

Simultaneous high-performance liquid chromatography analysis of 18 amino acids in special formula milk powder: development and validation

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Abstract: A high-performance liquid chromatography (HPLC) method was developed and validated for the simultaneous determination of 18 amino acids in special formula milk powder. This method was based on pre-column derivatization with phenyl isothiocyanate, separation on a C₁₈ column, ultraviolet detection, and quantification using external standards. Acid hydrolysis using 6 mol/L HCl under autoclaving at 120 °C for 2 h was optimized and compared with conventional oven hydrolysis at 110 °C for 24 h. The results revealed that all 18 amino acids yielded linear correlation coefficients exceeding 0.999. The limits of detection and quantification ranged from 0.07 to 0.32 µg/mL and from 0.23 to 1.07 µg/mL, respectively. The precision, repeatability, and stability were 0.06%–2.44%, 0.32%–2.22%, and 0.19%–2.77%, respectively, and the spiked recoveries ranged between 85.92% and 107.55%. The developed method allowed the analytical run time for all amino acids to be reduced to 35 min. Another advantage was that the proposed method enabled quantification of tryptophan, asparagine, and glutamine, which are not detected by the standard method defined in GB 5009.124–2016 *National food safety standards–determination of amino acids in food*. Overall, the obtained results demonstrate that our method allowed the simultaneous quantification of 18 amino acids in special formula milk powder with improved precision, sensitivity, and efficiency after optimization of the hydrolysis and detection conditions. This method is expected to prove suitable for the quality control of foods for special medical purposes.

Keywords: high-performance liquid chromatography; foods for special medical purposes; amino acids; pre-column derivatization; determination

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1 Introduction

Internationally, the development of foods for special medical purposes (FSMPs) started from infant food, which is first approved by the U.S. Food and Drug Administration (FDA) to be marketed as an orphan drug for the dietary treatment of phenylketonuria. The U.S. FDA further defines the classification of medical nutrition, keeping intravenous medical nutrition in the category of drugs, and nutrients absorbed through the digestive tract in the category of FSMPs^[1]. These products are different from functional foods, focusing on energy provision and nutritional support. FSMPs must be administered under the guidance of a physician or clinical dietitian and are taken alone or in combination with other foods. Among FSMPs used for cow's milk protein allergy in France, cow's milk protein-based extensively hydrolyzed formulas account for 48.4% in volume of the total, followed by formulas based on hydrolyzed rice-protein and free amino acids for 39.3% and 12.3%, respectively^[2]. With the aging of the global population and the incidence of chronic diseases increasing year by year, FSMPs hold great market potential. It was reported that more than 267 million people in China were over the age of 60 in 2021, and the market for

FSMPs in China is estimated to be worth approximately 110 billion yuan^[3]. To date, 92 FSMP products have been approved in China, and a variety of FSMPs containing proteins, short peptides, and amino acids are currently available on the market^[4]. Consequently, it is important to develop appropriate standards and regulations for FSMPs. To guide and regulate the production and sales of FSMPs, China has established a series of manufacturing practice and safety standards for these products since 2010. However, suitable detection standards for the nutritional components in FSMPs specifically tailored to these products have yet to be developed.

The raw materials used in FSMPs are rich in nutrients and should comply with corresponding regulations and standards. There are 19 types of amino acids that can be used in infant formulas for special medical purposes according to GB 25596–2010 *National food safety standard–general rules for infant formula food for special medical purposes*^[5], and the types and contents of amino acids in formula foods for amino acid metabolism disorders are strictly limited as stipulated in GB 29922–2013 *National food safety standards–general rules for food formulas for special medical purposes*^[6]. Conventional milk powder is designed for healthy individuals as a routine nutritional product. It has no special

restrictions on the types or amounts of amino acids and is produced under general food standards. In contrast, FSMPs are specifically formulated for patients with metabolic disorders such as phenylketonuria, cow's milk protein allergy, or amino acid metabolism disorders. The composition and content of amino acids in FSMPs must strictly comply with GB 25596–2010 and GB 29922–2013. Moreover, FSMPs generally contain hydrolyzed proteins or free amino acids instead of intact proteins to improve absorption and reduce allergenicity. These fundamental differences in target population, regulatory requirements, and ingredient composition mean that analytical methods validated for conventional milk powder are not directly applicable to FSMPs. Therefore, developing dedicated detection methods is essential.

Currently, however, no corresponding detection method is specified. The present determination method described in GB 5009.124–2016 *National food safety standards–determination of amino acids in food*^[7] only allows the detection of 16 amino acids, while tryptophan (Trp), asparagine (Asn), and glutamine (Gln) cannot be detected. FSMPs feature a complex matrix and various sample states, and matrix interference can prevent the detection of individual nutrients and lead to inaccurate quantification. Consequently, it is difficult to directly apply amino acid detection methods used for conventional foods or infant formula milk powders to the field of FSMPs. Moreover, there have been few reports specifically investigating the performance characteristics and validation of amino acid determination methods for use with FSMPs. Thus, there is an urgent need to develop methods for the analysis and detection of amino acids in FSMPs.

Currently used amino acid analysis methods mainly include liquid chromatography with tandem mass spectrometry^[8], capillary electrophoresis^[9], automated amino acid analysis^[10], and high-performance liquid chromatography (HPLC)^[11]. Among these, HPLC analysis possesses numerous advantages, such as broad applicability, rapid analysis, high sensitivity, and simple operation^[12]. Moreover, HPLC was chosen for this study because it is cost effective and widely accessible, and it allows simultaneous quantification of 18 amino acids in FSMPs including Trp, Asn, and Gln, which are not covered by GB 5009.124–2016. Amino acid hydrolysis is crucial to the success of the entire process and has a direct impact on the accuracy and precision of the subsequent amino acid analysis. Another important aspect is pre-column derivatization, for which commonly used reagents include 6-aminoquinoline-*N*-hydroxysuccinimidyl carbamate^[13], *o*-phthalaldehyde^[14], phenyl isothiocyanate (PITC)^[15], and dansyl chloride^[16]. PITC provides advantages with respect to sensitivity and time, especially for the detection of proline (Pro) and hydroxyproline. Pre-column derivatization using PITC requires only 10–20 min, after which the excess reagent can be easily removed without affecting the assay sensitivity. A previous report demonstrated that the PITC-amino acid derivatives are generated under mild conditions and the dried samples remain stable for four weeks at 20 °C^[17].

The distinct and complex nature of FSMPs necessitates the development of specific pretreatment and analysis methods. In this study, a procedure for amino acid hydrolysis under autoclaving at 120 °C for 2 h was developed and evaluated in comparison with the conventional method. The obtained results are expected to prove useful for both quality control during manufacturing and government regulation.

2 Materials and methods

2.1 Samples and reagents

Amino acid standards (> 98%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China), including the following 18 amino acids: phenylalanine (Phe), leucine (Leu), isoleucine (Ile), valine (Val), methionine (Met), threonine (Thr), tyrosine (Tyr), lysine (Lys), alanine (Ala), arginine (Arg), aspartic acid (Asp), Asn, glutamic acid (Glu), Gln, glycine (Gly), Pro, serine (Ser), and Trp. PITC, hydrochloric acid (HCl), acetic acid, and sodium acetate were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Triethylamine, phenol, *n*-hexane, and sodium hydroxide (NaOH) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were obtained from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was prepared using a water purification system (Millipore S.A.S, Massachusetts, USA). Unless otherwise stated, all chemicals and reagents were of analytical grade. All standards and materials were stored under appropriate conditions (refrigerator or freezer).

2.2 Preparation of standard amino acid solutions

The 18 amino acids standards were accurately weighed (15 mg each) and dissolved in 0.01 mol/L HCl solution (9.730 mL) to obtain a mixed amino acid solution where each amino acid had a concentration of 1.5 mg/mL; this solution was stored in a brown reagent bottle at –80 °C for use in subsequent experiments. The mixed amino acid solution was diluted as required in subsequent experiments. All of the solutions were mixed well and stored at 4 °C.

2.3 PITC derivatization of amino acids

Amino acid derivatization was performed according to the method described by Hao et al.^[18] with some modifications. Briefly, the amino acid standards (0.8 mL), 0.1 mol/L PITC in acetonitrile (0.4 mL), and 0.1 mol/L triethylamine in acetonitrile (0.4 mL) were mixed in a 4 mL centrifuge tube and shaken well for 5 min, and then kept in the dark at room temperature for 1 h. Next, the reaction mixture was extracted with *n*-hexane (1.6 mL) and shaken vigorously for 5 min then left to stand for 15 min. This afforded an upper *n*-hexane layer containing the remaining PITC and a lower acetonitrile/water layer containing the PITC-derivatized amino acids, where the latter was obtained and passed through a 0.45 µm membrane prior to HPLC analysis.

2.4 Hydrolysis of samples

Two sets of amino acid hydrolysis conditions were investigated, namely, 110 °C for 24 h in an oven and 120 °C for 2 h in an autoclave. Samples of special medical purposes (0.5 g, accurate to 0.000 1 g), purchased from a local market in Jinan, Shandong Province, were weighed into amino acid hydrolysis tubes and 6 mol/L HCl solution containing 1% (V/V) phenol (25 mL) was added. The tubes were then transferred to an oven at 110 °C for 24 h or an autoclave at 120 °C for 2 h. Each reaction was performed in triplicate. The reaction mixtures were allowed to cool to room temperature then diluted to 50 mL with ultrapure water and filtered through filter paper to obtain a clear solution followed by lyophilization. The lyophilized powder was redissolved in ultrapure water (20 mL) and the resulting solution was again filtered through filter paper before adjusting the pH to neutral using 10 mol/L

NaOH solution (200 μ L was found to be necessary). The obtained solutions were diluted fivefold then subjected to PITC derivatization and filtered through a 0.45 μ m membrane prior to HPLC analysis.

2.5 Sample pre-treatment

Samples of special medical purposes were accurately weighed (5 g, accurate to 0.0001 g) and suspended in ultrapure water (50 mL), and the resulting suspensions were magnetically stirred at 500 r/min for 30 min. Next, the samples were centrifuged at $3\,000 \times g$ and 4 °C for 15 min, removing the upper fat layers, and filtered through a double layer of gauze (this process was repeated two more times), then subjected to lyophilization. The resulting samples were subjected to amino acid hydrolysis under autoclaving at 120 °C for 2 h, as described in Section 2.4. The amino acid hydrolysis solutions were diluted twofold then derivatized, filtered, and analyzed by HPLC.

2.6 HPLC conditions

The PITC-derivatized amino acids were analyzed on an Agilent 1100 series HPLC system equipped with a ultraviolet (UV) detector (Tokyo, Japan) and a C_{18} column (250 mm $\times$ 4.6 mm, 5 μ m; item No. 00211-31043, Yuexu Technology Co., Ltd., Shanghai, China). Mobile phase A consisted of 1.26% aqueous sodium acetate trihydrate-acetonitrile (93:7, V/V, pH 6.35). Mobile phase B consisted of acetonitrile-water (8:2, V/V). The following gradient elution procedure was used: 0–3.5 min, 0%–7% B; 3.5–10.8 min, 7%–10% B; 10.8–13.9 min, 10%–12% B; 13.9–14 min, 12%–15% B; 14–29 min, 15%–34% B; 29–32 min, 34%–70% B; 32–35 min, 70%–100% B; 35–42 min, 100% B; 42–45 min, 100%–0% B; and 45–60 min, 0% B. Both mobile phases were filtered through 0.45 μ m membrane filters under vacuum and degassed prior to use. The column temperature was maintained at 30 °C. The wavelength was set to 254 nm, and the analysis time was 60 min. The flow rate and injection volume were 1.0 mL/min and 20 μ L, respectively.

2.7 Data analysis

All experiments were performed in triplicate. The experimental results were recorded and analyzed in Microsoft Excel 2016. Origin 2018 was used for plotting and analysis. The limits of detection (LODs) and limits of quantitation (LOQs) were determined from the chromatographic data based on the signal-to-noise ratio (R_{SN}). The LOD and LOQ were defined as the lowest analyte concentrations producing R_{SN} of approximately 3.3:1 and 10:1, respectively, with the noise measured from the baseline adjacent to the corresponding analyte peak.

3 Results and discussion

3.1 Selection and optimization of hydrolysis conditions

Hydrolysis is an important step in amino acid analysis that directly affects the identification and quantification of the amino acids present in peptide and protein samples^[19]. Three main methods, namely, acidic hydrolysis, alkaline hydrolysis, and oxidative hydrolysis, have been reported^[20–23]. Acidic hydrolysis is generally performed with 6 mol/L HCl at 110 °C for 24 h, but this process tends to partially or completely destroy both Trp and sulfur-containing amino acids (Met and Cys)^[24–26]. In this work, an amino acid hydrolysis method under autoclaving at 120 °C for 2 h was explored using samples of special medical purposes.

As shown in Figure 1, the autoclaving method (120 °C, 2 h) resulted in larger HPLC peak areas for more amino acids compared to the conventional oven method (110 °C, 24 h). Lamp et al.^[27] reported that only half of the 20 proteinogenic amino acids were hydrolyzed without losses during conventional acidic hydrolysis, which occurred because some amino acids are not fully hydrolyzed even after 24 h. It can be concluded from Figure 1 that the vast majority of the 18 amino acids afforded larger peak areas under the proposed autoclave hydrolysis conditions than under the conventional oven conditions. The obtained results indicate that the hydrolysis reaction under autoclaving at 120 °C for 2 h was not only much faster than that under the conventional conditions but also increased the amount of amino acid hydrolysis owing to the effect of raising the water temperature to 120 °C under autoclave pressure. Thus, the proposed amino acid hydrolysis conditions could provide an alternative method to the traditional approach.

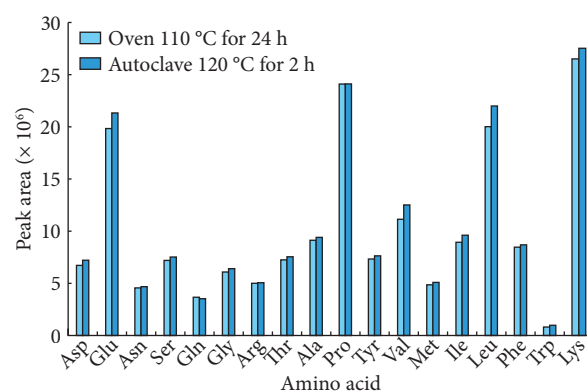


Figure 1 HPLC peak area comparison of 18 amino acids under the two sets of hydrolysis conditions.

Although the proposed hydrolysis method (120 °C, 2 h) improved the recovery of several amino acids, the potential degradation of sensitive amino acids such as Trp, Met, and Cys under acidic and high-temperature conditions remains a concern. Conventional acid hydrolysis (110 °C, 24 h) is known to partially or completely destroy Trp and sulfur-containing amino acids^[24–26]. In the study, Trp and Met were successfully detected with good linearity ($R^2 = 0.999\,9$ for Trp) and acceptable recoveries (107.55% for Trp, 107.25% for Met) using the autoclaving method. The shorter exposure time (2 h vs. 24 h) may limit oxidative degradation. Nevertheless, further validation of the stability and recovery of these sensitive amino acids under the proposed conditions is warranted, and the results should be interpreted with caution when analyzing samples rich in Trp or sulfur-containing amino acids.

3.2 Identification and elution order of PITC-derivatized amino acids

Amino acids react with PITC under basic conditions at room temperature to generate phenylthiocarbamyl (PTC), which can be detected using UV light. The elution sequence of the amino acids present in the mixed reference solution is shown in Figure 2. Baseline separation was achieved for all 18 amino acids based on their retention times (RT), and all of the amino acids eluted within 35 min. Compared with most reported HPLC methods for amino acid determination, the procedure developed in this study achieved higher analysis efficiency with a shorter elution time and a wider analyte coverage. All 18 amino acids were completely eluted within

35 min. For example, Gwatidzo et al.^[28] reported the detection of only 12 amino acids within 40 min, and Shi et al.^[29] detected 16 amino acids within 55 min. Therefore, it can be seen that the method developed in this study has the advantage of high efficiency, that is, the detection time is shorter and more amino acids can be detected.

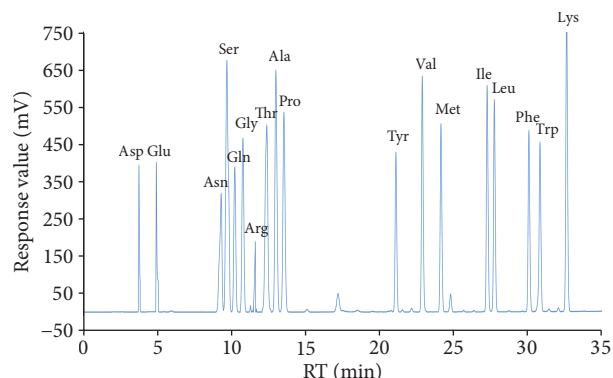


Figure 2 Elution order of the 18 amino acids after PITC derivatization by HPLC.

3.3 Methodological investigation

The parameters used to validate the amino acid detection method established in this study include the specificity, precision, linear equations, LODs, LOQs, repeatability, stability, and spiked recovery.

3.3.1 Specificity

Specificity refers to the ability of an analytical method to accurately detect the target analyte in the presence of other components, such as in the case of a complex sample environment. In this study, the specificity was assessed using a blank (solvent) sample (0.01 mol/L HCl), which was analyzed according to the derivatization procedure described in Section 2.3 and the HPLC conditions described in Section 2.6. The analysis results for the specificity are shown in Figure 3. No peaks that interfered with the determination of the main peaks were observed in the blank sample. This result demonstrates the good specificity of the method and the lack of interference with the HPLC peaks of interest. PITC derivatization followed by HPLC analysis has been shown to be highly specific for most amino acids^[30–32].

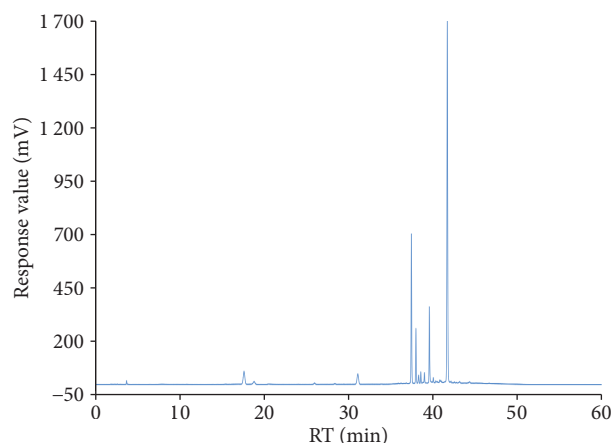


Figure 3 HPLC chromatogram of a blank (solvent) sample after PITC derivatization.

3.3.2 Precision, repeatability, and stability

A series of experiments were performed to evaluate the precision, repeatability, and stability of the proposed method. Precision is defined as the degree of dispersion of data collected from a set of parallel experiments. The amino acid mixed standard solution was injected six times in a single day and the chromatograms were recorded. The results are presented as the relative standard deviation (RSD), as shown in Table 1. The obtained results revealed that the precision for the 18 amino acids was between 0.06% and 2.44%, which are much lower than the values of 1.5%–6.0% reported by Palomino-Vasco et al.^[33] for the fluorescence-based chromatographic detection of amino acids in beer. Thus, the detection method developed in this study exhibited good precision.

Table 1 Precision, repeatability, and stability of the developed method for the 18 amino acids ($n = 6$).

Amino acid	Precision (%)	Repeatability (%)	Stability (%)
Asp	2.44	1.20	0.32
Glu	2.17	0.62	0.32
Asn	2.26	1.48	1.99
Ser	1.09	2.05	1.99
Gln	1.52	2.08	0.78
Gly	0.73	1.89	0.78
Arg	0.94	2.07	1.73
Thr	0.94	0.66	0.67
Ala	1.14	0.38	0.95
Pro	0.36	0.32	0.19
Tyr	0.07	1.50	1.69
Val	0.06	1.40	1.87
Met	0.20	2.07	2.15
Ile	0.13	0.88	1.56
Leu	0.21	1.53	1.57
Phe	0.16	2.22	1.10
Trp	0.21	0.48	2.77
Lys	0.20	0.92	2.24

The repeatability of the method was evaluated by analyzing samples of the same concentration. Finally, the stability was assessed by taking an appropriate amount of sample solution, injected samples at 0, 2, 4, 8, 12, and 24 h. The repeatability and stability of the developed method were assessed by the RSD values of samples of special medical purposes in six replicates. As shown in Table 1, the repeatability values ranged from 0.32% to 2.22%, indicating that the developed method was highly reproducible. Meanwhile, the stability values were in the range of 0.19%–2.77%, demonstrating that the sample solution exhibited good stability over 24 h. The stability RSD for Trp was 2.77%, which was slightly higher than those of the other amino acids, possibly indicating minor degradation over 24 h. Nevertheless, this value remains within acceptable limits ($< 3\%$) for routine analysis. For optimal quantification of Trp, it is recommended that derivatized samples be analyzed within 12 h. On the basis of these results, the developed method showed good performance for the simultaneous identification and quantitative analysis of 18 total amino acids in FSMPs.

3.3.3 Linearity, LOD, LOQ, and spiked recoveries

The linearity of the method was evaluated by analyzing a six-point calibration curve ($n = 6$) obtained from the mixed amino acid standard solutions. The concentrations of the standard solutions were in the range of 1.465–93.75 $\mu\text{g/mL}$. Linear regression equations for the 18 amino acids were calculated using the mass concentration ($\mu\text{g/mL}$) as the abscissa (x) and the peak area as the ordinate (y), and the R^2 was used to assess the linearity. The developed method was found to afford good linear equations, as demonstrated by the R^2 values of the calibration curves summarized in Table 2. The R^2 values were in the range of 0.999 3–0.999 9, all of which were greater than 0.999. Thus, good linear relationships were obtained for all amino acids compared with other studies ($R^2 > 0.995$)^[34–35].

Based on the R_{SN} criteria described above, the LODs ranged from 0.07 to 0.32 $\mu\text{g/mL}$, whereas the LOQs ranged from 0.23 to 1.07 $\mu\text{g/mL}$ for the 18 amino acids (Table 2). Vakili et al.^[36] reported LODs of 0.01–0.28 $\mu\text{g/mL}$ using HPLC with diode-array detection for the analysis of free amino acids in infant formulas and medical foods, which were comparable to the values obtained in the present study. The LOQs obtained herein are also comparable to those reported for pre-column derivatization HPLC methods in previous studies^[37], further confirming that the proposed method is highly sensitive and suitable for quantifying the 18 amino acids in FSMPs.

The average recovery was determined by spiking the samples of special medical purposes with 100 μL of the 93.75 $\mu\text{g/mL}$ mixed amino acid standard, where six replicates were analyzed in parallel on a single day. The samples were then subjected to hydrolysis, derivatization, and HPLC analysis and the average recovery for each of the 18 amino acids was evaluated by the external standard method. As shown in Table 2, the average spiked recoveries of the 18 amino acids ranged from 85.92% to 107.55%, while the corresponding RSDs were between 0.49% and 3.21%. The slightly higher recoveries for Asn, Tyr, Met, and Trp (106.70%–107.55%) may be due to matrix effects or minor co-elution, but are acceptable for complex matrices given that all RSDs were below 3.21%. All of

the results described above were obtained in accordance with the guidelines of the Association of Analytical Communities International^[38]. Taken together, the results demonstrate the successful development of an accurate, effective, and reliable method for the simultaneous detection of 18 total amino acids in FSMPs. The complex matrix of FSMPs may cause matrix effects. However, the spiked recoveries indicate that matrix interference does not significantly compromise accuracy. Therefore, external standard calibration is acceptable for routine analysis of total amino acids in FSMPs.

3.4 Application

The developed method was applied to six different types of amino acid-based medical food formulas to determine the amino acid concentrations in these products. The six tested products were commercially available amino acid-based medical food formulas designed for individuals with various metabolic disorders, such as phenylketonuria, maple syrup urine disease, and cow's milk protein allergy. The protein sources in these formulations comprise either free amino acid mixtures or extensively hydrolyzed whey or casein proteins. According to the product labels, the degree of hydrolysis (where applicable) exceeded 90%, and the total protein mass fraction ranged from 10%–15%. The declared amino acid profiles varied among products, reflecting different therapeutic targets.

The variety and concentrations of the detected amino acids are listed in Table 3. The results showed that the measured amino acid concentrations in the FSMPs were consistent with those indicated on the product labels (data not shown). The proportion of essential amino acids ranged from 36.72% to 46.23% of the total amino acid content, indicating relatively high variation. The variations in the amounts of amino acids may be attributable to the different functions of the medical foods. Notably, the Trp content varied markedly across the six products (0.73–16.16 mg/g prot), reflecting differences in formulation and protein source. Accurate Trp quantification is important because Trp is an essential amino acid and a precursor of serotonin and niacin, key regulators of neurological and metabolic health.

Table 2 Linear equations, coefficients of determination, LODs, LOQs, and spiked recoveries for the 18 amino acids ($n = 6$).

Amino acid	Linear equation	R^2	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Average recovery (%)	RSD (%)
Asp	$y = 459\,227x + 67.862$	0.999 8	0.15	0.50	94.67	1.05
Glu	$y = 478\,661x - 80.910$	0.999 3	0.15	0.50	90.87	1.05
Asn	$y = 875\,413x - 189.560$	0.999 6	0.19	0.63	106.70	2.00
Ser	$y = 831\,067x + 51.194$	0.999 5	0.09	0.30	85.92	2.00
Gln	$y = 531\,164x - 158.520$	0.999 3	0.15	0.50	86.60	1.17
Gly	$y = 661\,445x - 355.530$	0.999 3	0.13	0.43	92.83	1.17
Arg	$y = 445\,024x - 39.716$	0.999 6	0.32	1.07	92.83	2.80
Thr	$y = 608\,138x + 150.080$	0.999 6	0.12	0.40	100.98	1.08
Ala	$y = 999\,911x - 543.210$	0.999 5	0.09	0.30	92.66	0.62
Pro	$y = 839\,103x + 442.250$	0.999 6	0.11	0.37	93.00	0.49
Tyr	$y = 505\,873x - 181.730$	0.999 5	0.14	0.47	107.08	3.21
Val	$y = 781\,435x - 111.220$	0.999 6	0.09	0.30	94.92	1.52
Met	$y = 638\,510x - 301.200$	0.999 4	0.12	0.40	107.25	2.41
Ile	$y = 741\,052x - 200.180$	0.999 6	0.10	0.33	106.19	1.47
Leu	$y = 703\,584x + 283.830$	0.999 6	0.11	0.37	106.27	1.79
Phe	$y = 593\,875x - 138.140$	0.999 5	0.12	0.40	107.17	2.89
Trp	$y = 517\,592x + 8\,731.400$	0.999 9	0.13	0.43	107.55	1.46
Lys	$y = 103\,234x - 68.277$	0.999 4	0.07	0.23	91.04	1.13

Table 3 Concentrations (mg/g prot) of the 18 amino acids in the six amino acid-based medical food formulas obtained using the proposed HPLC method.

Amino acid	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Asp	105.01 ± 0.09	121.16 ± 0.12	137.32 ± 0.14	153.47 ± 0.13	111.16 ± 0.11	105.01 ± 0.09
Glu	193.86 ± 0.15	226.17 ± 0.13	258.48 ± 0.12	282.71 ± 0.16	204.25 ± 0.08	201.94 ± 0.07
Asn	24.23 ± 0.01	24.23 ± 0.01	32.31 ± 0.01	40.39 ± 0.02	32.31 ± 0.02	25.04 ± 0.01
Ser	24.23 ± 0.02	32.31 ± 0.02	48.47 ± 0.03	36.54 ± 0.02	30.39 ± 0.01	33.93 ± 0.02
Gln	32.31 ± 0.03	30.39 ± 0.02	36.54 ± 0.02	42.70 ± 0.03	36.54 ± 0.02	44.43 ± 0.03
Gly	24.23 ± 0.01	22.31 ± 0.01	28.47 ± 0.02	34.62 ± 0.02	28.47 ± 0.02	37.16 ± 0.03
Arg	32.31 ± 0.02	30.39 ± 0.01	36.54 ± 0.02	40.78 ± 0.03	44.62 ± 0.03	46.04 ± 0.03
Thr	62.70 ± 0.05	62.70 ± 0.04	56.54 ± 0.03	61.16 ± 0.04	65.01 ± 0.04	88.85 ± 0.05
Ala	48.47 ± 0.04	38.47 ± 0.03	36.54 ± 0.03	56.54 ± 0.04	48.47 ± 0.03	46.04 ± 0.03
Pro	88.85 ± 0.06	91.16 ± 0.05	91.16 ± 0.05	142.33 ± 0.08	129.63 ± 0.06	129.24 ± 0.06
Tyr	40.39 ± 0.05	38.47 ± 0.03	36.54 ± 0.02	48.85 ± 0.03	44.62 ± 0.02	50.89 ± 0.01
Val	56.54 ± 0.04	54.62 ± 0.06	60.78 ± 0.08	73.47 ± 0.07	61.16 ± 0.06	96.93 ± 0.08
Met	24.23 ± 0.01	22.31 ± 0.01	24.23 ± 0.02	28.47 ± 0.01	30.39 ± 0.03	30.69 ± 0.03
Ile	46.54 ± 0.02	46.54 ± 0.04	48.47 ± 0.04	53.09 ± 0.05	48.85 ± 0.03	79.97 ± 0.06
Leu	113.09 ± 0.08	111.16 ± 0.11	119.24 ± 0.15	118.09 ± 0.12	119.63 ± 0.10	137.32 ± 0.16
Phe	40.39 ± 0.03	46.54 ± 0.05	36.54 ± 0.04	61.16 ± 0.06	40.78 ± 0.04	59.77 ± 0.05
Trp	1.62 ± 0.01	0.73 ± 0.01	1.62 ± 0.02	0.81 ± 0.01	16.16 ± 0.02	14.54 ± 0.02
Lys	88.85 ± 0.04	95.01 ± 0.07	95.01 ± 0.11	93.86 ± 0.13	105.40 ± 0.12	101.16 ± 0.11
Total	1 047.85	1 094.67	1 185.00	1 369.00	1 198.00	1 329.00

4 Conclusions

In this study, an alternative method for amino acid acidic hydrolysis under autoclaving conditions was explored, which shortened the hydrolysis time from 24 to 2 h and improved the detection efficiency. The proposed method for the detection of amino acids in FSMPs exhibited high sensitivity, accuracy, repeatability, stability, and convenient operation and is expected to prove useful for simultaneous determination of the contents of 18 amino acids in FSMPs. In particular, the developed method allows quantification of Trp, Asn, and Gln, which are not detected by the method specified in GB 5009.124–2016. The obtained results should help overcome the current lack of standardized amino acid detection techniques suitable for use with FSMPs, which is expected to be invaluable for ensuring the nutritional qualities and safety of FSMPs and protecting the interests of consumers.

Conflict of interest

The authors report there are no competing interests to declare.

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Author contributions

Zhimeng Yuan: Formal analysis, writing-original draft, and writing-review & editing. Fuhao Du: Investigation, methodology. Xinrui Wang: Conceptualization, formal analysis, and data curation. Junfeng Dong: Resources and investigation. Xiaoning Zhang: Data curation and writing-review & editing. Cunfang Wang: Funding acquisition, conceptualization, and supervision.

Data availability

The data that support this article are available from the authors upon reasonable request.

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