



## Short communication

## Rapid resolution liquid chromatography (RRLC) analysis of amino acids using pre-column derivatization

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## ABSTRACT

A rapid resolution liquid chromatography (RRLC) method was developed for the simultaneous determination of 23 amino acids in rat serum after pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). The amino acid derivatives were separated on an Agilent Zorbax Eclipse Plus C<sub>18</sub> (4.6 mm × 50 mm, 1.8 μm) column at 45 °C. Ultraviolet (UV) detection was set at 360 nm. Good separation of 23 amino acids was achieved within 10 min with a ternary gradient elution of mobile phase at a flow rate of 1.5 mL min<sup>-1</sup>. Calibration curves were linear over the range from 1 to 500 μmol L<sup>-1</sup> with coefficients 0.9962 or better for each amino acid. The lower limits of quantification (LLOQ) of all 23 amino acids were 1 μmol L<sup>-1</sup> with signal-to-noise (S/N) ratio ≥ 4. Intra- and Inter-day precisions, expressed as relative standard deviation (RSD) percentages, were ranged from 0.32% to 3.09% and 0.67% to 5.82%, respectively. Finally, it was successfully applied to the determination of amino acids in rat serum with recoveries ranged from 90.8% to 106.0% and RSD percentages ranged from 1.78% to 4.68%, respectively. The results showed that the proposed method provided a shorter elution time, better resolution and sharper peak shapes for all amino acids. Compared with the conventional high performance liquid chromatography (HPLC) methods, even some ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), the established RRLC method was superior performance.

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## 1. Introduction

In yeast, animals and plants, amino acids play fundamental roles in a multitude of processes. They are not only the basic building blocks of proteins, but also the precursors for biosynthesis of numerous important biological and physiological compounds, which play essential roles in energy metabolism, neurotransmission and lipid transport. Amino acids analyses in many fields, such as in foods, feedstuff, drug quality control, biotechnology and biomedicine are very important [1–4].

Free amino acids can be analyzed by liquid chromatography (LC) [5–7], gas chromatography (GC) [8,9] and capillary electrophoresis (CE) [10–12]. However, the high polarity, low volatility and zwitterionic character of amino acids make simultaneous separation and detection of that so many rather similar compounds more difficult. Usually, derivatization approaches are developed to improve their separation and detection by increasing analytes volatility (for GC–FID/MS analysis) [8,9] or creating amino acid derivatives with strong chromophore/fluorophore groups (for LC or CE–ultraviolet (UV)/fluorescence analysis) [6,10]. Although a number of alternative analytical methods such as CE [11,12], electrochemical [13] and

evaporative light scattering detection [14] have been proposed for the determination of underivatized amino acids, these approaches have not been widely used due to limitations including low sensitivity, incompatibility with gradient elution, requiring a volatile mobile phase and inability of analyzing complex biological sample. To some extent, using liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) [15–17] has been shown to be a very sensitive technique for the determination of underivatized amino acids, but apart from involving shorter analysis time and not subjecting to chromatographic interference from the co-eluting compounds, the LC–MS/MS technique is very expensive and not available in many research laboratories. Owing to these reasons, high performance liquid chromatography (HPLC) based on pre- or post-column derivatization is also the most commonly used method for amino acids analysis.

In recent years, with the development of LC technology, LC system has been greatly improved on the basis of traditional HPLC system, i.e., ultra high pressure liquid chromatography (UPLC) and rapid resolution liquid chromatography (RRLC) [18,19]. The latter two systems are greatly superior to the former, and leads to equivalent separations at greatly reduced analysis time and solvent consumption by the combination of shorter columns and smaller particles. RRLC and UPLC methods have shown promising developments in the area of fast chromatographic separations and become the most frequently applied approaches, especially in the field of

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complex biologically derived mixtures analysis, where it is important to increase throughput and reduce analysis costs. Thus, it is advisable to select UPLC or RRLC as chromatographic separation method for the analysis of amino acids.

In the current study, a RRLC method was performed for the simultaneous determination of 23 amino acids in rat serum after pre-column derivatization with 2,4-dinitrofluorobenzene (DNFB). To the authors' knowledge, it was the first time that the RRLC method based on DNFB derivatization used for amino acids analysis. The proposed method provided a shorter elution time, better resolution and sharper peak shapes for all amino acids, and it was successfully applied to the quantitative analysis of 23 amino acids in rat serum with a total elution time of 10 min.

## 2. Experimental

### 2.1. Chemicals and reagents

L-Amino acids were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). 2,4-Dinitrofluorobenzene (DNFB) was purchased from Gracia Chemical Technology Co., Ltd. (Chengdu, China). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals of analytical-reagent grade were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ultrapure water used to prepare all aqueous solutions was from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.2. Preparation of standard and derivatization solution

A stock solution containing a mixture of 23 amino acid standards was prepared in water at an individual concentration of  $1.0 \text{ mmol L}^{-1}$ . Six calibration solutions at different concentrations ( $1, 10, 25, 100, 250$  and  $500 \mu\text{mol L}^{-1}$ ) were prepared by serially diluting appropriate amounts of the stock solution with  $0.2 \text{ M}$  borate buffer (pH 9.0). Similarly, quality control (QC) samples were prepared at four concentrations:  $1, 10, 250$  and  $400 \mu\text{mol L}^{-1}$  (lower limit of quantification (LLOQ), low, middle and high).

The derivatization solution was prepared by dissolving  $0.6052 \text{ g}$  DNFB in  $50 \text{ mL}$  acetonitrile to a concentration of  $72 \text{ mmol L}^{-1}$ .

### 2.3. Derivatization

The pre-column derivatization of amino acids with DNFB was a modified version of the reported Refs. [20–22]. To  $200 \mu\text{L}$  of the sample (serum or amino acids standard solutions),  $600 \mu\text{L}$  of acetonitrile was added and vigorously vortexed for 1 min. After the mixture was centrifuged at  $13,000 \text{ rpm}$  for 10 min at  $4^\circ\text{C}$ ,  $600 \mu\text{L}$  of the supernatant was evaporated to dryness at  $45^\circ\text{C}$  under a gentle stream of nitrogen. To the residue,  $200 \mu\text{L}$  of  $0.2 \text{ M}$  borate buffer (pH 9.0) and  $100 \mu\text{L}$  of DNFB solution were added, and the derivatization reaction was carried out on a water bath ( $60^\circ\text{C}$ ) for 60 min in the dark. After cooling in ice-cold water,  $700 \mu\text{L}$  of phosphate buffer solution (pH 7.0) was added to the reaction solution. The resulting solution of DNFB derivatives was then briefly vortexed again and transferred through a  $0.45 \mu\text{m}$  nylon filter into auto-sampler vials for injection.

### 2.4. Rapid resolution liquid chromatography

The RRLC analysis was carried out on an Agilent 1260 series rapid resolution liquid chromatography system (Agilent Technologies, Waldbronn, Germany) which consisted of a G1311B quaternary pump equipped with on-line vacuum degasser, a G1329B refrigerated model SL auto-sampler, a G1316A column

**Table 1**

Gradient elution program used for the separation of amino acids derivatives under RRLC conditions.

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0	90	5	5
4	78	17	5
5	78	22	0
7	67	33	0
9	50	50	0
10	90	5	5

oven and a G1315C diode array detector. Chromatographic separations were performed on an Agilent ZORBAX Eclipse Plus  $\text{C}_{18}$  column ( $4.6 \text{ mm} \times 50 \text{ mm}$ ,  $1.8 \mu\text{m}$ ; Agilent Technologies). Column temperature was maintained at  $45^\circ\text{C}$ . The mobile phase consisting of  $10 \text{ mM}$  ammonium acetate solution (A), acetonitrile (B) and methanol (C) was carried out as Table 1 in a gradient elution mode with a flow rate of  $1.5 \text{ mL min}^{-1}$ . UV detection was set at  $360 \text{ nm}$ , and the injection volume was  $5 \mu\text{L}$ .

## 3. Results and discussion

### 3.1. Derivatization procedure

DNFB was chosen for derivatization since it allows detection in UV region and can react with primary and secondary amines to form yellow colored products through nucleophilic aromatic substitution reaction in aqueous borate buffer and the excess reagent does not interfere. Moreover, DNFB amino acid derivatives have been found to be very stable under common laboratory conditions. Due to these advantageous performances, pre-column DNFB derivatization has been used in our study.

### 3.2. Optimization of chromatographic separation conditions

Analytical conditions for the analysis of 23 amino acid derivatives using RRLC and traditional HPLC system were optimized and compared, respectively. The best chromatographic separation conditions were presented and discussed here.

The separation of the derivatized amino acids was initially carried out on a Varian 210 high performance liquid chromatograph (Palo Alto, CA, USA) equipped with a 325 UV-vis detector and a Varian Star Chromatographic workstation. Chromatographic separations were carried out on an Agilent Zorbax Eclipse Plus  $\text{C}_{18}$  ( $4.6 \text{ mm} \times 250 \text{ mm}$ ,  $5 \mu\text{m}$ ) and a Waters XBridge  $\text{C}_{18}$  ( $4.6 \text{ mm} \times 250 \text{ mm}$ ,  $5 \mu\text{m}$ ) column, respectively. The latter showed better performance. A satisfactory resolution of the derivatized amino acids was obtained with a binary mobile phase of  $25 \text{ mM}$  ammonium acetate solution (A) and acetonitrile (B) (Table 2) at a flow rate of  $1.0 \text{ mL min}^{-1}$ . A total analysis time of 50 min was obtained at  $45^\circ\text{C}$  after injection of  $20 \mu\text{L}$ .

The subsequent study was focused on optimizing the separation of the derivatized amino acids under RRLC. Chromatographic separations were carried out on an Agilent Zorbax Eclipse XDB  $\text{C}_{18}$

**Table 2**

Gradient elution program used for the separation of amino acids derivatives under HPLC conditions.

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
25	78	22
35	72	28
40	65	35
45	62	38
46	50	50
50	90	10

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