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Determination of Free Amino Acids in Isatidis Radix By HILIC-UPLC-MS/MS

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A rapid, accurate and precise method for the determination of 22 amino acids in Isatidis Radix by Hydrophilic Interaction Ultra-High-Performance Liquid Chromatography Coupled with Triple-Quadrupole Mass Spectrometry (HILIC-UPLC-MS/MS) was established. Chromatographic separation was carried out on a Acquity UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) with gradient elution of acetonitrile (containing 0.05% formic acid and 2 mM ammonium formate) and water (containing 0.15% formic acid and 10 mM ammonium formate) at a flow rate of 0.4 mL/min; Waters Xevo^{TM} TQ worked in multiple reaction monitoring mode. All components were separated in 17 min. All calibration curves were linear ($R^2 > 0.991$) over the tested ranges. The limits of detection (LOD) and limits of quantitation (LOQ) for these compounds were 0.21-79.55 and 0.72-294.23 ng/mL, respectively. The average recoveries were in the range of 93.75-104.16% with RSD value less than 6.56%. Therefore, this method could be an alternative assay for the determination of 22 amino acids in Isatidis Radix due to its rapidness, sensitivity, less sample and solvent consumption.

Key Words : HILIC-UPLC-MS/MS, Isatidis radix, Amino acids

Introduction

Isatidis Radix, the root of *Isatis indigotica* Fortune, called Banlangen in Chinese, is widely distributed in China, especially in Jiangsu, Anhui, Hebei, *etc*. As a traditional Chinese medicine (TCM), Isatidis Radix was firstly documented to have medicinal use in Shengnongbencaojing which is the earliest existing medicine monographs in China. Studies showed that Isatidis Radix possess the properties of antioxidant, antitumor, antiviral activities and immune enhancing activities.[1-3] Chemical studies showed that Isatidis Radix contains various constituents, including alkaloids, lignans, phenylpropanoids, steroids and organic acid.[4-7] In addition, it was also found that Isatidis Radix contained amino acids which are arguably the most important compounds from a biological point of view.[8] They are not only the basic structural units of proteins, but also a source of energy and serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide.[9] Furthermore several studies have reported that the profiling of free amino acids can be used to discriminate the origin of a substance and its shelf life.[10,11] Therefore, the determination of these compounds is important in improving their therapeutic value and could be convenient for quality evaluation of Isatidis Radix.

To date, various analytical methods have been used to analyze amino acids, including high performance liquid chromatography (HPLC),[12-14] fluorescence detection or coulometric detection, thin-layer chromatography,[15] gas chromatography-mass spectrometry (GC-MS),[16,17] liquid chromatography-mass spectrometry (LC-MS)[18] and capillary electrophoresis,[19] which require pre- or post-column derivatization. However, most of the derivatization methods are affected by some disadvantages, including derivative instability, insufficient reproducibility of derivative yield, interferences caused by the reagent, or time-consuming and laborious derivatization procedures.[9,20,21] Therefore, some analysis techniques that eliminate the need for derivatization procedures have been established, such as HPLC-mass spectrometry (HPLC-MS) and HPLC-evaporative light scattering detection (HPLC-ELSD) with using an ion-pairing reagent such as trifluoroacetic acid, tetrabutylammonium hydroxide, heptafluorobutyric acid and perfluorinated acid and so on.[22-24] Addition of ion-pair reagents to mobile phase improves separation of underivatized amino acids on a reverse-phase silica-based C8 or C18 columns.[20,23,25,26] They also increase the MS signal of amino acids and improve peak shape. However, ion-pair reagents frequently affect assay durability,[22] increase the retention times of amino acids[20] and decrease the sensitivity of the MS detector.[27] All these problems limited the application of the above methods.

For the above reasons, a sensitive and reliable method for identification and quantification of amino acids needs to be developed and validated. As the highly polar compounds, amino acids can obtain good retention and separation on hydrophilic interaction chromatography (HILIC). In contrast with RP-HPLC, HILIC is a kind of chromatographic separation technology for highly polar compounds which employs traditional polar stationary phases such as silica, amino or cyano, but the mobile phase used is similar to those employed in the RP-HPLC mode.[28,29] It was first introduced by Alpert in 1990,[30] and was known to partly solve some issues that normal phase or polar-embedded columns pose for mass detectors, specifically the need to use ion-pairing agents combined with highly aqueous mobile phases, both of which altered the MS sensitivity. With HILIC columns, the retention of polar compounds was improved by increasing the percentage of organic solvents contained in the aqueous mobile phases.[31] So it is suitable for the separation of a

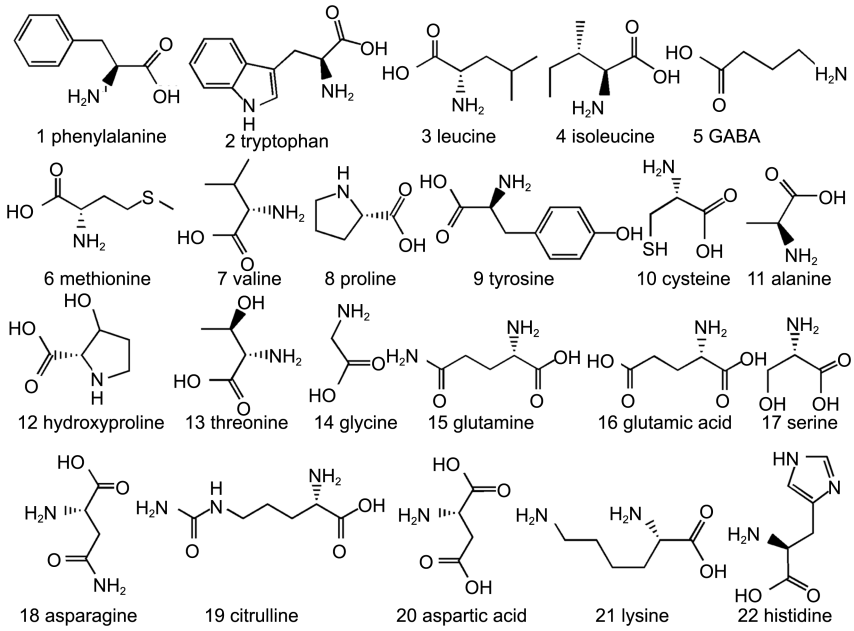

Figure 1. Chemical structures of 22 compounds.

broad spectrum of highly polar compounds, including peptides, amino acids, carbohydrates, nucleobase and nucleosides as well as many other biologically important compounds.[21,32,33] In addition, Ultra-high-performance liquid chromatography coupled with a triple quadrupole electrospray tandem mass spectrometry (UPLC–MS/MS) is a powerful tool to solve the problems of above methods because of its high sensitivity and rapid resolution. Due to the high selectivity of multiple reaction monitoring (MRM) mode, optimization of chromatographic separation is greatly simplified. Furthermore, precursor and product ion monitoring can be used to increase specificity of detection and identification of the known molecules.

In this study, an efficient and sensitive method based on the above notion was developed and validated, using HILIC-UPLC-MS/MS, for simultaneous, identification and quantification of amino acids in Isatidis Radix. To our knowledge, this is the first time the HILIC-UPLC-MS/MS method has been employed to analyze simultaneously 22 amino acids in Isatidis Radix. In this paper, amino acids were determined in 10 samples of Isatidis Radix. The profiles of the amino acids in Isatidis Radix would be extraordinary helpful for improving their potential values and also may be used as markers for their quality control. In addition, compositional analysis of free amino acids, especially in the food and medicines science field, is receiving plenty of attention. So multivariate statistical analysis was used to investigate the differences in free amino acid profiles among the samples.

Experimental

Materials and Reagents. The chemical structures of amino acids including phenylalanine (Phe, 1), tryptophan (Trp, 2), leucine (Leu, 3), isoleucine (Ile, 4), γ-amino-butyric acid (GABA, 5), methionine (Met, 6), valine (Val, 7), proline (Pro, 8), tyrosine(Tyr, 9), cysteine (Cys, 10), alanine (Ala, 11), hydroxyproline (Hpro,12), threonine (Thr, 13), glycine (Gly, 14), glutamine (Gln, 15), glutamic acid (Glu, 16), serine (Ser, 17), asparagine (Asn, 18), citrulline (Cit, 19), aspartic acid (Asp, 20), lysine (Lys, 21) and histidine (Hit, 22) were shown in Figure 1 which were purchased from Sigma-Aldrich (St. Louis, MO). The purity of each compound was > 98%, determined by HPLC analysis. The ten batches of samples of Isatidis Radix were collected from Anhui (S1), Shanxi (S2), Henan (S3), Hebei (S4), Heilongjiang (S5), Neimengu (S6), Gansu (S7), Gansu (S8), Sichuang (S9) and Anhui (S10) provinces, PR China. Its botanical origin was identified by Professor Jianwei Chen, Nanjing University of Chinese Medicine. The voucher specimens were deposited at the Herbarium in Nanjing University of Chinese Medicine (Nanjing, China). Acetonitrile was HPLC-grade from Merck (Darmstadt, Germany), and deionized water (H_2O) was purified by a superpurification system (Eped Technology Development Co., Ltd., Nanjing, China). Other reagent solutions, such as ammonium formate and formic acid, were of analytical grade (Sino pharm Chemical Reagent Co., Ltd., Shanghai, China).

Instrumentation and Chromatographic Conditions. Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters, Corp., Milford, MA, USA), consisting of a quaternary pump solvent management system, an online degasser, and an autosampler. An Acquity UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm, Waters) with an ACQUITY UPLC BEH Amide 1.7 μm VanGuard precolumn was applied for all analyses. The raw data were acquired and processed with MassLynx 4.1 software. The mobile phase was composed of A (water, which containing 0.15% formic acid and 10 mM ammonium formate) and B (acetonitrile, which containing 0.05% formic acid and 2 mM ammonium formate) with a gradient elution: 0-6 min, 15-20%A; 6-10

Table 1. The molecular weights (MW), MRM transitions, cone voltage, collision energies, and retention times (Rt) of 22 amino acids

Analytes	Retention time (min)	$[M+H]^+$ (*m/z*)	MRM transitions (Precursor → product)		Cone voltage (V)	Collision energy (eV)
1 Phe	2.37	166.09	166.1	120.1	18	14
2 Trp	2.38	205.10	205.1	146.0	16	18
3 Leu	2.41	132.10	132.1	86.1	16	10
4 Ile	2.62	132.10	132.1	86.1	16	10
5 GABA	2.78	104.07	104.1	87.0	16	10
6 Met	2.94	150.06	150.1	104.0	14	10
7 Val	3.26	118.09	118.1	72.1	12	10
8 Pro	3.40	116.07	116.1	70.0	20	10
9 Tyr	3.45	182.08	182.1	136.0	16	16
10 Cys	3.49	122.03	122.0	76.0	14	17
11 Ala	4.01	90.06	90.1	44.0	16	10
12 Hpro	4.75	132.07	132.1	68.0	18	16
13 Thr	4.89	120.07	120.1	74.0	38	20
14 Gly	5.40	76.04	76.0	30.0	12	6
15 Gln	5.68	147.08	147.1	84.0	8	16
16 Glu	6.18	148.06	148.1	84.0	12	14
17 Ser	6.54	106.05	106.1	60.0	14	8
18 Asn	6.67	133.06	133.1	74.0	12	14
19 Cit	7.03	176.1	176.1	70.0	16	20
20 Asp	7.53	134.05	134.1	88.0	14	10
21 Lys	10.59	147.11	147.1	84.0	14	14
22 Hit	11.20	156.08	156.1	110.0	20	16

min, 20-30%A; 10-17 min, 30-40%A. The flow rate was set at 0.4 mL/min. The column temperature and injection volume were set at 35 °C and 1 μL, respectively. Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source (ESI). High-purity nitrogen was used as the nebuliser and auxiliary gas, argon was used as the collision gas. The ESI-MS spectra were acquired in positive ion multiple reaction monitoring (MRM) mode with a capillary voltage of 3.0 kV, a source temperature of 150 °C, a desolvation temperature of 550 °C, a desolvation gas flow of 1000 L/h, a cone gas flow of 50 L/h, a collision gas flow of 0.15 mL/min. The retention time (RT) and MS information for each analyte including molecular weight (MW), MRM transitions, cone voltage (CV) and collision energies (CE) were shown in Table 1.

Standard Preparation. The standard stock solutions of **1**, 21.60 μg/mL; **2**, 30.80 μg/mL; **3**, 20.00 μg/mL; **4**, 21.20 μg/mL; **5**, 17.20 μg/mL; **6**, 21.60 μg/mL; **7**, 23.20 μg/mL; **8**, 51.60 μg/mL; **9**, 22.80 μg/mL; **10**, 29.20 μg/mL; **11**, 16.40 μg/mL; **12**, 18.00 μg/mL; **13**, 22.40 μg/mL; **14**, 19.20 μg/mL; **15**, 31.60 μg/mL; **16**, 29.20 μg/mL; **17**, 31.60 μg/mL; **18**, 24.00 μg/mL; **19**, 16.68 μg/mL; **20**, 22.80 μg/mL; **21**, 20.40 μg/mL and **22**, 24.80 μg/mL were accurately prepared in water and stored at 4 °C before use. The standard solutions were diluted with water to a series of appropriate concentrations and used to construct calibration curves. The standard solutions were filtered through a 0.22 μm membrane prior to injection.

Sample Preparation. The dried samples were ground using a pestle and mortar. They were then sieved through a 40 mesh stainless-steel sieve before extraction. 1.0 g of each sample powder was accurately weighed into a 50 mL conical flask, and 20 mL of distilled water was added to the conical flasks. All of the mixture was precisely weighed and placed into an ultrasonic bath (40 kHz) for 60 min at room temperature, and then the same solvent was added to compensate for the weight lost during the extraction. After centrifugation (13,000 rpm, 10 min), the supernatant was stored at 4 °C and filtered through a 0.22 μm polytetrafluoroethylene filter and transferred to a glass vial for the UPLC-MS/MS system injection.

Data Processing and Statistical Analysis. Data were processed using the MassLynx 4.1 software application manager for the quantification of compounds. Principle component analysis (PCA) was performed using SPSS 16.0 software.

Results and Discussion

Optimization of Extraction Procedure. To achieve optimal extraction conditions, extraction variables such as extraction methods (refluxing and sonication), extraction solvent (water, 25% aqueous methanol, and 50% aqueous methanol), solvent volume (20, 40, and 60 mL), and extraction time (15, 30, 60, and 90 min) were investigated on 1.0 g, 40 mesh of different kinds of samples. When one of the parameters was determined, the others were set at the default. The results revealed that ultrasonic extraction was better than reflux extraction, and it was selected for use in further experiments. Furthermore, extraction solvent were investigated which showed that water was ideal solvent for all samples. In addition, the volume of solvent was chosen as 20 mL which was sufficient for the samples extraction. Various time of extraction were also optimized which revealed that sixty minutes was the best choice, thereafter, a further increase of extraction time did not result in a significant increase in amount. Finally, suitable extraction conditions were optimized as follows: each sample was extracted by sonication with 20 mL of water for 60 min, which was adequate and appropriate for the analysis.

Optimization of the UPLC Chromatographic Conditions. In our preliminary test, two brands of analytical columns, Acquity UPLC BEH HILIC (2.1 × 100 mm, 1.7 mm) and Acquity UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) were compared. The results showed the second one obtain chromatograms with better resolution of adjacent peaks within shorter time for the former lack of separation resulted in significant peak interferences. Hence, the Acquity UPLC BEH Amide column was chosen. For mobile phase, acetonitrile was proven to be better than methanol. In addition, the presence of ammonium formate in the mobile phase could improve the separation of amino acids. To further optimization, it is found that high-resolution and sharp peaks were achieved for the 22 target compounds by

adjusting the formic acid concentration in 10 mM ammonium acetate solutions and acetonitrile respectively. As a result, a mixed solution including acetonitrile which containing 0.05% formic acid and 2 mM ammonium formate and water which containing 0.15% formic acid and 10 mM ammonium formate were chosen as the preferred mobile phase, and gradient elution was applied. It was also suggested that the separation was better when the flow rate was 0.4 mL/min and the column temperature was kept at 35 °C.

MS spectra were studied in both positive and negative ion modes. It was found that compared to the negative ion mode, amino acids had not only higher sensitivity but also clearer mass spectra in the positive ion mode, which made it easier to confirm molecular ions or quasi-molecular ions in the identification of each peak. As such, the positive MS ion mode was selected. The chemical structures of 22 components were characterized based on their retention behavior and MS information, such as quasi-molecular ions $[M+H]^+$ and fragment ions. The ion pairs of precursor → product ion for MRM detection were generated by the Intellistart procedure which was embedded in the MassLynx 4.1 software. The signal of each compound was optimised by altering CV and CE. Under the optimized UPLC and MS/MS conditions, all 22 compounds in Isatidis Radix were identified and quantified. Retention time (RT) and MS information for each analyte including molecular weight (MW), MRM transitions, CV and CE are shown in Table 1, and representative MRM chromatography of 22 markers in Figure 2.

Method Validation. Method validation is an important requirement in the practice of chemical analysis. The purpose is to study the method performance parameters. The parameters for this validation included a linear range, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy.

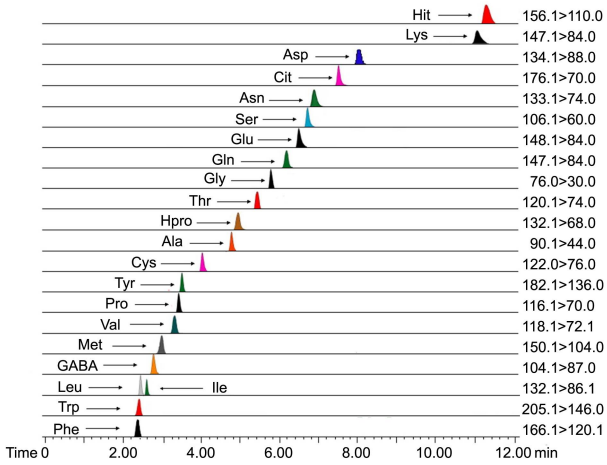

Figure 2. Representative MRM chromatograms of the 22 amino acids in Isatidis Radix.

Linearity, LOD and LOQ: Standard calibration curves were generated for standard solutions at different concentrations of free amino acids in Isatidis Radix. As shown in Table 2, all the analytes showed good linearity ($R^2 > 0.991$) in a relatively wide concentration range.

The LOD and LOQ for each analyte under present chromatographic conditions were determined at the signal-to-noise ratio (S/N) for each compound of about 3 and 10, respec-

Table 2. Regression equations, correlation coefficients, linearity ranges, and limits of detection (LOD) and quantitation (LOQ) of the 22 amino acids

Analyte	Calibration curve	R^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
1 Phe	y = 12329x-2532.8	0.9986	10.00-21600.00	0.63	2.31
2 Trp	y = 6526.5x-1988.4	0.9989	10.00-30800.00	1.05	4.18
3 Leu	y = 25667x-4297.9	0.9993	10.00-20000.00	2.00	5.69
4 Ile	y = 16654x-2928.8	0.9988	10.00-21200.00	2.70	8.16
5 GABA	y = 18355x-1128.3	0.9998	10.00-34400.00	0.21	0.72
6 Met	y = 4124.8x-799.83	0.9991	10.00-21600.00	1.06	4.34
7 Val	y = 18919x-2155.3	0.9995	50.00-58250.00	7.46	29.18
8 Pro	y = 36832x+2067.6	0.9999	500.00-51600.00	0.29	1.26
9 Tyr	y = 2830.5x+406.76	0.9999	20.00-22800.00	5.92	17.91
10 Cys	y = 4375.9x-2432.8	0.9911	300.00-29200.00	57.21	199.85
11 Ala	y = 2478.3x-76.861	0.9997	80.00-16400.00	23.6	74.14
12 Hpro	y = 2524.4x-292.92	0.9995	90.00-18000.00	4.55	15.92
13 Thr	y = 87.033x-152.22	0.9907	560.00-22400.00	74.02	294.23
14 Gly	y = 334.21x-416.93	0.9955	480.00-19200.00	48.20	144.19
15 Gln	y = 3889.5x-1051.6	1.0000	300.00-31600.00	7.28	22.09
16 Glu	y = 2948.8x-2836	0.9914	300.00-29200.00	78.02	271.93
17 Ser	y = 2419.4x-895.21	0.9996	320.00-31600.00	36.20	109.16
18 Asn	y = 2015.6x-989.85	0.9995	240.00-24000.00	60.89	235.08
19 Cit	y = 3321.1x-292.89	1.0000	170.00-16800.00	5.05	18.05
20 Asp	y = 97.633x+103.73	0.9906	230.00-22800.00	62.34	219.03
21 Lys	y = 2173.9x-2675.5	0.9913	510.00-20400.00	50.04	200.08
22 Hit	y = 3479.8x - 3127.0	0.9924	250.00-24800.00	79.55	247.58

Table 3. Precision, repeatability, stability and recovery of 22 amino acids

Analyte	Precision (RSD, %)		Repeatability (RSD, %, n=6)	Stability (RSD, %, n=6)	Recovery (%, n=3)	
	intraday (n=6)	interday (n=6)			mean	RSD,%
1 Phe	1.30	4.01	0.79	1.31	101.10	2.18
2 Trp	1.31	6.29	4.29	1.39	95.41	3.12
3 Leu	1.21	3.83	2.61	1.25	97.92	3.19
4 Ile	0.79	2.60	4.03	2.52	98.98	2.56
5 GABA	1.03	4.81	5.14	3.68	102.41	5.53
6 Met	0.80	3.31	5.90	6.12	98.45	5.04
7 Val	0.91	1.29	3.92	3.71	93.98	4.18
8 Pro	1.26	1.16	3.51	2.57	98.67	2.15
9 Tyr	2.11	5.09	4.93	3.62	95.09	4.03
10 Cys	3.12	6.11	5.09	4.06	94.76	4.96
11 Ala	1.61	2.73	2.00	1.82	96.94	3.11
12 Hpro	2.02	3.68	2.25	1.72	98.07	3.65
13 Thr	4.81	2.04	4.5	6.42	95.28	5.12
14 Gly	3.83	6.41	6.03	7.12	104.16	4.95
15 Gln	4.14	4.33	5.76	5.55	95.18	4.89
16 Glu	2.83	1.98	5.99	1.11	93.75	3.88
17 Ser	3.11	4.23	4.53	2.41	100.11	5.12
18 Asn	2.97	2.85	4.91	4.17	97.34	4.24
19 Cit	1.32	2.76	10.58	3.23	98.51	2.98
20 Asp	4.22	3.65	5.22	5.21	94.23	6.56
21 Lys	2.84	6.71	4.06	5.8	96.98	4.87
22 Hit	3.68	6.63	2.21	2.97	94.18	5.17

tively. The LOD and LOQ values for the 22 analytes were determined, which are listed in Table 2.

Precision, Repeatability and Stability: The intra- and inter-day precisions were investigated by determining a mixed standard solution in six replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD). To further evaluate the repeatability of the developed assay, the sample of Isatidis Radix (collected from S1) was analyzed in six replicates with the above established method and variations were expressed by RSD. One of the sample solutions mentioned above was injected into the apparatus at 0, 2, 4, 8, 12, and 24 h, respectively, to evaluate the stability of the solution. As shown in Table 3, the intraday precisions, interday precisions, repeatability, and stability RSD values of the 22 compounds were < 4.81%, < 6.71%, < 10.58% and < 7.12% respectively. These results suggest that good accuracy and precision can be obtained using our method.

Recovery: A recovery test was used to evaluate the accuracy of the method. Known amount of the 22 standards were added into a certain amount of S1. Then the spiked samples were extracted and analyzed as described above. Three replicates were performed for the test. As shown in Table 3, the recovery ranged from 93.75% to 104.16%, and the RSD ranged from 2.15% to 6.56%, which further indicated the established method was accurate enough for the determination of the 22 amino acids in Isatidis Radix.

Sample Analysis. The developed HILIC-UPLC-MS/MS method was applied to analyse the 22 amino acids in 10 samples collected from different locations in China. All the contents were summarised in Table 4. The results showed that all these Isatidis Radix samples were rich in the free amino acids, especially those essential amino acids such as Trp, Phe, Leu, Ile, Met, Val, Thr, and Lys. This result indicated that Isatidis Radix is a good medicine for the supplement of free amino acids. As shown in Table 4, the contents of these amino acids obviously varied in the different samples. The total content of these 22 amino acids in S5 reached as high as 34.93 mg/g, whereas it was only 6.75 mg/g in S1. As for the individual compounds determined in the experiments, Pro was found to be the most abundant free amino acid in all samples, and its average content in these investigated samples was 7.28 mg/g, which accounted for more than 33% of the total amino acids tested in this study. Howere, the Hpro was the lowest except for not detected. In addition, without the protein amino acids, three nonprotein amino acids, including GABA, Hpro, and Cit, were found in the Isatidis Radix samples.

PCA of the Samples. In this study, PCA was performed on the basis of the contents of 22 tested compounds from UPLC profiles to evaluate the variation of Isatidis Radix.

Table 4. Contents of 22 amino acids in Isatidis Radix (μg/g, n = 3, RSD < 2%)

Sample	Contents of analytes (μg/g)																						Total
	Phe	Trp	Leu	Ile	GABA	Met	Val	Pro	Tyr	Cys	Ala	Hpro	Thr	Gly	Gln	Glu	Ser	Asn	Cit	Asp	Lys	Hit	
S1	63.09	12.18	58.77	57.57	269.24	10.06	175.15	3759.09	9.81	6.89	173.61	2.33	596.58	52.00	166.19	93.64	129.53	316.45	76.98	73.15	352.98	295.33	6750.61
S2	380.51	49.30	287.95	111.91	595.41	18.65	347.79	8859.23	45.31	14.58	568.01	12.98	439.05	282.29	389.37	487.08	226.03	660.17	530.08	221.22	832.25	484.16	15843.32
S3	403.80	88.49	321.95	437.96	1219.32	19.68	1021.08	6922.53	59.96	13.97	539.85	5.90	1857.22	180.28	1916.12	860.45	736.94	928.49	747.73	370.06	4052.40	517.77	23221.96
S4	125.01	29.44	84.19	112.16	843.57	10.84	302.04	4794.31	34.52	14.83	223.32	–[a]	689.63	91.40	622.87	100.39	214.20	299.68	30.62	25.42	1332.58	373.57	10354.57
S5	459.22	96.82	462.84	462.65	1416.36	21.59	1177.36	8584.93	76.02	16.28	870.17	11.86	2726.38	237.88	2904.84	1831.84	977.14	1739.74	1169.74	1295.16	6301.86	2087.44	34928.15
S6	298.07	60.31	235.21	235.21	969.47	18.52	565.96	8089.80	42.51	16.07	398.42	5.52	1496.39	105.20	1136.79	495.77	376.18	801.78	2187.74	–	2393.37	1316.37	21244.64
S7	280.29	71.94	224.85	416.39	1074.05	20.29	868.46	4733.94	64.81	14.95	500.69	5.29	902.13	84.02	998.65	315.73	339.65	12.03	251.02	–	2048.96	921.54	14149.69
S8	421.19	80.55	334.04	374.83	1452.14	25.67	1041.96	8622.86	66.51	16.36	619.20	11.28	1686.63	158.17	3800.10	1293.55	815.59	1540.71	445.14	+[b]	8181.42	2236.75	33224.65
S9	420.46	95.22	370.55	682.39	2225.87	13.30	1514.03	7188.29	134.36	20.13	926.10	8.66	3976.02	294.63	2954.56	1005.64	661.16	3175.32	631.21	467.73	6220.27	1516.95	34502.85
S10	312.60	40.70	282.12	289.86	915.51	18.95	792.39	11240.97	108.65	19.08	993.36	13.45	3220.42	447.26	503.90	537.90	533.73	1768.00	331.89	–	1118.71	2384.84	25874.28

[a]Not detected. [b]+: Detected, not quantified. S1: Anhui provinces; S2: Shanxi provinces; S3: Henan provinces; S4: Hebei provinces; S5: Heilongjiang provinces; S6: Neimengu provinces; S7: Gansu provinces; S8: Gansu provinces; S9: Sichuang provinces; S10: Anhui provinces, PR China.

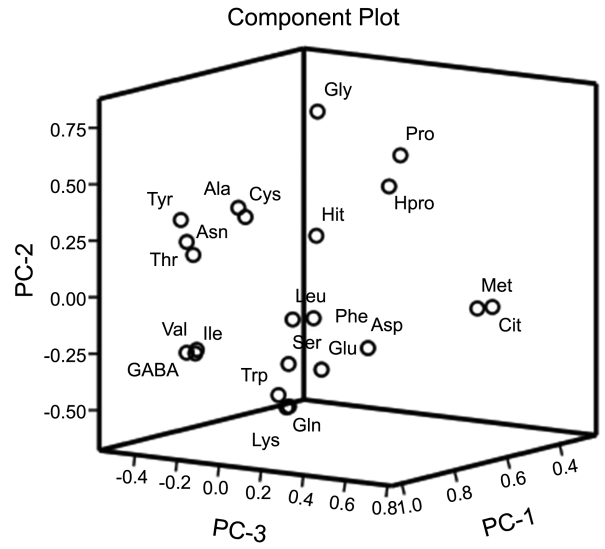

Figure 3. Loading plot obtained by PCA of the 22 amino acids.

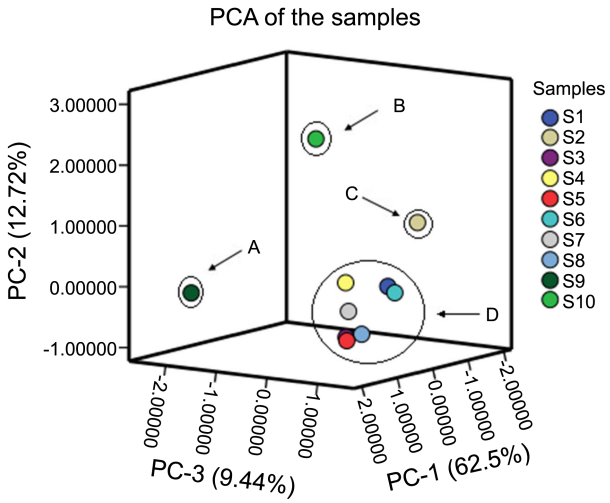

Figure 4. Scatter plot obtained by PCA of the 10 samples of Isatidis Radix. S1: Anhui provinces; S2: Shanxi provinces; S3: Henan provinces; S4: Hebei provinces; S5: Heilongjiang provinces; S6: Neimengu provinces; S7: Gansu provinces; S8: Gansu provinces; S9: Sichuang provinces; S10: Anhui provinces, PR China.

The first three principal components (PC 1, PC 2 and PC 3) with > 84.67% of the whole variance were extracted for analysis. Among them, PC 1, PC 2 and PC 3 accounted for 62.51%, 12.72% and 9.44% of the total variance respectively. The remaining principal components, which had a minor effect on the model, were discarded. The component loading matrix is shown in Figure 3. According to their loadings, PC 1 had good correlation with all of the analytes except for Gly, Cit, and Met, which exhibited their main relationship with PC 2 and PC 3. The results mentioned above suggested that all 22 compounds may contribute to the classification of the samples. The sample scatter plot is shown in Figure 4, where each sample is represented as a marker. It was noticeable that the samples were clearly clustered into four domains, with sample 9 in domain A, sample 10 in domain B, sample 2 in domain C and the others in domain D. These results indicated that samples with similar chemical profiles were commonly divided into one domain. As for sample 9, the contents of Thr, Ile, Val, Asn, Cys, Tyr and GABA were the highest in all the samples. For sample 10, the contents of Gly, Hpro, Pro, were the highest in all the samples. For sample 2, the contents of the amino acids were relatively lower than other samples. These properties may be due to the factors of species, local environment and climate.

Conclusion

In summary, a HILIC-UPLC–MS/MS method for simultaneous qualification and quantification of free amino acids without derivatization was established. The method was simple, reliable and fast with high precision, sensitivity and repeatability, which can be used to analysis of 22 free amino acids in 10 Isatidis Radix samples. Compared with the existing methodology for amino acids analysis, the sample preparation was very simple, greatly reducing the laborious and time-consuming derivatization procedures required by most other methods. Moreover, this method might also be utilized for the quality control of other herbal medicines, in which highly polar compounds were rich. According to the analytical results, Isatidis Radix is a healthy medicine rich in free amino acids, and our work could provide some useful information for rational utilization of Isatidis Radix resources.

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