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RESEARCH PAPER

Direct Detection of Amino Acids Using Extractive Electrospray Ionization Tandem Mass Spectrometry

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Abstract: A novel method was developed for the direct detection of amino acids in biological fluids by extractive electrospray ionization (EESI) tandem mass spectrometry (MS) with minimal sample pretreatment. Based on our previous research, a novel EESI ion source was constructed to improve analytical performance and operation safety. The EESI-MS conditions were optimized using representative amino acid standards. The methanol-water reagent solvent (1:1, v/v) was electrosprayed at 5 μL min^{-1} at a high voltage (+4 kV, positive ion detection mode). The temperature of the heated capillary was optimized to be 150 °C. Collision induced dissociation (CID) experiments were performed by applying 17%–25% of the collision energy to the precursor ions isolated with a window width of 1.5 mass/charge (*m/z*) units. The limit of detection (LOD) for these amino acids was in the range of 0.14–26.2 μg L^{-1}, and the linear dynamic range was larger than two orders of magnitude. The average time for a single amino acid analysis using a typical human urine sample was less than 0.5 min. The recovery rates for different concentrations of amino acids ranged from 82.6% to 105.6%, and the relative standard deviations (RSD) ranged from 2.2% to 11.4%. The results showed that EESI-MS is a powerful tool for the rapid, sensitive, and quantitative detection of amino acids in complex biological samples.

Key Words: Extractive electrospray ionization; Ambient mass spectrometry; Amino acids; Urine

1 Introduction

Recent research in life sciences suggests that small bio-molecules are not only the substrates and/or the end products of genes and proteins, but also the biological markers that indicate and respond to various perturbations to biological systems[1–3]. Moreover, small molecules, together with other biological species, participate in regulating a large variety of physiological and pathological processes. Notably, amino acids are well known as the metabolites of great importance, and they are the basic units constituting peptides and proteins. Meanwhile, many studies have shown that amino acids play important roles in the occurrence and development of a number of diseases[4,5]. Jain *et al*[6] found that glycine is an important metabolite for human breast cancer, since it is strongly correlated with the rate of proliferation of cancer cells. Asiago *et al*[7] predicted the recurrence of breast cancer earlier than 13 months on average before the clinical diagnosis using the metabolomics assay including glutamic acid, histidine, proline, and tyrosine. Therefore, it is important to further study amino acids for both qualitative and quantitative purposes, which may lead to significant discoveries in many research fields including disease diagnosis, drug metabolism, biological sciences, etc.

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Common traditional methods to measure amino acids are gas chromatography mass spectrometry (GC-MS)[8], liquid chromatography mass spectrometry (LC-MS)[9,10], capillary electrophoresis (CE)[11–14], etc. However, all these methods require manual sample preparation steps, which are tedious and time-consuming even with assistance from automatic robot systems. Therefore, it is difficult to achieve high-throughput analysis of massive samples. Meanwhile, extractive electrospray ionization mass spectrometry (EESI-MS) is a novel ambient mass spectrometry method. EESI uses an electrospray ionization (ESI) channel to provide primary ions, which then transfer the charges to the neutral compounds (from the sample introduction channel) in a 3D ionization space through collision and extraction. Its main advantage is that it can analyze complex matrix samples, such as biological fluids, with minimal sample pretreatment[15–17]. In this study, we first improved our previous EESI ionization source, with a semi-enclosed rectangular structure, so that it has a specific pipe to discharge liquid/gas waste for enhancing the experimental stability and security. The EESI-MS platform was optimized using various polar amino acid standards, and it was further developed for the fast detection of small biomolecules from complex matrix samples (urine). The results demonstrated that EESI-MS is a powerful tool for the rapid, sensitive, and quantitative detection of amino acids in complex biological samples.

2 Experimental

2.1 Instruments and reagents

Figure 1 shows an EESI device for the analysis of amino acids in complex samples[15–17]. To ensure that the device has high security and stability in the experiments, we designed and manufactured the EESI-MS system with a semi-enclosed interface that could provide flexible and continuous adjustments. To be noticed, useless gases and liquids can be discharged from the pipes on the upper and bottom surfaces of the EESI ion source. Moreover, the height of the EESI ion source can be adjusted to match the position of the MS inlet. The interface materials do not affect the mass spectrometry signals if provided sufficient ionizing space, thus we used the stainless steel to construct the EESI-MS interface. All the experiments were carried out on a commercial linear ion trap mass spectrometer (LTQ-XL, Finnigan, San Jose, CA, USA) installed with a home-made EESI ion source. The Xcalibur software was used to record the mass spectrum, followed by a background subtraction.

Methanol (HPLC grade) was purchased from the TEDIA Company (USA). Amino acid standards (analytical grade) including proline, serine, asparagine, isoleucine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, leucine, alanine, valine, tryptophan, tyrosine, threonine, glycine, methionine, and phenylalanine were bought from the Shanghai Lanji High-Tech Company (Shanghai, China). Ultrapure water (resistivity 18.2 MΩ cm) was supplied by the chemistry facility of the East China Institute of Technology (ECIT) equipped with a Barnstead Nanopure Ultrapure Water Purification System from the Thermo Scientific Company (USA).

2.2 EESI-MS experiments

The EESI ion source and the LTQ mass spectrometer were set to work in the positive ion detection mode. The corona discharge voltage was +4.0 kV for the ESI channel, and a mixture of methanol and water (1:1, *V/V*) was electrosprayed with an infusion rate of 5 μL min^{-1} to produce primary ions. The flow rate for sample infusion in the sample introduction channel was also 5 μL min^{-1}. The gas pressure (nitrogen with a purity of 99.9999%) in both channels was 1 MPa; the temperature of the heated capillary of the LTQ instrument was maintained at 150 °C. Collision induced dissociation (CID) experiments were performed by applying 17%–25% of the

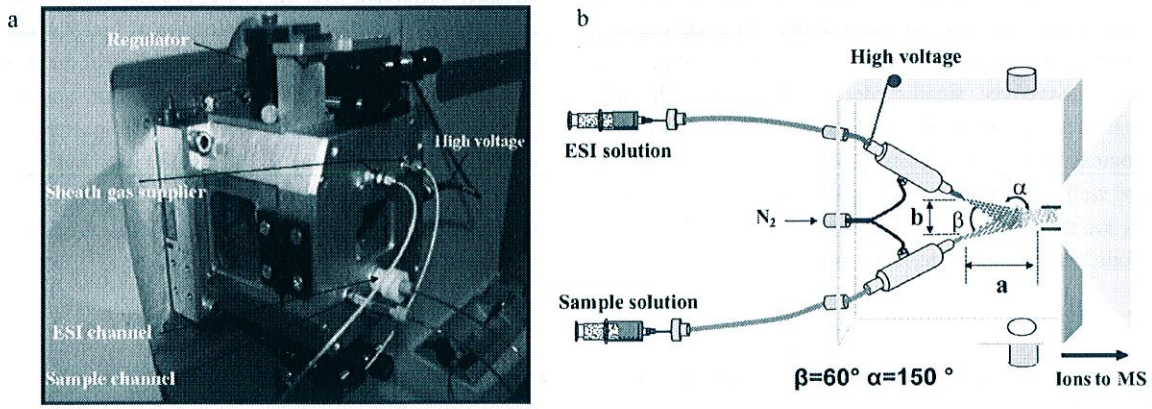


Fig.1 (a) EESI ion source for the high-throughput analysis of amino acids in biological samples with minimal pretreatment, and (b) schematic diagram of EESI ion source, the parameters were optimized and determined by amino acid standards

collision energy to the precursor ions that were isolated with a mass/charge (*m/z*) window of 1.5 units. Each mass spectrum was recorded with a scan time of 0.5 min, and the data were averaged for the output.

3 Results and discussion

3.1 Optimizing conditions of EESI ion source

As shown in Fig.1, the EESI ion source has a unique design utilizing two sprayers that are aligned along a certain angle with respect to the mass spectrometer inlet, forming the ion-molecular interaction area/ionization area. The neutral sample molecules are ionized in a relatively large three-dimensional space through the extraction and the energy/charge transfer process. We investigated the effect of physical parameters of the EESI source and the mass spectrometer on the MS signal intensities. For example, the angle β is formed between the sample outlet and the electrospray beam; the angle α is between the sample outlet and the heated capillary of the LTQ instrument (Fig.1). The angles of α and β were optimized to be 150° and 60°, respectively (data not be shown). Under this condition (α = 150°, β = 60°), to obtain the best MS sensitivity, the distance (a) between the inlet of the LTQ instrument and the gas outlets should be 5 mm and the distance (b) between the two spray tips was selected to be 1 mm (Fig.2). To be noticed, arginine ($M=C_6H_{14}N_4O_2$, *m/z* 174) was selected as the amino acid standard in the optimization step since it could be easily detected as the protonated molecule ion $[M + H]^+$ which loses NH_3 to produce the fragment ions at *m/z* 158, loses H_2O to yield the fragment at *m/z* 157, loses $NH=C(NH_2)_2$ to generate the peak at *m/z* 116, loses NH_3 and CO to produce the peak at *m/z* 130 in MS/MS experiments.

3.2 Qualitative detection of amino acids

It is important to exclude false positive results during the direct detection of targeted compounds in complex samples, therefore, in this study, tandem mass spectrometry was used to improve the detection selectivity. For instance, protonated arginine generated a MS peak at *m/z* 175 in the EESI-MS spectrum (Fig.3). The assignment was based on the tandem mass spectrum of the precursor ions of *m/z* 175. The major fragments were those at *m/z* 158, 157, 130 and 116; the precursor ions (*m/z* 175) lost NH_3 to produce the fragment ions at *m/z* 158, lost H_2O to yield the fragment at *m/z* 157, and lost $NH=C(NH_2)_2$ to generate the peak at *m/z* 116. The peak at *m/z* 130 was generated by the loss of NH_3 and CO from the precursor ions (*m/z* 175). The fragment ions at *m/z* 116 further lost H_2O and CO to yield the peak at *m/z* 70 (Fig.3). Furthermore, the human urine samples from a healthy volunteer were analyzed by using the same protocols as above after diluting the urine 100 times. The results in Fig.4 showed that the peak at *m/z* 175 was clearly detected, and that the fragment ions at *m/z* 158, 157, 130 and 116 were also observed in the EESI-MS/MS experiment (the insert in Fig.4). The fragmentation patterns of amino acids in our tandem mass spectrometry analysis consistently agree with that in previous studies[18]. For example, we summarized the characteristic MS/MS fragmentation of the 10 typical amino acids in Table 1. The data demonstrated that EESI-MS can detect and identify amino acids from standard solutions as well as complex biological samples with minimal pretreatment.

3.3 Quantitative detection of amino acids

To obtain the calibration curves, a series of standard solutions were prepared by dissolving each amino acid into water with different concentrations (0.001, 0.002, 0.005, 0.010, 0.050, 0.1, 0.5, 1.0, 3.0 and 5.0 mg L^{-1}). In each calibration curve (Fig.5), the *X*-axis represents the amino acid concentrations, and the *Y*-axis shows the EESI-MS signal intensities. The LOD of this method was calculated to be 0.14–26.2 μg L^{-1} (*S/N* = 3) based on the measurements of a series of amino acid standards by using the following equation[19]:

$$LOD = c3\sigma/S \quad (1)$$

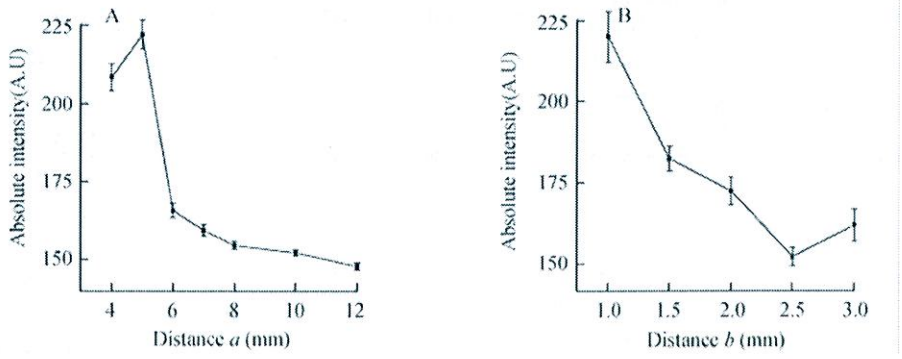


Fig.2 (a) Effects of distance a in Fig.1 on the signal intensity of arginine detected by EESI-MS/MS, and (b) effects of distance b in Fig.1 on the signal intensity of arginine detected by EESI-MS/MS

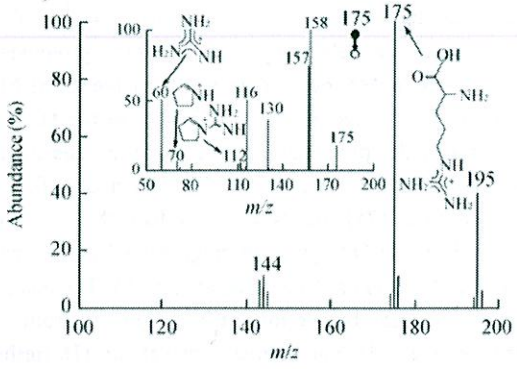


Fig.3 EESI-MS spectrum of arginine
Inset:EESI-MS/MS spectrum of arginine

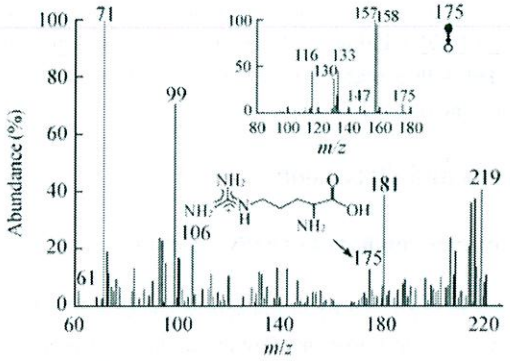
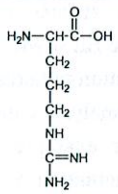
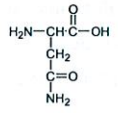
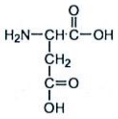
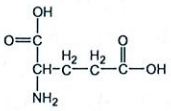
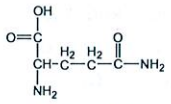
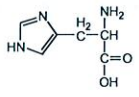
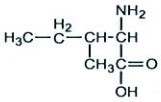
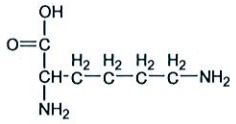
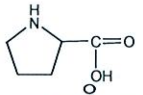
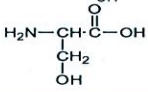


Fig.4 EESI-MS spectrum of a human urine sample (diluted 100 times)
Inset: EESI-MS/MS spectrum of arginine

Table 1 Summary of EESI-MS/MS spectra for 10 typical amino acids

No.	Amino acid	Molecular structure	M_W	$[M+H]^+$	CE (%)	Fragments (MS/MS)
1	Arginine		174	175	22	116 $[M+H-NH=C(NH_2)_2]^+$ 130 $[M+H-NH_3-CO]^+$ 70 $[M+H-NH=C(NH_2)_2-H_2O-CO]^+$ 158 $[M+H-NH_3]^+$ 157 $[M+H-H_2O]^+$
2	Asparagine		132	133	19	87 $[M+H-CO-H_2O]^+$ 116 $[M+H-NH_3]^+$
3	Aspartic acid		133	134	24	88 $[M+H-CO-H_2O]^+$ 116 $[M+H-H_2O]^+$
4	Glutamic acid		147	148	25	102$[M+H-CO-H_2O]^+$ 130$[M+H-H_2O]^+$
5	Glutamine		146	147	22	101 $[M+H-CO-H_2O]^+$ 130 $[M+H-NH_3]^+$
6	Histidine		155	156	22	110 $[M+H-CO-H_2O]^+$
7	Isoleucine		131	132	22	69 $[M+H-CO-H_2O-NH_3]^+$ 86 $[M+H-CO-H_2O]^+$ 115 $[M+H-NH_3]^+$
8	Lysine		146	147	19	84 $[M+H-CO-H_2O-NH_3]^+$ 130 $[M+H-NH_3]^+$ 129 $[M+H-H_2O]^+$
9	Proline		115	116	19	70 $[M+H-CO-H_2O]^+$
10	Serine		105	106	21	60 $[M+H-CO-H_2O]^+$ 88 $[M+H-H_2O]^+$

where, c is the amino acid concentration, σ is the standard deviation of all the measurements (n = 6), and S is the mean value of the 6 signals measured. Notably, for each data pointed out in Fig.5 and Table 2, six measurements were repeated and the relative standard deviation (RSD) values were 3.6%–9.5%. In general, the linear dynamic range was higher than 2 orders of magnitude.

3.4 Urine sample analysis

Generally, the urine volume from a healthy adult is 2 L per 24 h, in which the content of serine is approximately 777 μmol per 24 h (40.83 mg L^{-1}) and the content of arginine is approximately 26 μmol per 24 h (2.264 mg L^{-1})[20]. However, the exact concentrations of amino acids are affected by several factors such as collection time and health status. In this study, urine samples of healthy adults were diluted 100 times; the serine concentration was measured to be 125 μg L^{-1}, and the arginine level was determined to be 57 μg L^{-1}. These values are within the normal physiological range. For recovery experiments, a series of serine standard solutions at different concentrations of 100, 300 and 500 μg L^{-1} were respectively added into the urine samples (diluted 100 times). Serine was measured to be 225 μg L^{-1} (RSD = 2.2%, n = 6), 372 μg L^{-1} (RSD = 2.4%, n = 6), and 642 μg L^{-1} (RSD = 5.2%, n = 6), with the recovery of 100.7%, 82.6%, and 103.4%, respectively. The different concentrations of 50, 100, and 500 μg L^{-1} of arginine were also respectively added into the urine samples (diluted 100 times). The arginine was measured to be 98.8 μg L^{-1} (RSD = 11.4%, n = 6), 162.6 μg L^{-1} (RSD = 5.1%, n = 6), and 528 μg L^{-1} (RSD = 4.6%, n = 6) with the recoveries of 83.7%, 105.6% and 94.2%, respectively. The experimental data showed that EESI-MS/MS provides a relatively good reproducibility for measuring trace levels of amino acids in urine samples. More importantly, each measurement in this study can be finished within 0.5 min.

4 Conclusions

A high-throughput approach based on EESI tandem mass spectrometry was developed for the rapid detection of amino acids in complex biological samples with minimal pretreatment. We constructed and optimized an EESI ion source, and the qualitative and quantitative performance of our method was examined with both amino acid standards and human urine samples. The results showed that EESI-MS/MS provides the reliable, sensitive, and rapid detection of a wide concentration range of amino acids, which may lead to important discoveries in many research fields such as biological sciences.

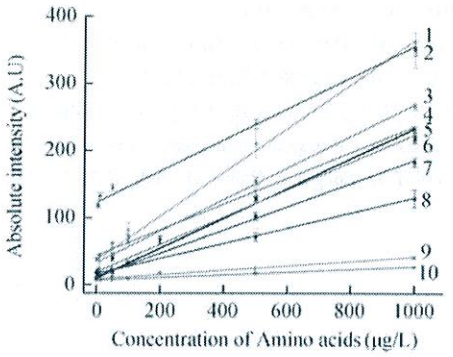


Fig.5 Calibration curves of 10 typical amino acids determined by EESI-MS/MS

Table 2 Summary of performance of EESI-MS/MS for quantitative detection of amino acids

Amino acid	Linear equation	R^2	Linear dynamic range (μg L^{-1})	LOD (μg L^{-1})	RSD (%, n = 6)
Lysine	$y = 36.287 + 0.328x$	0.984	10–1000	3.5	8.8
Proline	$y = 122.70 + 0.233x$	0.980	5–1000	0.5	3.6
Histidine	$y = 32.540 + 0.236x$	0.991	10–1000	4.0	4.6
Glutamine	$y = 43.234 + 0.193x$	0.974	1–1000	0.2	4.6
Arginine	$y = 9.413 + 0.224x$	0.992	10–1000	3.4	6.0
Isoleucine	$y = 18.789 + 0.205x$	0.991	2–1000	0.4	4.1
Serine	$y = 16.070 + 0.114x$	0.985	10–1000	6.3	9.5
Asparagine	$y = 11.752 + 0.174x$	0.994	1–1000	0.1	6.3
Aspartic acid	$y = 8.531 + 0.033x$	0.980	10–1000	2.0	5.0
Glutamic acid	$y = 6.536 + 0.021x$	0.999	10–1000	2.0	4.6
Glycine	$y = 1.163 + 0.0009x$	0.985	100–3000	26.2	5.7
Alanine	$y = 8.720 + 0.005x$	0.986	50–5000	6.9	7.1
Valine	$y = 118.681 + 0.073x$	0.994	10–1000	1.7	4.3
Leucine	$y = 24.278 + 0.181x$	0.986	10–1000	2.0	5.9
Threonine	$y = 4.609 + 0.006x$	0.982	1–3000	0.22	4.7
Phenylalanine	$y = 20.910 + 0.031x$	0.940	50–5000	4.3	4.0
Tryptophan	$y = 70.167 + 0.024x$	0.995	50–5000	2.5	6.1
Tyrosine	$y = 23.33 + 0.117x$	0.955	10–1000	2.0	5.5
Methionine	$y = 33.53 + 0.043x$	0.990	50–5000	7.0	7.1

References

[1] Xu G W, Yang J. *Chinese Journal of Chromatography*, **2003**, 21(4): 316–320

[2] Tang H R, Wang Y L. *Chinese Bulletin of Life Sciences*, **2007**, 19(3): 272–280

[3] Kaddurah-Daouk R, Kristal B S, Weinshilboum R M. *Pharmacology and Toxicology*, **2008**, 48: 653–683

[4] Chace D H, Kalas T A. *Clinical Biochemistry*, **2005**, 38(4): 296–309

[5] Wang T J, Larson M G, Vasan R S, Cheng S, Rhee E P, McCabe E, Lewis G D, Fox C S, Jacques P F, Fernandez C, O'Donnell C J, Carr S A, Mootha V K, Florez J C, Souza A, Melander O, Clish C B, Gerszten R E, *Nat. Med.*, **2011**, 17(4): 448–453

[6] Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza A L, Kafri R, Kirschner M W, Clish C B, Mootha V K. *Science*, **2012**, 336(6084): 1040–1044

[7] Asiago V M, Alvarado L Z, Shanaiah N, Gowda G A N, Owusu-Sarfo K, Ballas R A, Raftery D. *Cancer Research*, **2010**, 70(21): 8309–8318

[8] Kaspar H, Dettmer K, Gronwald W, Oefner P J. *J. Chromatogr. B*, **2008**, 870(2): 222–232

[9] Li P F, Wang Y, Tao B B, Wang J, Luo J, Zhang X D, An Z L, Liu L H. *Chinese J. Anal.Chem.*, **2012**, 40(5): 762–767

[10] Wang Y H, Feng J L, Pan Z Q, Fang X X, Li B R. *Chinese Journal of Health Laboratory Technology*, **2006**, 16(2): 161–163, 239

[11] Waterval W A H, Scheijen J L J M, Ortmans-Ploemen M M J C, Habets-van der Poel C D, Bierau J. *Clinica Chimica Acta*, **2009**, 407(1-2): 36–42

[12] Min J Z, Hatanaka S, Yu H f, Higashi T, Inagaki S, Toyo'oka T. *Analytical Methods*, **2010**, 2(9): 1233–1235

[13] Zhai H Y, Cai P X, Chen Z G, Li G H, Ye X, Mo J Y. *Chem. J. Chinese Universities*, **2004**, 25(6): 1037–1039

[14] Strieglerová L, Kubáň P, Bocek P. *J. Chromatogr. A*, **2011**, 1218(37): 6248–6255

[15] Ding J H, Yang S P, Liu Q, Wu Z Z, Chen H W, RenY L, Zheng J, Liu Q J. *Chem. J. Chinese Univesities*, **2009**, 30(8): 1533–1537

[16] Chen H W, Yang S P, Li M, Hu B, Li J Q, Wang J. *Angew. Chem. Int. Ed.*, **2010**, 49(17): 3053–3056

[17] Law W S, Wang R, Hu B, Berchtold C, Meier L, Chen H W, Zenobi R. *Anal. Chem.*, **2010**, 82(11): 4494–4500

[18] Piraud M, Vianey-Saban C, Petritis K, Elfakir C, Steghens J P, Morla A, Bouchu D. *Rapid Communications in Mass Spectrometry*, **2003**, 17(12): 1297–1311

[19] Ding J H, Gu H W, Yang S P, Li M, Li J Q, Chen H W. *Anal. Chem.*, **2009**, 81(20): 8632–8638

[20] Gong D Y. *Clinical Laboratory Medicine*. Beijing: Higher Education Press, **2007**: 101–181