Simultaneous Determination of 34 Amino Acids in Tumor Tissues from Colorectal Cancer Patients Based on the Targeted UHPLC-MS/MS Method

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A targeted ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was established and validated for the simultaneous determination of 34 amino acids in tissue samples from colorectal cancer (CRC) patients. The chromatographic separation was achieved on an Agilent ZORBAX SB-C18 column (3.0 × 150 mm, 5 μm) with a binary gradient elution system (A, 0.02% heptafluorobutyric acid and 0.2% formic acid in water, v/v; B, methanol). The run time was 10 min. The multiple reaction monitoring mode was chosen with an electrospray ionization source operating in the positive ionization mode for data acquisition. The linear correlation coefficients were >0.99 for all the analytes in their corresponding calibration ranges. The sample was pretreated based on tissue homogenate and protein precipitation with a 100 mg aliquot sample. The average recovery and matrix effect for 34 amino acids and 3 internal standards were 39.00%∼146.95% and 49.45%∼173.63%, respectively. The intra- and interday accuracy for all the analytes ranged from −13.52% to 14.21% (RSD ≤8.57%) and from −14.52% to 12.59% (RSD ≤10.31%), respectively. Deviations of stability under different conditions were within ±15% for all the analytes. This method was applied to simultaneous quantification of 34 amino acids in tissue samples from 94 CRC patients.

1. Introduction

Amino acids play an essential role in both metabolism and proteome. Proper amino acid management is critical for maintaining cell function and organismal viability, particularly under metabolic disturbance. With the alterations in tumor cell metabolism recognized as a hallmark of cancer, there have been more research studies on amino acids, which are involved in the primary tumor formation, growth, and metabolism. As shown in Table 1 [1–18], studies in the field of colorectal cancer (CRC) metabolism were routinely performed to elucidate the difference of amino acid profiles in blood, urine, and feces between CRC patients and healthy volunteers or between CRC tissue and normal tissue. Recent findings are related to (1) glycemic and/or ketogenic amino acids that are involved in the energy metabolism by entering tricarboxylic acid cycle pathway; (2) amino acids that are synthetic precursors of pyrimidine bases or provide nitrogen sources for the synthesis of purine bases; and (3) amino acids that are the decomposition products from cytosine, uracil, and thymine nucleotides and that can reflect the severity of malignant tumor and the therapeutic effect of chemotherapy for malignant tumor [19]. To sum up, details about the association of amino acids with the formation, growth, and
metabolism of CRC are presented in Figure 1. Besides, as a higher level of sarcosine was observed in the urine of patients with prostate cancer [20–22], sarcosine was believed to be closely related to cancer and also investigated in our study.

Despite the overlap between the results of the targeted and nontargeted metabolomic assays, there were also substantial inconsistencies. Whenever the assessment of a specific pathway such as amino acids became the focus of interest, a targeted metabolomic assay would seem preferable to a nontargeted one [23]. Generally, gas chromatography-mass spectrometry (GC-MS) was available for the amino acid analysis in some laboratories [24–26], but it required a derivatization step for nonvolatile analytes, which was important for the analyte detection and quantification, but could be avoided when a liquid chromatography-mass spectrometry (LC-MS) method was adopted. Amino acids, as a group of polar chemical compounds, should also be modified by using a derivatization reagent to make them volatile and thus accessible for GC-MS [27]. Over the past two decades, many researchers had described the ultrahigh-performance liquid chromatography-tandem mass spectrometry- (UHPLC-MS/MS-) based method for a targeted metabolomic study with increasing levels of sophistication [28–30]. Out of these considerations, thirty-four endogenous amino acids in human biological samples were involved in our study by using the LC-MS technique.

Amino acid metabolomics in human serum has been studied on a larger scale due to its potential diagnostic value in patients with breast, lung, ovarian, head and neck, gastric, and pancreatic cancers or in CRC patients [31], suggesting that the amino acid profiling in plasma/serum or in other body fluids or selected tissue samples might be a new tool for early diagnosis of cancers [32]. As different cancer subtypes show distinct metabolic phenotypes, the present paper aimed to evaluate the amino acid profile in clinical samples from CRC patients by means of a targeted metabolomic assay based on a UHPLC-MS/MS method, which was established and validated for the quantitative analysis of 34 amino acids in tumor tissues. So far, no research has ever evaluated the ability of the amino acid metabolic phenotype of CRC patients. Additionally, this was the first experiment to show the amino acid profile differences between cancerous, paracancerous, and normal tissue of CRC patients, which may shed light on the metabolic stability of amino acids in humans and provide biological information to find and evaluate a new diagnostic tool for the further study of CRC.

### 2. Materials and Methods

#### 2.1. Chemicals and Reagents

Thirty-four amino acids, including glycine (Gly), L-alanine (Ala), L-serine (Ser), L-valine (Val), L-threonine (Thr), L-leucine (Leu), L-isoleucine (Ile), L-aspartic acid (Asp), L-lysine (Lys), L-glutamine (Gln), L-glutamic acid (Glu), L-phenylalanine (Phe), L-arginine (Arg), L-tyrosine (Tyr), L-proline (Pro), L-asparagine (Asn), L-methionine (Met), L-tryptophan (Trp), L-cysteine (Cys), L-histidine (His), L-citrulline (Cit), asymmetric dimethylarginine (ADMA), L-cystine (Cys), sarcosine (Sar), β-alanine (3-aminopropanoic acid, Apa), β-aminoisobutyric acid (3-amino-2-methylpropanoic acid, Amp), γ-aminobutyric acid (4-aminobutyric acid, Aba), 5-oxo-L-proline (Opr), 4-hydroxy-L-proline (Hpr), L-ornithine (Orn), 2-amino-L-hexanoid acid (α-aminoacidic acid, Ahd), hippuric acid (Hia), symmetric dimethylarginine (SDMA), and L-kynurenine (Kyn) were purchased from the National Institutes for Food and Drug Control of China (Beijing, China), Dalian Meilun Biotech Co., Ltd. (Dalian, China), Shanghai Yuanye Biotech Co., Ltd. (Shanghai, China), and Sigma-Aldrich LLC. (Darmstadt, Germany). L-Alanine-d4 (Ala-d4), L-methionine-d3 (Met-d3), and L-phenylalanine-d5 (Phe-d5) were chosen as internal standards (ISs), which were all provided by Toronto Research Chemicals Inc., (Toronto, Canada). The information about the 34 standards and 3 ISs is shown in Supplementary Material Table S1.

Methanol and acetonitrile of HPLC grade were purchased from Merck Inc., (Darmstadt, Germany). Phosphate-buffered solution (PBS) (10x, namely, 0.1 mol/L) of the cell culture grade (Lot. MA0016-May-19B) was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China).

| Amino acids | Variations | References |
|-------------|------------|-----------|
| Gly         | ↓          | [1, 2]    |
| Ala         | ↑          | [3–5]     |
| Ser         | ↑          | [3, 6, 7] |
| Val         | ↓          | [2, 6, 7] |
| Thr         | ↓          | [2]       |
| Leu         | ↑          | [3, 5–7]  |
| Ile         | ↑          | [6–8]     |
| Asp         | ↑↑         | [3, 4, 6, 7, 9] |
| Gln         | ↑          | [3, 8]    |
| Glu         | ↑          | [4, 8, 9] |
| Phe         | ↑          | [3, 10]   |
| Arg         | ↑          | [6, 7]    |
| Tyr         | ↑          | [3, 10]   |
| Pro         | ↑          | [3, 11, 12] |
| Met         | ↓          | [5–7]     |
| Trp         | ↑          | [3]       |
| Cys         | ↑          | [4, 11]   |
| His         | ↓          | [3, 6, 7] |
| Cit         | ↑          | [3]       |
| ADMA        | ↑          | [13–15]   |
| Cyss        | ↑          | [9]       |
| Aba         | ↑↑         | [16, 17]  |
| Opr         | ↑          | [4]       |
| Hpr         | ↓          | [3]       |
| Orn         | ↑          | [3]       |
| Ahd         | ↓          | [5]       |
| Hia         | ↑↓         | [5, 18]   |
| SDMA        | ↑          | [14, 15]  |
| Kyn         | ↓          | [9, 18]   |

*Note.* *CRC patients vs. healthy volunteers; ↑: increase; ↓: decrease; ↑↓: increase and decrease. *The cancerous tissue of CRC patients vs. the normal tissue of CRC patients.*
Heptafluorobutyric acid (HFBA) (Lot. P11933) was obtained from Adamas Reagents Co., Ltd., (Basel, Switzerland). Formic acid (FA) (Lot. C10009619) was gained from Shanghai Macklin Biochemical Co., Ltd., (Shanghai, China). Hydrochloric acid (HCl) of analytical grade was the product of Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Sodium chloride injection (0.9% saline, 1000 ml/bag, Lot. 160613) was produced by the pharmaceutical preparation factory of Shanghai Changzheng Hospital, the Second Military Medical University (Shanghai, China). Water was deionized and further purified by a Milli-Q Plus water purification system (Darmstadt, Germany) in our laboratory. The other reagents and solvents were of analytical grade.

2.2. Liquid Chromatography and Mass Spectrometry Conditions. An Agilent 1290 UHPLC coupled to an Agilent 6460 triple-quadrupole tandem mass spectrometer (Agilent Inc, USA) was used to establish the method. Chromatographic separation was performed on an Agilent ZORBAX SB-C<sub>18</sub> column (3.0 x 150 mm, 5 μm), whose temperature was maintained at 50°C. Binary gradient elution was used by mixing the mobile phase A (0.02% HFBA and 0.2% FA in water, v/v) and B (methanol) at a flow rate at 0.4 mL/min. The mobile phase elution procedure was as follows (A/B, v/v): 0 min, 98:2; 1 min, 85:15; 4 min, 85:15; 5 min, 80:20; and 10 min, 20:80. The run time was 10 min for each sample, and the post time was set at 4.0 min to equilibrate the column pressure. The autosampler temperature was maintained at 4°C. The injected volume was 2 μL with needle wash.

The ionization of analytes was performed based on an electrospray ionization (ESI) source under the positive ionization mode, and the data acquisition was carried out in a multiple reaction monitoring (MRM) mode. Optimized mass spectrometer conditions were as follows: capillary voltage, 5.0 kV; dwell time, 40 ms; collision gas (high-purity nitrogen) pressure, 0.2 MPa; and nebulizer gas (nitrogen) pressure, 50 psi. The dry gas temperature was 325°C and...
delivered at 10 L/min. The sheath gas temperature was 350°C at the flow rate of 12 L/min.

All data were acquired and processed using Agilent Mass Hunter workstation software (version B.07.00). The optimized MRM parameters of 34 amino acids and 3 ISs are summarized in Table 2.

2.3. Preparation of Calibration Standards and Quality Control Samples. Stock solutions (2.5 mg/mL) for each analyte were prepared separately and stored at −80°C. Gly, Ala, Ser, Val, Thr, Leu, Ile, Lys, Phe, Arg, Pro, Met, His, Cit, Sar, Apa, Amp, Aba, Opr, Hpr, Orn, Hia, and Kyn were dissolved in 5% methanol aqueous solutions, and Glu, Glu, and Trp were prepared in 0.2% FA aqueous solutions. Asp, Tyr, Asn, Cys, Cys, Ahd, ADMA, and SDMA were in 4% HCl aqueous solutions. Stock solutions were further diluted with 5% methanol aqueous solution to obtain the following 4 groups of working solutions. Group A included Ala, Val, Thr, Leu, Ile, Lys, Glu, Phe, Arg, and Tyr (250 μg/mL for every analyte). Group B included Gly, Ser, Asp, and Glu (250 μg/mL for every analyte) and Pro, Asn, Met, and Trp (125 μg/mL for every analyte). Group C included Cys, His, Cit, ADMA, and Cys (250 μg/mL for every analyte). Group D included Sar, Apa, Amp, Aba, Opr, Hpr, Orn, Ahd, Hia, SDMA, and Kyn (125 μg/mL for each analyte).

The highest calibration standard solution was prepared by adding appropriate volumes of working solutions group A–D into PBS (Ix, namely, 0.01 mol/L) using previously reported methods [33–35]. Then, the other 8 calibration standard solutions were obtained by diluting the highest calibration standard solution with PBS. The final concentrations of calibration standard solutions were 1000, 2000, 4000, 8000, 10000, 20000, 40000, 60000, and 80000 ng/mL for Gly, Ala, Ser, Val, Thr, Leu, Ile, Asp, Lys, Glu, Glu, Phe, Arg, and Tyr; 500, 1000, 2000, 4000, 5000, 10000, 20000, 30000, and 400000 ng/mL for Pro, Asn, Met, and Trp; 100, 200, 400, 800, 1000, 2000, 4000, 6000, and 80000 ng/mL for Cys, His, Cit, ADMA, and Cys; and 50, 100, 200, 400, 500, 1000, 2000, 3000, and 4000 ng/mL for Sar, Amp, Aba, Opr, Hpr, Orn, Ahd, Hia, SDMA, and Kyn, respectively.

Quality control (QC) samples were also separately prepared in the same way and at low, medium, and high concentrations (QC1~3). The low, medium, and high concentrations of the QC samples were 2000, 10000, and 60000 ng/mL for Gly, Ala, Ser, Val, Thr, Leu, Ile, Asp, Lys, Glu, Glu, Phe, Arg, and Tyr; 1000, 5000, and 30000 ng/mL for Pro, Asn, Met, and Trp; 100, 200, 400, 800, 1000, 2000, 4000, 6000, and 80000 ng/mL for Cys, His, Cit, ADMA, and Cys; and 100, 500, and 3000 ng/mL for Sar, Amp, Aba, Opr, Hpr, Orn, Ahd, Hia, SDMA, and Kyn, respectively. All solutions were stored at −20°C.

2.4. Sample Pretreatment. Each tissue sample with a mass of about 100 mg was precisely weighed and added with a 5-fold mass of 0.9% saline before being homogenized by a superfine homogenizer at 15000 r/min for 2 min in the ice water bath to obtain tissue homogenate, and then the mixture was centrifuged for 15 min at 19060 × g at 4°C for five minutes of ultrasonic treatment in the ice water bath. Then, a 50 μL aliquot of the supernatant was transferred to a micro-centrifuge tube, and 150 μL of 0.2% FA acetonitrile solution (containing 400 ng/mL ISs) was added. The mixture was centrifuged again under the same conditions after being rested for 3 min and vortex-mixed for 2 min, and 2 μL of supernatant was injected into the UHPLC-MS/MS system for analysis.

2.5. Method Validation. Method validation was performed according to Chinese Pharmacopoeia [36] and US Food and Drug Administration (FDA) guidance [37] and with reference to our previous report [38]. The selectivity was evaluated by comparing six different batches of the blank matrix to the corresponding spiked samples, and the responses of interferents in the blank matrix less than 20% of the low limit of quantitation (LLOQ) samples and 5% of ISs were considered acceptable.

The calibration standards were prepared in triplicates and measured three times on different days (at least 2 days). The calibration curve was regressed from the IS-adjusted peak area versus the nominal concentration under a 1/X^2 weighting factor. LLOQ was defined as the lowest concentration point of the calibration curve. A deviation of backcalculation for each calibration standard within ±15% was thought to be acceptable, and for LLOQ, the deviation should be within ±20%.

The recovery and matrix effect were assessed by preparing six replicates of the QC sample at low and high concentration levels. The matrix effect was the ratio of the peak area in the spiked postextraction samples to solvent-substituted samples at the same concentration, and the recovery was the ratio of the peak area in the spiked samples to spiked postextraction samples at the same concentration.

The intra- and interday accuracy and precision were assessed using the QC samples at LLOQ, low, medium, and high concentration levels (n = 5). Samples were analyzed in three analytical lots on separate days (at least 2 days), and the relative standard deviation (RSD) % for inter- and intraday precision not more than 15% was regarded as acceptable (for LLOQ, not more than 20%). For intra- and interday accuracy, the relative error (RE) % within ±15% (for LLOQ, within ±20%) was considered reasonable.

The stability of each analyte was assessed at three concentration levels (low, medium, and high) using the QC samples (n = 3) under four different conditions: room temperature stability was evaluated after exposing samples at room temperature for 6 h; three freeze-thaw cycles stability was evaluated after freeze and thaw of samples from −20°C to room temperature three times; short-term stability was assessed by analyzing samples kept in the autosampler (4°C) for 24 h; and long-term stability was evaluated after the samples were stored at −20°C for 90 days.

The dilution effect of all the analytes was assessed by diluting the sample with a blank matrix into the calibration range and comparing the measured concentrations to the nominal concentrations. Each dilution factor should be assessed at least five times, and the RSD% and RE% should be less than 15% and within ±15%, respectively.
2.6. Study Population and Sample Collection. The experimental protocol was reviewed and approved by the Ethical Committee of Changzheng Hospital prior to specimen collection, and it was conducted in accordance with the Helsinki Declaration of 1964, as revised in 2013, and according to regulatory guidance. Informed consent was obtained from all participants enrolled in this study.

Between July 2016 and December 2017, 94 patients (male 56, female 38) with CRC were enrolled from Changzheng Hospital. None of the patients received neoadjuvant treatment. The median age of these patients was 60 (ranging from 32 to 87). 10 patients were diagnosed with stage I CRC, 33 patients with stage II, 45 patients with stage III, and 6 patients with stage IV. The demographic and clinical chemistry characteristics of these CRC patients are shown in Table 3.

The sample set including cancerous, paracancerous, and normal tissue samples was collected from each of these CRC patients and was named Tc, Tp, and Tn, respectively. All samples were immediately washed using 0.9% icy saline solution, and the surfaces were subsequently dried by the filter tissue. The samples were stored at −80°C within cryotubes until analysis.

2.7. Data Analysis. Data were analyzed statistically, and graphs were generated by GraphPad Prism 6.01 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). A nonparametric test (Friedman test) was performed to compare the content differences of 34 amino acids between sample sets. The p value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Method Development. Many studies have published the quantitative analyses of amino acids in plasma [23, 24, 28, 31, 32], but these methods have never been applied in tissue homogenate. In this study, we developed a robust method for the quantitative analysis of underivatized amino acids in human tissue by UHPLC-MS/MS.

| Table 2: The optimized MRM parameters of 34 amino acids and 3 ISs (ESI positive). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Analyte | Molecular weight | Precursor ion | Product ion | Fragmentor (V) | Collision energy (eV) |
|---------|-----------------|----------------|----------------|-----------------|-----------------|
| Gly     | 75.07           | 76              | 30             | 50              | 13              |
| Ala     | 89.09           | 90              | 44             | 50              | 8               |
| Ser     | 105.09          | 106             | 60             | 65              | 9               |
| Val     | 117.15          | 118             | 72             | 60              | 7               |
| Thr     | 119.12          | 120             | 74             | 65              | 9               |
| Leu     | 131.17          | 132             | 86             | 65              | 7               |
| Ile     | 131.17          | 132             | 86             | 65              | 7               |
| Asp     | 133.10          | 134             | 74             | 65              | 12              |
| Lys     | 146.19          | 147             | 84             | 70              | 11              |
| Gln     | 146.14          | 147             | 84             | 65              | 12              |
| Glu     | 147.13          | 148             | 84             | 70              | 12              |
| Phe     | 165.19          | 166             | 120            | 65              | 10              |
| Arg     | 174.20          | 175             | 70             | 90              | 16              |
| Tyr     | 181.19          | 182             | 136            | 70              | 11              |
| Pro     | 115.13          | 116             | 70             | 70              | 15              |
| Asn     | 132.12          | 133             | 74             | 60              | 11              |
| Met     | 149.21          | 150             | 56             | 65              | 13              |
| Trp     | 204.23          | 205             | 188            | 70              | 5               |
| Cys     | 212.16          | 122             | 59             | 60              | 21              |
| His     | 155.15          | 156             | 110            | 80              | 13              |
| Cit     | 175.19          | 176             | 159            | 70              | 7               |
| ADMA    | 202.25          | 203             | 46             | 90              | 16              |
| Cyss    | 240.30          | 241             | 152            | 80              | 12              |
| Sar     | 89.09           | 90              | 44             | 55              | 10              |
| Apa     | 89.09           | 90              | 30             | 60              | 12              |
| Amp     | 103.12          | 104             | 30             | 60              | 11              |
| Aba     | 103.12          | 104             | 87             | 65              | 9               |
| Opr     | 129.11          | 130             | 84             | 75              | 12              |
| Hpr     | 131.13          | 132             | 86             | 75              | 13              |
| Orn     | 132.16          | 133             | 70             | 65              | 10              |
| Ahd     | 161.16          | 162             | 98             | 65              | 11              |
| Hia     | 179.17          | 180             | 105            | 65              | 6               |
| SDMA    | 202.25          | 203             | 172            | 90              | 14              |
| Kyn     | 208.21          | 209             | 192            | 70              | 7               |
| Ala-2nd | 93.12           | 94              | 48             | 50              | 9               |
| Met-d3  | 152.23          | 153             | 56             | 65              | 13              |
| Phe-d5  | 170.22          | 171             | 125            | 70              | 11              |
high ionization efficiency were obtained in the positive ionization mode for all the analytes.

The selection of an appropriate matrix for calibration samples and the QC samples preparation was an important part of methodological development when LC-MS was used for the quantitative analysis of endogenous compounds in biological samples. There were two main approaches to this problem: the first was to dissolve alternative analytes in the real matrix and the other was to use real analytes in an alternative matrix [35]. The ideal substitutive matrix should be completely analyte-free and identical to the real matrix in terms of analyte solubility and extractability, but it was unpractical for the detection of endogenous compounds. In our experiment, plasma was processed using neutral decolorizing carbon for stripping some endogenous carbohydrates [40]. This approach was effective for carbohydrates but not for all amino acids because the prepared plasma still contained a high concentration of amino acids. Therefore, the calibration and the QC samples could be prepared in an artificial matrix only as it was impossible to make an “analyte-free” matrix. As human biofluid usually contains a variety of proteins, fatty acids, and electrolytes, which is hard for simulation, some research studies documented that the PBS [33, 34] or mobile phase [41] could be treated as the matrix when the calibration and the QC samples were prepared. In addition, the pH value, osmotic pressure, and ion concentration of PBS were closer to those of the biofluid of humans than the mobile phase [35]. As such, PBS was applied as the “mimic tissue fluid” to prepare both the calibration and the QC samples.

When it came to optimization of sample preparation, treatment of the tissue homogenate commonly involved protein precipitation (PPT), liquid-liquid extraction, and solid phase extraction. The PPT method was considered the best method in that it was user-friendly, inexpensive, and suitable for high-throughput biological sample pretreatment. It was also validated in our previous study for the assay of 18 plasma amino acids by a UHPLC-MS/MS method [38]. By adding a 3-fold volume of precipitator (0.2% FA acetonitrile solution containing each IS of 400 ng/mL), stable and optimal recovery as well as matrix effect was achieved, so this pretreatment method was applied in this improved method.

In all, the quantification of amino acids in human plasma by both GC-MS- and LC-MS-based mass spectroscopy was well established [23, 24, 28, 31, 32] and would ideally suit our validation study after improvement. As the key differences for LC-MS were often in terms of sample preparation and separation parameters, the comparison of those published

| Items                        | Total | Male  | Female |
|------------------------------|-------|-------|--------|
| Number of patients           | 94    | 56    | 38     |
| Age (median, range)          | 60, (32–87) | 58, (32–87) | 61, (38–80) |
| Number of patients with TNM stage |       |       |        |
| Stage I                      | 10    | 3     | 7      |
| Stage II                     | 33    | 18    | 15     |
| Stage III                    | 45    | 33    | 12     |
| Stage IV                     | 6     | 2     | 4      |

As for optimization of ESI-MS/MS conditions, this study highlighted the importance of quantifying the isomeric analytes using two strategies. For compounds that shared the same MRM transitions, such as Ala and Sar, modifications of the mobile phase and its gradient, as well as the column optimization, were tried to make sure they were completely separated in chromatography. The coelution of the other two pairs of standards, such as Leu, Ile, and Hpr and Lys and Gin, was also avoided at the same time. If the isomeric analytes had a similar retention time, another MRM transition was chosen to separate them on different mass spectrometer channels. For example, the MRM transition for ADMA was set at 203/46 instead of 203/70 and 203/172 for SDMA. The representative MRM chromatograms of Ala, Sar, Leu, Ile, Hpr, Lys, Gln, ADMA, and SDMA are shown in Figure 2.

During the selection of ISs, the ideal condition was an isotope-labeled internal standard for each analyte, but the major problem was the high expense and longer delivery time. Besides, those amino acids were of the same structure, so it was acceptable to use a structural analog as the internal standard for analytes. Furthermore, method verification in our experiment was currently acceptable as specified by Chinese Pharmacopoeia and FDA guidelines. Hence, the practical value was the availability of three isotopically labeled amino acids, which could facilitate application while making research less expensive.

In terms of optimization of chromatographic conditions, generally, analysis using sub-2 μm columns yielded a greater (S/N) due to the reduction in band broadening and thus an increase in sensitivity. We actually conducted the analysis by using reverse-phase LC columns with 1.8 and 5.0 μm packing materials, which generated a similar chromatographic peak resolution. Moreover, ion suppression from the coeluting peak was alleviated when the 5.0 μm column was used. Since gradual accumulation of small amounts of protein and/or particulates might occur and become noticeable after injection of a large number of samples owing to the incomplete efficiency of protein removal (only about 95%–99%) [39], a wash step of the column was set between different analysis batches. Besides, the use of a column of 5 μm particle size was considered to be less vulnerable pollution than one of 1.8 μm particle size. Based on our previous reports [38], the adding of HFBA could lead to the best performance separation for amino acids. Therefore, we found that 0.02% HFBA and 0.2% FA aqueous solution with methanol could result in better separation of compounds and chromatographic peaks shapes and higher signal response (S/N) for most analytes. Also, great abundance and
Figure 2: The representative MRM chromatograms of Ala, Sar, Leu, Ile, Hpr, Lys, Gln, ADMA, and SDMA: (a) blank matrix; (b) blank matrix spiked with 34 amino acid and 3 ISs; (c) cancerous tissue sample.
equations were regressed to calculate the measured concentrations in all samples within the analytical runs. Good correlation coefficients ($r > 0.99$) were observed for all analytes in their corresponding calibration ranges. The regression equations, coefficients, calibration ranges, and LLOQ for the 34 amino acids and ISs are shown in Table 4.

3.2. Method Validation Results

3.2.1. Specificity. The representative total ion current chromatograms and MRM chromatograms of blank sample, blank sample spiked with 34 amino acids and 3 ISs, and real CRC samples are shown in Supplementary Material Figure S1. The retention time of the 34 amino acids is shown in Table 4. No interfering peaks from endogenous matrix substances were shown at the retention time of 34 amino acids and 3 ISs, suggesting satisfactory separation and selectivity.

3.2.2. Linearity of Calibration Curves and LLOQ. The linear equations were regressed to calculate the measured concentrations in all samples within the analytical runs. Good correlation coefficients ($r > 0.99$) were observed for all analytes in their corresponding calibration ranges. The regression equations, coefficients, calibration ranges, and LLOQ for the 34 amino acids and ISs are shown in Table 4.

3.2.3. Recovery and Matrix Effect. The average recovery results of the 34 amino acids and 3 ISs using QC1 and QC3 samples ranged from 39.00% to 146.95% (RSD 0.44%–7.40%). The average matrix effect results using the same two samples ranged from 49.45% to 173.63% (RSD 0.61%–12.97%), indicating that the extraction procedure was consistent and stable (Supplementary Material Table S2).

3.2.4. Inter- and Intraday Accuracy and Precision. The intra- and interday accuracy and precision of this method were assessed using the LLOQ, QC1, QC2, and QC3 samples. The deviations (RE%) of intraday ranged from $-13.52$% to $14.21$%, and the RSD were less than $8.57$%, while the deviations of interday ranged from $-14.52$% to $12.59$%, and the RSD was not more than $10.31$%. The intra- and interday
Figure 3: The contents of 34 amino acids in cancerous, paracancerous, and normal tissue from 94 CRC patients (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), and **** \( p < 0.0001 \)).
Table 5: The median and SD values of 34 amino acids in cancerous, paracancerous, and normal tissue from 94 CRC patients.

| Analyte | Cancerous tissue (µg/g) | Paracancerous tissue (µg/g) | Normal tissue (µg/g) |
|---------|-------------------------|----------------------------|---------------------|
| Gly     | 244.454                 | 110.611                    | 91.526              |
| Ala     | 330.693                 | 163.616                    | 145.35            |
| Ser     | 143.072                 | 92.541                     | 127.721            |
| Val     | 141.541                 | 58.259                     | 107.203            |
| Thr     | 104.922                 | 63.748                     | 73.603              |
| Leu     | 181.020                 | 94.785                     | 122.750            |
| Ile     | 97.020                  | 58.074                     | 71.391              |
| Asp     | 107.197                 | 67.386                     | 69.694              |
| Lys     | 284.995                 | 231.061                    | 278.033            |
| Gln     | 159.779                 | 77.801                     | 170.369            |
| Glu     | 335.986                 | 145.846                    | 268.418            |
| Phe     | 127.176                 | 70.195                     | 85.905             |
| Arg     | 150.227                 | 97.943                     | 127.102            |
| Tyr     | 113.370                 | 60.918                     | 88.310             |
| Pro     | 141.950                 | 152.552                    | 68.731             |
| Asn     | 54.833                  | 42.362                     | 42.362             |
| Met     | 76.329                  | 45.541                     | 63.348             |
| Trp     | 32.690                  | 19.826                     | 27.032             |
| Cys     | 15.593                  | 16.903                     | 10.482             |
| His     | 6.044                   | 5.366                      | 4.033              |
| Cit     | 16.222                  | 16.414                     | 16.281             |
| ADMA    | 17.082                  | 11.799                     | 7.941              |
| Cyss    | 26.370                  | 33.970                     | 10.720             |
| Sar     | 0.611                   | 0.490                      | 0.520              |
| Apa     | 1.934                   | 1.456                      | 0.529              |
| Amp     | 0.525                   | 0.474                      | 0.469              |
| Aba     | 2.341                   | 2.356                      | 2.069              |
| Opr     | 2.982                   | 3.705                      | 2.637              |
| Hpr     | 2.390                   | 1.385                      | 1.425              |
| Orn     | 1.551                   | 2.266                      | 1.043              |
| Ahd     | 0.481                   | 0.390                      | 0.745              |
| Hia     | 0.656                   | 0.103                      | 0.546              |
| SDMA    | 1.525                   | 1.093                      | 1.116              |
| Kyn     | 7.065                   | 8.108                      | 2.043              |

The accuracy of the LLOQ sample ranged from −18.03% to 16.99%, and precision was less than 13.25%. The results are shown in Supplementary Material Table S3.

3.2.5. Stability. The stability results of different conditions are shown in Supplementary Material Table S4. It demonstrated that all the analytes were stable with the accuracy within ±15% under different conditions.

3.2.6. Dilution Effect. The dilution effect results showed that the accuracy and precision for 8-time dilution were acceptable (RE ranged from −12.86% to 14.42%, RSD ≤5.46% for all the analytes). The results are shown in Supplementary Material Table S5.

3.3. Application in Determination of Clinical Samples. This targeted UHPLC-MS/MS method was successfully applied to simultaneously determine 34 amino acids in tumor tissue samples. Figure 3 and Table 5 depict the contents, median, and SD values of 34 amino acids in cancerous, paracancerous, and normal tissue from 94 CRC patients. As expected, the contents of some amino acids were significantly different between sample types. For example, Gly in Tc sample was markedly higher than that in Tp and Tn samples (each p < 0.0001). In addition, significantly increased contents were also found in Tc sample for the Asp, Glu, Pro, Cys, ADMA, Cyss, Kyn, Orn, Aba, Apa, and Hpr (each p < 0.0001). This supported the trait of altered metabolism of cancer, and it was encouraging that the candidate metabolites varied so much between cancer and normal tissue. However, it was not enough to diagnose the CRC by the amino acid profiles alone because the samples in our study were limited, and further research was needed to evaluate their potential as biomarkers for CRC diagnosis and treatment.

4. Conclusion

A simple, rapid, sensitive, and efficient targeted UHPLC-MS/MS method was developed for the determination of 34 amino acids with analytical time less than 10 min in tumor tissues, which was validated for selectivity, linearity, extraction recovery, matrix effect, intra- and interday accuracy and precision, and stability. The use of diluted PBS as the "mimic tissue fluid" could prevent serious interference from endogenous amino acids, which was proved to be simple and efficient. Enough retention was achieved for the highly polar amino acid analytes in the C18 column by using the HFBA, an ion-pairing reagent, as the mobile phase additive, without any derivatization procedure. The one step PPT method for 100 mg cancer tissue supported the high-throughput testing. In summary, this UHPLC-MS/MS method was successfully applied to plot the profiles of 34 amino acids in cancerous, paracancerous, and normal tissue from CRC patients, which may be of help for the diagnosis and treatment of CRC in the future.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| Aba          | 4-Aminobutyric acid |
| ADMA         | Asymmetric dimethylarginine |
| Ahd          | 2-Amino-L-hexanoic diacid |
| Ala          | L-Alanine |
| Ala-d4       | L-Alanine-d4 |
| Amp          | 3-Amino-2-methylpropanoic acid |
| Apa          | 3-Aminopropanoic acid |
| Arg          | L-Arginine |
| Asn          | L-Asparaginase |
| Asp          | L-Aspartic acid |
| Cit          | L-Citrulline |
| CRC          | Colorectal cancer |
| Cys          | L-Cysteine |
| Cyss         | L-Cystine |
| ESI          | Electrospray ionization |
| FA           | Formic acid |
| FDA          | Food and drug administration |
| GC-MS        | Gas chromatography-mass spectrometry |
Gln: L-Glutamine
Glu: L-Glutamic acid
Gly: Glycine
HCl: Hydrochloric acid
HFBA: Heptafluorobutyric acid
Hia: Hippuric acid
His: L-Histidine
Hpr: 4-Hydroxy-L-proline
HPLC: High-performance liquid chromatography
Ile: L-Isoleucine
IS: Internal standard
Kyn: L-Kynurenine
LC-MS: Liquid chromatography-mass spectrometry
Leu: Leucine
LLOQ: Low limit of quantitation
Lys: L-Lysine
Met: L-Methionine
Met-d3: L-Methionine-d3
MRM: Multiple reaction monitoring
Opr: 5-OxO-L-proline
Orn: L-Ornithine
PBS: Phosphate-buffered solution
Phe: L-Phenylalanine
Phe-d5: L-Phenylalanine-d5
PPT: Protein precipitation
Pro: L-Proline
QC: Quality control
RE: Relative error
RSD: Relative standard deviation
Sar: Sarcosine
SDMA: Symmetric dimethylarginine
Ser: L-Serine
TCA: Tricarboxylic acid
Thr: L-Threonine
Trp: L-Tryptophan
Tyr: L-Tyrosine
UHPLC-MS/MS: Ultrahigh-performance liquid chromatography-tandem mass spectrometry
Val: L-Valine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

TABLE S1: the control substances of 34 amino acids and 3 ISs. TABLE S2: the extraction recovery and matrix effect of 34 amino acids and 3 ISs (n = 6). TABLE S3: the intra- and interday accuracy and precision of 34 amino acids (n = 5). TABLE S4: the stability results of 34 amino acids (n = 3). TABLE S5: the dilution effect results of 34 amino acids and 3 ISs (n = 3). FIGURE S1: the representative total ion current chromatograms and MRM chromatograms of 34 amino acids and 3 ISs—(a) blank matrix; (b) blank matrix spiked with 34 amino acid and 3 ISs; (c) cancerous tissue sample. (Supplementary Materials)

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