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**RESEARCH PAPER**

# Array-Based Two Dimensional Liquid Chromatography System for Proteomic Analysis of Human Plasma

**HUANG Zhi[1], DENG Nan[2], YAN Guo-Quan[1], GAO Ming-Xia[1], LIANG Zhen[2], ZHANG Li-Hua[2,*], ZHANG Xiang-Min[1,*], ZHANG Yu-Kui[2]**

[1] Department of Chemistry & Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China
[2] Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

**Abstract:** The human plasma proteome has the characteristics of complexity in component and large dynamic range of protein concentrations. Herein, an array-based online two dimensional liquid chromatography system combined with protein equalizer technology was developed for the large-scale depletion of high abundance proteins and enrichment of low abundance proteins in human plasma. The array-based online two dimensional liquid chromatography system could be used to separate the plasma at the intact protein level with good reproducibility and high throughput. The total separation time was only 4 h and the fast location of high abundance proteins was also achieved. After the high abundance protein fractions was treated by ampholine@PM polymer microsphere, the number of identified low abundance proteins increased ten-fold, which significantly decreased the loss of low abundance proteins in high abundance protein fractions. The techniques combined were then applied to perform the proteomic analysis of human plasma sample. The total number of identified proteins was 1474 and the dynamic range of protein concentration was 7. In this work, 252 proteins were identified in high abundance protein fractions, among which 61 proteins belonged to high abundance proteins. These results demonstrated that an array-based online two dimensional liquid chromatography system combined with protein equalizer technology could efficiently achieve the large-scale depletion of high abundance proteins and the enrichment of low abundance proteins in human plasma, with a remarkable improvement in protein identification and a great prospect in the proteomic research of other complex samples.

**Key Words:** Multidimensional liquid chromatography; Array-based column system; High abundance protein; Protein equalizer technology; Human plasma

## 1 Introduction

Nowadays, life science research has entered the functional genomics era after the completion of the Human Genome Project[1]. Human plasma is the most commonly used sample in clinical diagnosis, thus being a hot spot of proteomics research. However, the dynamic range of protein concentration spans over 10 orders of magnitude in human plasma, in which the top 20 high abundance proteins (HAPs) make up 99% of the total protein content[2,3]. As a result, the severe masking effect caused by HAPs leads to the significant interference for the MS detection of low abundance proteins (LAPs). Therefore, it is essential to deplete HAPs in human plasma to improve the performance of biomarker discovery[4,5].

Compared with antibody-immunoaffinity column for the HAP depletion[6–9], multidimensional liquid chromatography (MDLC) is a kind of universal sample pretreatment techniques, which could deplete dozens of unknown HAPs in a single assay[10,11]. Previously, an offline ion exchange (IEX)/RPLC 2D-LC system was developed to separate rat liver at intact

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* Corresponding author. Email: lihuazhang@dicp.ac.cn; xmzhang@fudan.edu.cn
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protein level, resulting in 58 HAPs depleted and three times increase in the number of LAPs identified[10,11]. Recently, a high-throughput online array-based 2D-LC system, constructed by our group, has been successfully used for the separation of intact protein in human plasma with a significantly less time for the whole 2-D separation, thus endowing a great potential for the depletion of HAPs and comprehensive proteomic research in human plasma[12].

Whatever kind of high abundance protein depletion strategy adopted, part of low abundance proteins may also exist in the depleted HAP fractions due to the interaction with high abundance proteins. Therefore, how to enrich low abundance proteins from the depleted HAP fractions is still a great challenge urgently needed to be addressed[13]. Protein equalizer technique has the characteristics of equal opportunity in the interaction with a variety of proteins, thus narrowing the dynamic range of high and low abundance protein concentrations and enriching low abundance proteins[14]. After treated by ampholine@PM polymer microspheres, the number of proteins identified doubled in human plasma[15], demonstrating the effectiveness of ampholine@PM polymer microspheres for the enrichment of low abundance proteins from the HAP fractions.

In this study, an online array-based 2D-LC system was adopted to separate human plasma at intact protein level with a shortened whole separation time, thus achieving high-throughput and fast separation for human plasma. To deal with the significant interference by HAPs for the MS detection of LAPs, fast location of high abundance protein fractions was achieved and then the HAP fractions and LAP fractions were further treated separately. Meanwhile, ampholine@PM polymer microspheres were used to enrich low abundance proteins from the HAP fractions to further improve the identification of low abundance proteins. In this way, this strategy provided a simple and promising technique for the discovery of new disease biomarkers in human plasma.

## 2 Experimental

### 2.1 Reagents and instruments

HPLC grade trifluoroacetic acid (TFA), iodoacetamide (IAA), dithiothreitol (DTT), l-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (bovine pancreas), bovine serum albumin (BSA) and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Milford, MA, USA). Bicinchoninic acid (BCA) concentration assay kit was provided by Beyotime Institute of Biotechnology (Haimen, China). Healthy human plasma was obtained from the Huashan Hospital (Shanghai, China). All other chemicals were of analytical-reagent grade.

Array-based 2D-LC system (Fig.1) was constructed on the basis of Shimadzu LC 2010A system (Shimadzu Co. Japan). A ten-port electrically actuated multi-position valve, a six-port two-position valve, a ten-channel flow splitter and three-way micro-splitter valves were purchased from Valco Instruments Corporation (Houston, TX, USA). The first dimensional column was a ProPac™ SAX-10 column (10 μm, non-porous, 250 mm × 4.0 mm i.d., Dionex, USA) with the corresponding guard column (50 mm × 4.0 mm i.d.). The second dimensional columns were eight Xtimate™ C8 columns (5 μm, 30 nm, 250 mm × 2.1 mm i.d., Welch Materials, Inc., Shanghai, China) with eight corresponding guard columns (10 mm × 2.1 mm i.d.). A Probot microfraction collector (LC Packings, Dionex, USA) was used to collect the second dimensional effluents. BCA concentration assay was measured by Epoch microplate spectrophotometer (BioTek Corp., USA).

### 2.2 Analytical conditions for human plasma

#### 2.2.1 SAX separation for human plasma

The solvent system of SAX separation consisted of mobile phase A (10 mM Tris-HCl, pH 8.0) and mobile phase B (10 mM Tris-HCl, 500 mM NaCl, pH 8.0). 100 μL of plasma solution (1 mg of proteins) was separated on the SAX column. The flow rate was set at 0.5 mL $min^{-1}$. The UV absorption wavelength was set at 215 nm. The linear gradient was shown as follows: 0–5 min, 100% A; 5–15 min, 0–10% B; 15–45 min, 10%–25% B; 45–70 min, 25%–40% B; 70–90 min, 40%–67% B; 90–91 min, 67%–100% B; 91–101 min, 100% B; 101–102 min, 100%–0 B; 102–150 min, 100% A.

The switching program for the fraction transfer to the second dimensional trapping columns was set as follows: 0–19 min, Fraction 1; 19–35 min, Fraction 5; 35–68 min, Fraction 2; 68–73 min, Fraction 6; 73–78 min, Fraction 7; 78–83 min, Fraction 8; 83–100 min, Fraction 3; and 100–130 min, Fraction 4.

#### 2.2.2 2D RP separation for SAX fractions

The solvent system of RPLC separation consisted of mobile phase A (5% ACN, 0.1% TFA) and mobile phase B (95% ACN, 0.1% TFA). The pump flow rate was 1.6 mL $min^{-1}$ and the split column flow rate was 0.2 mL $min^{-1}$ on average. The gradient was shown as follows: 0–5 min, 100% A; 5–15 min, 0–25% B; 15–25 min, 25%–33% B; 25–60 min, 33%–40% B; 60–80 min, 40%–54% B; 80–85 min, 54%–67% B; 85–85.1 min, 67%–100% B; 85.1–95 min, 100% B; 95–95.1 min, 100%–0 B; 95.1–110 min, 100% A. The effluents were collected at a 60-s interval for the further investigation.

### 2.3 BCA concentration assay for effluents