding concentration in μg mL⁻¹. The results showed good regression coefficients of 0.9839–0.9996 for all standard AAs over the concentration listed in Table 2. The LOQ and LOD were calculated by the determination of the signal-to-noise ratio (S/N) of 10 and 3, respectively. As shown in Table 2, the calculated LOD ranged from 4.37 to 44.61 μg mL⁻¹, and the calculated LOQ ranged from 13.26 to 135.21 μg mL⁻¹. These results indicate the high sensitivity of the developed method toward AAs.

The accuracy and precision of the proposed method were determined using three concentrations of each AA within the linearity range, measured three times each. Table 3 shows results as mean recovery percent ± RSD. The obtained results indicated that satisfactory accuracy and precision were obtained as the values of RSD did not exceed 2%, with additional mean recoveries within the range of 98–102%. The method’s robustness was estimated by investigating the deliberate change of flow rate and acetonitrile percentile by 0.2 units. The mentioned changes did not remarkably affect the results, indicating the satisfactory performance of the method for implementation in different laboratories.

3.5. Opportunities and Challenges of the Proposed Method. AA analysis is one of the most challenging analytical techniques as most AAs are structurally related, polar, and lack chromophores. The accurate quantitative determination of AAs has been a long historical project with continuous improvements and innovations have been achieved. Several publications have reported AA analysis in foods and biological fluids using GC–MS, capillary electrophoresis, HILIC–MS/MS, ion-paired LC–MS/MS, electrochemical detection (CE), ion exchange chromatography, and HPLC. Most of these methods depend on pre- or postcolumn derivatization to improve the detection sensitivity. However, elimination of derivatization steps attracts many researchers, and many studies have described the analysis of AAs without derivatization steps such as underivatized determination of free AAs in honey using LC–MS/MS, branched-chain AAs in maple syrup urine disease using LC–MS/MS, evaluation embryo viability using CE–MS, urine analysis by LC–MS/MS, ion exchange, CE–MS coupled to a porous layer-gold nanoparticle-modified chiral column, AAs and peptides in nutritive mixtures in the total parenteral nutrition solution, and online coupling of UV, fluorescence, and electrochemical detection. The elimination of the derivatization step is advantageous for many reasons as the selection of an appropriate derivatization reagent is challenging as the reagents differ in their selectivity toward each amino acid; also, additional peaks usually appear in chromatograms, side reactions usually occur, complete derivatization cannot be
4. CONCLUSIONS

In this study, a rapid chromatographic analytical method for the direct simultaneous quantitative determination of 10 AAs in dietary supplements for the application in quality control laboratories was proposed. Ten AAs (L-threonine, L-lysine, L-histidine, L-methionine, L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine, and L-tryptophan) were successfully separated using a C18 column and UV detection at 225 nm without derivatization taking the advantage of the presence of these AAs in high concentrations in the dietary supplements. The HPLC-UV method was carefully optimized, validated, and applied to isolate each amino acid from the mixtures on the same chromatogram within a relatively short duration of 30 min. The percentage recoveries of the AAs were achieved in the range of 98.91–100.77, which were within the acceptance criteria. The percentage RSD was not more than 2%, which proved that the developed method was precise. In comparison to the methods in the literature, the current method showed many advantages such as elimination of the derivatization steps in addition to short time of analysis, high sensitivity, direct determination, elimination of the derivatization steps in addition to short time of analysis, high sensitivity, direct determination, minimum sample preparation steps, simple analytical procedure, and less cost. Moreover, the developed method can be used for quality control of amino acid content in dietary supplements.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03228.

HPLC chromatogram of 10 AAs separated in the presence of the excipients (PDF)

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