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Research Article

Analysis of urinary metabolites for metabolomic study by pressurized CEC

A new approach for the metabolomic study of urinary samples using pressurized CEC (pCEC) with gradient elution is proposed as an alternative chromatographic separation tool with higher degree of resolution, selectivity, sensitivity, and efficiency. The pCEC separation of urinary samples was performed on a RP column packed with C_{18}, 5 μm particles with an ACN/water mobile phase containing TFA. The effects of the acid modifiers, applied voltage, mobile phase, and detection wavelength were systematically evaluated using eight spiked standards, as well as urine samples. A typical analytical trial of urine samples from Sprague Dawley (S.D.) rats exposed to high-energy diet was carried out following sample pretreatment. Significant differences in urinary metabolic profiles were observed between the high energy diet-induced obesity rats and the healthy control rats at the 6th wk postdose. Multivariate statistical analysis revealed the differential metabolites in response to the diet, which were partially validated with the putative standards. This work suggests that such a pCEC-based separation and analysis method may provide a new and cost-effective platform for metabolomic study uniquely positioned between the conventional chromatographic tools such as HPLC, and hyphenated analytical techniques such as LC-MS.

Keywords:
Metabolites / Metabolomics / Pressurized capillary electrochromatography / Urine
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1 Introduction

Metabolomics or metabonomics, defined as the measurement of multiparametric metabolic responses of a biological compartment or a living system to pathophysiological stimuli or genetic modification [1–3], is an effective tool for toxicological studies, potential biomarker identification of diseases, and identification of regulatory network pathways in plants and animals [4–8]. Selectively monitoring characteristic variation of endogenous metabolites (MW $<$ 1000 Da) in a single cell, biofluids, or tissue, coupled with widely ascribed multivariate statistical analysis techniques such as principal component analysis (PCA), partial least square (PLS), and artificial neural networks (ANN), enables such a state-of-the-art tool for probing network integrity, systems diversity, and complexity of human individuals [9–13].

Increasingly sophisticated analytical techniques have been generating mega datasets for -omics studies. Most of the pioneering works aiming to large-scale metabolite analysis have been initiated by NMR [4, 7, 14–17], HPLC-MS, and LC-MS-MS [18–22], CE-MS [12, 23–25], and GC-MS [26–30], either alone or in combination. However, there is so far no single analytical tool capable of capturing the entire composition of endogenous metabolites due to the large number of metabolic molecules with diverse chemical properties. In this context, CEC, a hybrid technique integrating best features of CE and capillary HPLC, *e.g.*, highly efficient separation of CE, and versatile selectivity and ideal sample capacity of cHPLC, has been developed for the analysis of complex samples with a wide variety of chemical compositions [31]. It has the ability to analyze both charged and neutral substance at relatively low concentration of analytes and consume relatively low volume of mobile phase. Compared to traditional HPLC and CE, mobile phase in pressurized CEC (pCEC) system is driven by a pressurized flow and an EOF simultaneously, reducing band broadening and improving separa-

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Abbreviations: capLC, capillary LC; **ECF,** ethyl chloroformate; **FA,** formic acid; **HAC**, acetic acid; **HED,** high-energy diet; **PA,** phosphoric acid; **PCA,** principal component analysis; **pCEC,** pressurized capillary electrochromatography; **PLS-DA,** projections to latent structures-discriminant analysis; **S.D.,** Sprague Dawley; **VIP,** variable importance

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tion efficiency. Such dual mechanisms (LC and CE) make CEC an ideal technique for the analysis of complicated samples such as biological fluids. Additionally, gradient elution is applied in pCEC analytical protocols to enhance the separation of small molecule metabolites with similar chemical properties.

The pCEC separation method was applied, for the first time, in metabolomic study with the primary aim of developing an alternative chromatographic method for metabolic profiling of biological fluids. Urinary samples from Sprague Dawley (S.D.) obesity rats induced by high-energy diet (HED) at predose and the 6th wk postdose were analyzed in comparison with healthy control rats. The system reproducibility, linearity, and robustness have been evaluated. The effects of the concentration of the acid modifiers, applied voltage, detection wavelength, and the type of acid modifiers on separation of the spiked standards and urine samples were also investigated.

2 Materials and methods

2.1 Reagents and materials

Ethyl chloroformate (ECF), pyridine, anhydrous ethanol, sodium hydroxide, chloroform, anhydrous sodium sulfate, phosphoric acid (PA), acetic acid (HAC), TFA, and formic acid (FA) were analytical grade from China National Pharmaceutical Group Corporation (Shanghai, China). L-2-Chlorophenylalanine (Shanghai Intechem Tech, China) was used as an internal standard. Homogentisate, methionine, melatonin, tyrosine, tyramine, tryptophan, phenylalanine, and dopamine were purchased from Sigma–Aldrich (St. Louis, MO, USA). ACN and methanol (CH_3OH) (HPLC grade) were commercially obtained from Merck (Darmstadt, Germany). Ultra-pure water was prepared with the Millipore Milli-Q SP water purification system (18.2 MΩ, Millipore, MA, USA).

Stock solutions of eight standards (homogentisate, methionine, melatonin, tyrosine, tyramine, tryptophan, phenylalanine, and dopamine) were prepared in ultra-pure water (1 mg/mL). Likewise, aqueous solutions of these compounds were employed to obtain the calibration curves and quantification limits.

2.2 Dosing and sample collection

After acclimation of 1 wk in metabolic cages, 13 male S.D. rats (190 ± 10 g) were randomly divided into two groups: the HED-treated group ($n = 7$) and the healthy control group ($n = 6$). HED-treated group rats were fed with HED (10 lard, 5 corn oil, 10 sucrose, 10 casein, and 65% standard rat chow) for 6 wk; meanwhile the healthy control rats were fed with standard rat chow. Twenty-four hours urine of these rats was collected at predose and the 6th wk, respectively. All the collected urine samples were centrifuged, and immediately stored at −80°C pending pCEC analysis.

2.3 Derivatization procedures

The derivatization of the urine samples was performed according to our previously developed method [32, 37]. Briefly, each 600 μL aliquot of standard mixture or diluted urine sample (urine:water = 1:1, v/v) was added to a screw-top glass tube. Followed by the addition of 100 μL of L-2-chlorophenylalanine (0.1 mg/mL), 400 μL of anhydrous ethanol, and 100 μL of pyridine, 50 μL of ECF were transferred into the mixtures for the first derivatization at 20.0 ± 0.1°C. Such a procedure was repeated with the addition of 50 μL of ECF into the products after sonication, chloroform extraction, and pH adjustment. The resulting mixtures were vortexed for 30 s, then centrifuged for 3 min at 3000 rpm. The aqueous layer was aspirated off, while the remaining chloroform layer containing derivatives was isolated, concentrated till dryness. The residue was then dissolved in methanol by ultrasonication (250 W) and subjected to pCEC analysis.

2.4 Gradient pCEC analysis

All pCEC separation was performed on a Trisep™-2100 CEC system (Unimicro Technologies, Pleasanton, CA) which comprised a Unimicro binary microsyringe pump, a high-voltage power supply (+30 kV and −30 kV), a Valco six-port injection valve, a UV–Vis variable wavelength detector equipped with a cell for on-column detection, and a Unimicro Trisep™ workstation 2003, as described in literature [33]. A continuous mobile phase was generated by merging two solvent flows in a mixer and entered Valco six-port injection valve with microsyringe pump. Samples injected were delivered to the injection valve and introduced in the external 5 μL sample loop, and then carried to the four-port split valve by the mobile phase flow. After splitting in the four-port valve (split ratio 400:1), the flow entered a capillary column. A constant pressure of 13 000 kPa was applied to the column inlet during the separation. A negative voltage was applied to the outlet of the column, and the inlet of the column was connected to the split valve and grounded.

A RP column (EP-150-30/50-5-C18) of 50 cm (of which 30 cm was packed) × 150 μm id packed with 5 μm C_{18} particles was supplied by Unimicro Technologies. Detection windows (~2 mm) were burned into the column walls. A 2 kV voltage was applied across the capillary to produce EOF. The flow-rate was 0.08 mL/min and the injection volume was 5 μL. The data was collected directly from the UV detector at wavelength of 214 nm and analyzed using the Unimicro Trisep workstation 2003.

The mobiles phase contained water (0.01% v/v TFA) (A) and 95% v/v ACN (0.01%TFA) (B), filtered by 0.22 μm HPLC filters and separation was achieved using the following gradient: 0–30 min, 0–50 % B; 30–40 min, 50–75% B. Prior to use, the samples and pCEC mobile phases were sonicated in an ultrasonic bath for 15 min at room temperature to remove any air bubbles. The injection volume was 5 μL. The electro-

chromatograms shown in Fig. 6 were obtained using the aforementioned commercial C_{18} packed column (EP-150-30/50-5-C18, Unimicro Technologies). All pCEC separations were performed using 13 000 kPa pressure applied to the end of the column to prevent bubble formation and the capillary was kept at a constant temperature of 25°C. The spiked standard solution and the urine samples were recorded using a UV detector operated at four different wavelengths (214, 220, 240, and 260 nm), respectively. Since the best S/N was observed at 214 nm, this wavelength was used throughout the remainder of this study.

2.5 Data processing and pattern recognition

Peak area paired retention time of each compound detected was obtained using the Unimicro Trisep workstation 2003. The detected peaks were normalized to the total sum of these peak areas in Matlab software 7.0 (The MathWorks) in order to eliminate the disparity of urine volume. PCA and projections to latent structures-discriminant analysis (PLS-DA) [34, 35] were carried out in SIMCA-P 11.0 (Umetrics, Umeå, Sweden) using mean-centered, auto-scaled (scaled to unit variance) data. The PCA scores plot represents the distribution of samples in multivariate space where each coordinate denotes each subject. The rank of variable importance (VIP) values of PLS-DA loading [36] is used to identify the differential metabolites between groups (the HED-treated group and the healthy control group). In order to validate the model against overfitting, a typical seven-round cross-validation was performed with 1/7th of the samples being excluded from the model in each round. This procedure was repeated in an iterative manner until each sample had been excluded once and the corresponding Q^2Y value was obtained from the results in SIMCA-P package. R^2Y provides an estimate of how well the model fits the Y data whereas Q^2Y is an estimate of how well the model to predict the Y. The cumulative values of R^2Y and Q^2Y close to 1 indicate an excellent model.

2.6 Univariate statistical analysis

Based on thresholds on the *p*-values of classical one-way ANOVA implemented in the SPSS 14.0 software (SPSS, USA), the differentially expressed metabolites from multivariate statistical analysis were further verified at a univariate analysis level. The critical *p*-value was set to 0.05 in this study.

3 Results and discussion

3.1 Derivatization procedure

It is essential to pretreat biological samples, *e.g.*, serum, urine, and tissue prior to pCEC analysis because the capillary column can easily be contaminated. Initial studies with direct injection of rat urine using HPLC system have shown that substances in urinary sample, mainly proteins, rapidly contaminated the column.

A simple, commonly used method for sample pretreatment is organic solvent precipitation, where loss of a variety of important metabolites may occur during the coprecipitation of proteins [37]. Our previous studies [38] indicated that GC-MS analysis with ECF derivatization was able to identify a wide range of endogenous metabolites involving amines, amino acids, fatty acids, and amino-alcohols. In addition, ECF-derivatization method improved the UV absorption of these compounds and changed their polarity, leading to a higher sensitivity and better separation. Such a sample pretreatment procedure involving chemical derivatization and the subsequent chloroform extraction can readily remove proteins in the urine sample and increase the concentration for detection. This is considered necessary for pCEC since its packed capillary column is more susceptible to contamination or blockage than the HPLC system. Therefore, an ECF derivatization procedure was adopted for our use with pCEC analysis of urinary samples. The derivatization process was thoroughly studied and optimized using the spiked standards and rat urinary samples available.

A total of 48 standards of amino acids, amines, and fatty acids were chosen as the references for identification of metabolites in the urine. Initial studies indicated that eight compounds present in the urine could be identified and confirmed by pCEC analysis. Thus, the separation was optimized on the basis of the selected eight standards. In addition, the influence of operating parameters such as type of acid modifiers, concentration of selected acid modifier, organic solvent composition, applied voltage, and elution condition was studied to optimize the pCEC separation of the rat urines.

3.2 Extraction and derivatization efficiencies

To study the derivatization efficiencies of the metabolites, test analytes involving the eight standards and urine samples were derivatized according to the method of Section 2. The chloroform solution was dried by adding a small portion of anhydrous sodium sulfate. The dry organic solution was concentrated and the residue was then dissolved in 500 μL of methanol by sonication. 5 μL was injected to the pCEC system directly. The extraction of the leftover aqueous solution by chloroform and subsequent pCEC analysis of this chloroform layer displayed no significant signal for the test analytes, indicating that the extraction efficiency was nearly 100%.

The aqueous solution remaining from the above derivatization process was rederivatized and extracted with chloroform. The pCEC analysis of the chloroform layer showed no significant signal for derivatized spiked standards and urinary metabolites and at the same time, examination of the remaining aqueous solution after the second derivatization process by pCEC indicated that the amount of remaining underivatized analytes was insignificant. This indicates that

almost all of the significant metabolites in the sample were derivatized through two derivatization processes.

3.3 Effects of acid modifiers

The effects of different acids, *e.g.*, HAC, FA, PA, and TFA on the separation of the eight spiked standards have been investigated. From Fig. 1, the addition of HAC into mobile phase decreased the peak sensitivity and resolution. In contrast, the general sensitivity, resolution, and peak shape of such eight test standards exhibited better when the pH of mobile phase was adjusted with FA, PA, or TFA, respectively. The satisfactory peak shape and resolution were achieved with TFA (Fig. 1). Additionally, the baseline noise was 50, 8, 5, and 5 μV using HAC, FA, PA, and TFA, respectively. To improve peak shape and resolution of the test standards, all other pCEC separation were performed using TFA as the acid modifier.

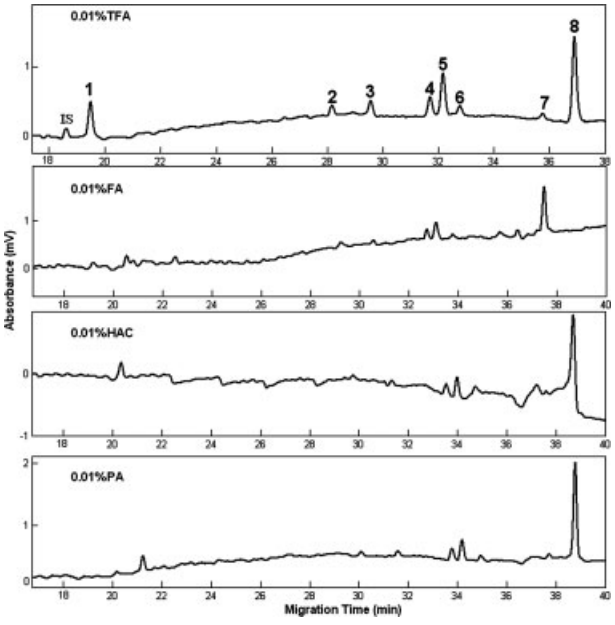

Figure 1. Effects of different acid modifiers on the pCEC separation of the eight spiked standard solution. pCEC conditions: column, 150 μm id, 50 cm total length, and 30 cm effective length packed with C_{18}, 5 μm particle size; applied voltage, 2 kV; injection volume, 5 μL; detection, UV at 214 nm. Key: 1, homogentisate; 2, methionine; 3, melatonin; 4, tyrosine; 5, tyramine; 6, tryptophan; 7, phenylalanine; 8, dopamine.

3.4 Effects of concentration of acid modifiers

Four mobile phases containing 0.005, 0.01, 0.03, and 0.05% of TFA have been studied (Fig. 2). The "apparent" pH value varied with the portion of acid in the mobile phase, which may influence separation of samples. For example, the pH became smaller with the increased acids, leading to worse separation of the spiked standards. However, the best peak shape and resolution was obtained by 0.01% TFA. In addition, the background current was 0.9, 1.3, 4.3, and 7.2 μA using 0.005, 0.01, 0.03, and 0.05% TFA, respectively. It is possible that the poor peak shape obtained from 0.03 and 0.05% TFA because of Joule heating caused by higher background current. To improve peak shape and resolution of the samples, 0.01% TFA was preferred in this work.

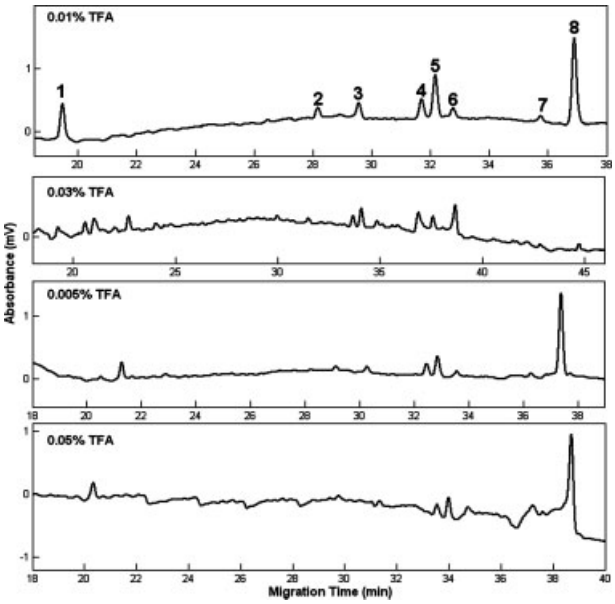

Figure 2. Effects of different concentrations of TFA on the pCEC separation of the eight spiked standard solution. Key: 1, homogentisate; 2, methionine; 3, melatonin; 4, tyrosine; 5, tyramine; 6, tryptophan; 7, phenylalanine; 8, dopamine.

3.5 Optimization of mobile phase composition

In order to obtain good resolution and peak shape, the effects of mobile phase composition were also investigated. Mobile phases including: (i) 95% v/v ACN (0.01% TFA)/water (0.01% TFA); (ii) 95% v/v ACN/water (0.01% TFA); (iii) 95% v/v ACN/water; (iv) 95% v/v CH_3OH (0.01%TFA)/water (0.01% TFA); (v) 95% v/v CH_3OH/water (0.01% TFA); (vi) 95% v/v CH_3OH/water were used. As shown in Fig. 3, the peak shape, resolution, and selectivity became better when using ACN instead of methanol with a reduced analysis time. Taking into consideration the resolution and speed of the analysis, 95% v/v ACN (0.01% TFA)/water (0.01% TFA) was used to further optimize separation conditions for the test standards and urine samples.

3.6 Effect of applied voltage

The influence of the applied voltage on the efficiency, resolution, and analysis time of the analytes was evaluated using a mobile phase of 95% v/v ACN (0.01% TFA)/water (0.01%

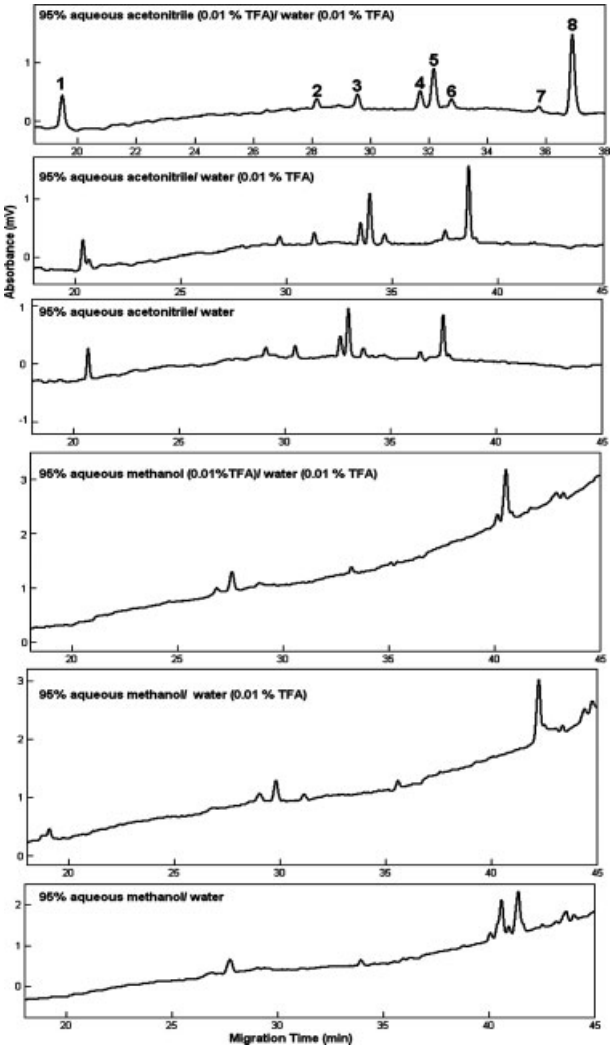

Figure 3. Effect of mobile phase composition on the pCEC separation of the eight spiked standard solution. (The details are denoted in the figure.)

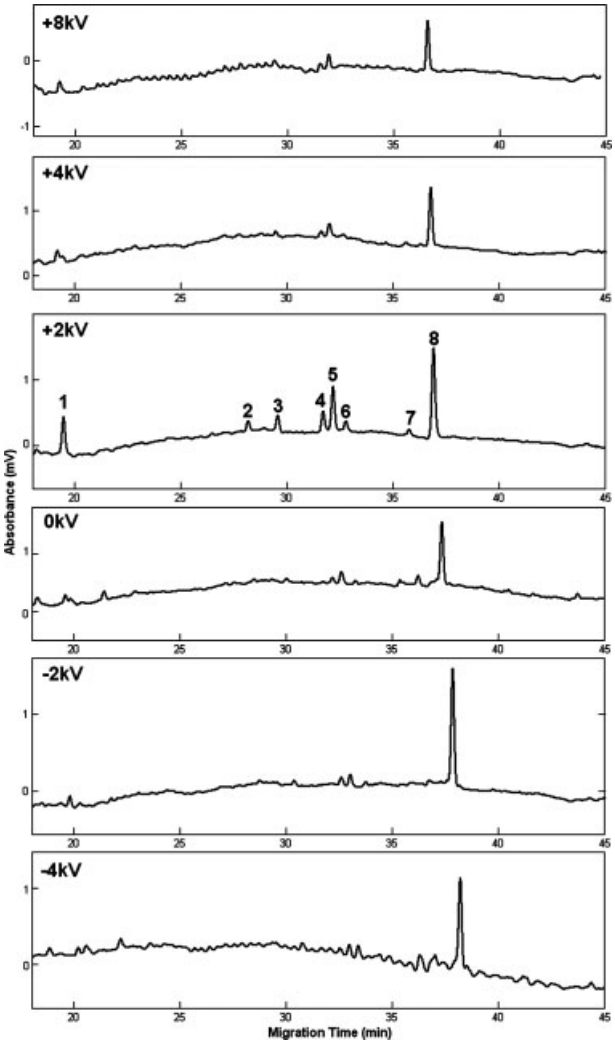

Figure 4. Influence of applied voltage on the pCEC separation of the eight spiked standard solution.

TFA), and the results are shown in Fig. 4. The separation of the spiked standards was improved by changing the applied voltage either from −4 to 2 kV or from 8 to 2 kV. A higher voltage reduces the retention time because of an increasing net velocity, but such high applied voltage makes the pCEC system unstable due to Joule heating caused by high background current. Separation at lower applied voltage, *e.g.*, negative voltage takes longer with less efficient peak separation. Based on these results, the best resolution with higher efficiency was obtained using 2 kV (see Fig. 4).

3.7 Condition of gradient elution

Since the above studies have demonstrated that the eight standards displayed better separation efficiency, ECF-derivatized urine samples were used to evaluate the influence of isocratic elution and different gradient elution conditions. As shown in Fig. 5, isocratic elution offers no obvious separation on the test solution. In contrast, gradient elution exhibits the better peak shape, resolution, and sensitivity, of which the best gradient elution condition was: 0–30 min, 0–50% v/v ACN (0.01% TFA); 30–40 min, 50–75% v/v ACN (0.01% TFA).

3.8 Method validation

The reproducibility, linearity, and sensitivity of the method were investigated (Table 1). The RSDs values ($n = 8$) were better than 1.8% for migration times and between 2.4 and 8.5% for peak areas, and little decrease in performance was observed over 100 consecutive runs. Calibration curves of peak area (y) *versus* concentration (x) were constructed. These curves showed good linearity over the concentration range

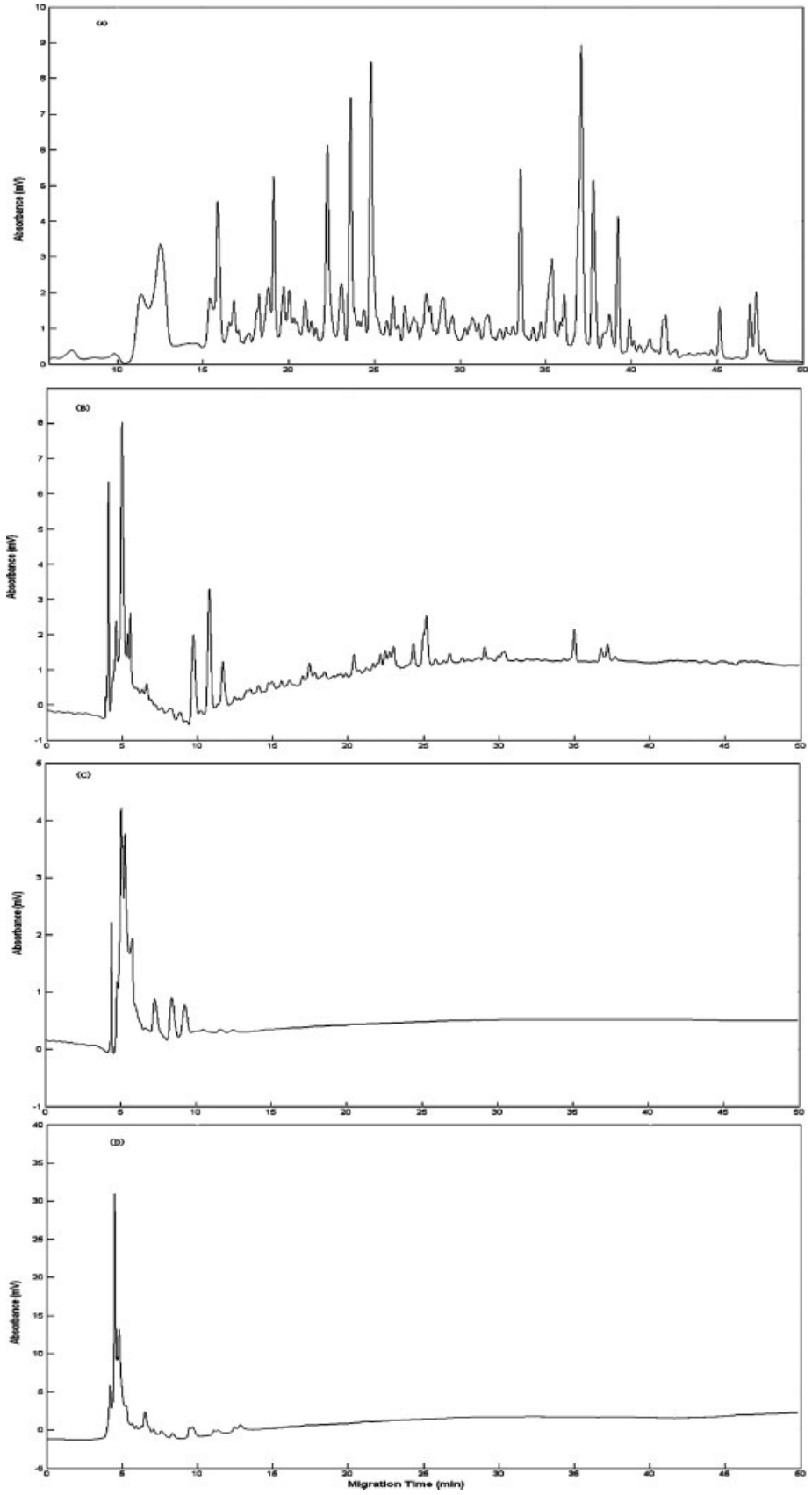

Figure 5. Capillary electrochromatograms (214 nm) of urine samples in the condition of isocratic and gradient elution. (A) Mobile phase: ACN (0.01%TFA)–water (0.01%TFA), gradient elution: 0–30 min, 0–50% v/v ACN (0.01% TFA), 30–40 min, 50–75% v/v ACN (0.01% TFA); (B) mobile phase: ACN (0.01%TFA)–water (0.01%TFA), gradient elution: 0–35 min, 25–75% v/v ACN (0.01%TFA); (C) isocratic elution (ACN (0.01%TFA)–water (0.01%TFA) = 70:30, v/v); (D) isocratic elution (ACN (0.01% TFA)–water (0.01% TFA) = 30:70, v/v).

Table 1. Lineary, reproducibility, and sensitivity

Compound	Linearity				RSD (n = 8, %)		LOQ (μg/mL)	S/N
	Range (μg/mL)	Calibration curves	n	R^2	Migration time	Peak area		
Homogentisate	1.25–500	$y = 701.75x + 5698.4$	9	0.9989	0.6	5.0	0.12	100
Methionine	1.25–500	$y = 278.47x + 762.93$	9	0.9988	1.1	2.9	0.10	30
Melatonin	1.25–500	$y = 388.87x + 1895.9$	9	0.9995	1.2	2.7	0.10	42
Tyrosine	1.25–500	$y = 541.58x + 2179.4$	9	0.9999	0.8	3.4	0.10	61
Tyramine	1.25–500	$y = 939.26x + 6114.8$	9	0.9990	1.3	6.3	0.15	130
Tryptophan	1.25–500	$y = 264.66x + 543.29$	9	0.9992	0.8	2.4	0.08	28
Phenylalanine	1.25–500	$y = 137.3x + 911.05$	9	0.9989	0.9	5.2	0.08	17
Dopamine	20–1000	$y = 239.1x + 1886.2$	6	0.9975	1.8	8.5	5.0	252

1.25–500 μg/mL for homogentisate, methionine, melatonin, tyrosine, tyramine, tryptophan, phenylalanine, and 20–1000 μg/mL for dopamine with correlation coefficients from 0.9975 to 0.9999. Quantification limit of each compound was determined using the S/N provided by Unimicro Trisep workstation 2003 (Table 1) and all the S/N values were higher than 10.0, allowing the individual calculation of each compound's quantification limit. The reproducibility was investigated using both the spiked standard solution and urine sample of a typical healthy rat. After the correction with internal standard, the RSDs of peak area for each standard spiked and standards (endogenous metabolites) detected in rat urine were comparable, RSDs below 10%. Additionally, the stability assay was evaluated using aforementioned spiked standards. For most spiked compounds, the RSDs were better than 6.5% within 24 h and less than 10% within 36 h. To further validate our methodology to analyze complex endogenous metabolites, each 20 μL aliquot of 8, 16, 24, 32, and 40 μg/mL of the spiked standard solution were mixed with 20 μL in pooled urine prior to ECF-derivatization. In general, the recovery was calculated through the aforementioned calibration curves, and the mean recoveries of all these compounds ranged from 90 to 110% with RSDs better than 10% (Table 2).

Table 2. Recovery of eight standards in urine analysis (n = 3)

Compounds	Recovery (%)[a)]	
	Average	RSD
Homogentisate	104.78	4.58
Methionine	107.74	4.07
Melatonin	93.58	3.09
Tyrosine	94.44	2.13
Tyramine	90.02	1.35
Tryptophan	94.74	3.57
Phenylalanine	95.5	2.17
Dopamine	94.06	3.20

a) Mean recovery was obtained by eight determinations (three parallel samples at five different concentrations).

3.9 pCEC analysis of urine samples

Typical pCEC-UV chromatograms of urine samples from the healthy control group and the HED-treated group at the 6th wk were shown in Figs. 6A and B, respectively, where the test spiked standards (Fig. 6C) were easily identified. Unlike chemically induced disease models, the urinary metabolic profiles from two groups of experimental rats shared a high degree of similarity since the HED is similar in composition to the normal rat food and does not exert a severe metabolic network perturbation. Using our optimized analytical protocol, clear difference between the pCEC profiles of control and treatment group were observed and further discerned using pattern recognition, indicating that the use of pCEC separation is potentially advantageous in differentiating subtle variations among metabolic profiles.

3.10 Pattern recognition

HED-induced obesity animal model was utilized to test the potential application of the pCEC method for metabolomic study. In this study, we identified about 150 different urine metabolites, of which eight compounds are confirmed *via* standard compounds. pCEC analysis of urine samples from the two groups (the HED-treated group, $n = 7$ *vs.* the healthy control, $n = 6$) at predose (Fig. 6E) and the 6th wk postdose (Fig. 6D) were illustrated. Visual examination of the chromatograms of pCEC displayed clear difference between groups. PCA scores plot (Fig. 6D) of pCEC data described the obvious separation between two different groups at the 6th wk postdose whereas little variation was observed at predose. PLS-DA revealed the differential metabolites in response to the diet, which were partially validated with the putative standards (Table 3). From Table 3, a number of marked changes were observed from the

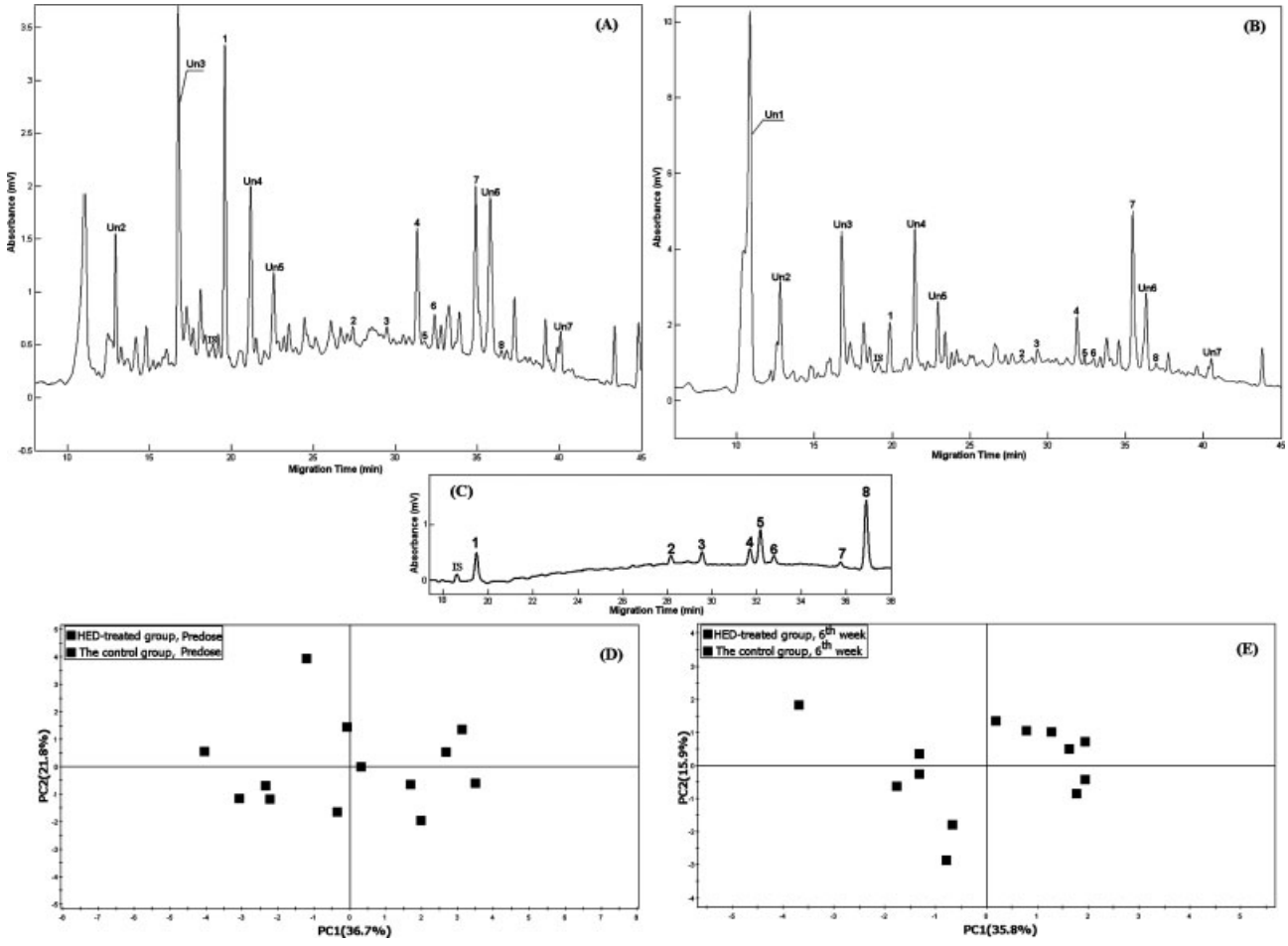

Figure 6. Comparison of pCEC-UV chromatograms of typical urine samples from the obesity rats treated with HED (B) or the control group (A). (C) pCEC-UV chromatograms of the eight spiked standards. (D) PCA scores plot of urine samples from the HED-treated and the healthy control rats at the 6th wk. (E) PCA scores plot of urine samples from the HED-treated and the healthy control rats at predose.

pCEC chromatograms of urine samples following diet intervention. The urinary expression levels of homogentisate, tyrosine, and tryptophan in the diet-induced group were significantly decreased as compared to the control group at the 6th wk, which is in good agreement with other reports [39, 40]. Meanwhile, the accumulation of urinary phenylalanine may be a result of HED on metabolic pathways through inhibiting the bio-transformation of phenylalanine into tyrosine as suggested in the literature [41].

The analysis of the endogenous urinary metabolites from these experimental rats indicated a general and significant change in metabolic activities. That is, after the HED intervention the animals entered into a state of "fat accumulation", involving a series of changes in metabolic network, such as disordered lipid metabolism, internal secretion, and energy metabolism. The "fat accumulation" and the subsequent "low-consumption" of the energy led to a state of obesity as evidence by animals' decreased activity, increased body weight, and serum lipid.

3.11 Comparison of HPLC, capillary LC, and pCEC

The urine and serum samples were performed on the three analytical instruments: HPLC, capillary LC (capLC), and pCEC. Results of HPLC, capLC, and pCEC analyses of urine (Fig. 7A, B, C) and serum (Fig. 7D, E, F) samples are shown. It is readily seen that, as compared to the conventional HPLC and capLC systems, pCEC is able to capture significantly more urinary metabolites with good sensitivity and resolution.

4 Concluding remarks

A convenient, quantitative analytical method using gradient pCEC with ECF derivatization for the analysis of *in vivo* metabolites has been developed. The method has been optimized and validated over an array of standards and urine samples, and applied for characterizing the metabolic pro-

Table 3. Summary of the differential metabolites from VIP values of two-component PLS-DA model ($R^2Y = 0.932$, $Q^2Y = 0.814$) in the HED-treated group at the 6th wk

Peak no.	Retention time	Metabolites identification	VIP rank
1	19.895	Homogentisate	1(↓)
2	27.655	Methionine	3(↑)
3	29.326	Melatonin	7(↓)
4	31.810	Tyrosine	8(↓)
5	32.287	Tyramine	10(↓)
6	32.869	Tryptophan	4(↓)
7	35.409	Phenylalanine	14(↑)
8	36.945	Dopamine	2(↑)
Un1	10.890	Unknown	9(↑)
Un2	12.850	Unknown	5(↑)
Un3	16.760	Unknown	11(↓)
Un4	21.478	Unknown	15(↓)
Un5	22.941	Unknown	13(↑)
Un6	36.276	Unknown	12(↓)
Un7	40.510	Unknown	6(↑)

(Compared to the healthy control group, ↑ represents significantly elevated concentration, whereas ↓ represents significantly lowered concentration).

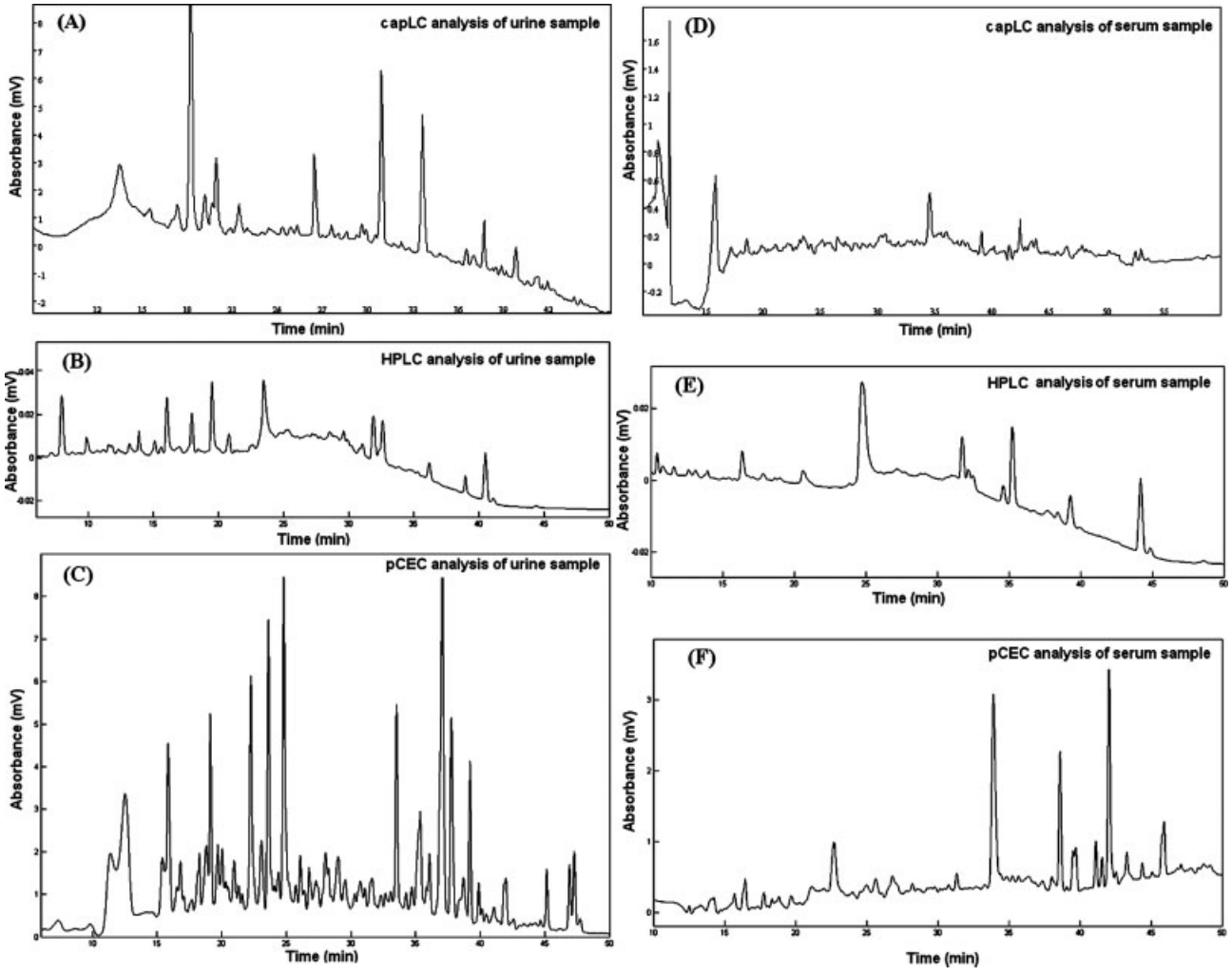

Figure 7. Comparison of HPLC, capLC, and pCEC analysis of urine (A, B, C) and serum (D, E, F) samples in the optimized condition. HPLC conditions: Waters Symmetry Shield RP18, 4.6 mm × 250 mm 5 μm; water (0.02% TFA) (A) and 95% aqueous methanol (0.02%TFA) (B) in the gradient mode as follows: 0–30 min, 50% B; 30–40 min, 50–75% B; 40–55 min, 75–90% B. UV at 214 nm; column temperature, 25°C; flow rate, 1 mL/min. capLC conditions: column, 150 μm id, 50 cm total length, and 30 cm effective length packed with C_{18}, 5 μm particle size; injection volume, 5 μL; detection, UV at 214 nm.

files of the obese rat model. Based on its simplicity, robust performance, and potentially wide applicability, this method is expected to play an important role in metabolomic study as a unique platform technology, which is positioned between the conventional chromatographic tools, such as HPLC, and the hyphenated analytical techniques such as LC-MS and LC-MS-MS. Further work is in progress to develop the hyphenated techniques, *e.g.*, pCEC coupled with MS (or Qtof-MS) which may provide more powerful approaches for the metabolomic study of biological systems with subtle variations in metabolic profiles.

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5 References

[1] Fiehn, O., *Comp. Funct. Genomics* 2001, *2*, 155–168.

[2] Nicholson, J. K., Lindon, J. C., Holmes, E., *Xenobiotica* 1999, *29*, 1181–1189.

[3] Stitt, M., Fernie, A. R., *Curr. Opin. Biotechnol.* 2003, *14*, 136–144.

[4] Brindle, J. T., Antti, H., Holmes, E., Tranter, G. *et al., Nat. Med.* 2002, *8*, 1439–1444.

[5] Lindon, J. C., Holmes, E., Nicholson, J. K., *Curr. Opin. Mol. Ther.* 2004, *6*, 265–272.

[6] Fiehn, O., Kopka, J., Dörmann, P., Altmann, T. *et al., Nat. Biotechnol.* 2004, *4*, 189–199.

[7] Nicholls, A. W., Nicholson, J. K., Haselden, J. N., Waterfield, C. J., *Biomarkers* 2000, *5*, 410–423.

[8] Taylor, J., King, R. D., Altmann, T., Fiehn, O. *et al., Bioinformatics* 2002, *18*, 241–248.

[9] Chen, M., Zhao, L., Jia, W., *J. Proteome Res.* 2005, *4*, 2391–2396.

[10] Gavaghan, C. L., Wilson, I. D., Nicholson, J. K., *FEBS Lett.* 2002, *530*, 191–196.

[11] Ott, K. H., Aranibar, N., Singh, B., Stockton, G. W., *Phytochemistry* 2003, *62*, 971–985.

[12] Sugimoto, M., Kikuchi, S., Arita, M., Soga, T. *et al., Anal. Chem.* 2005, *77*, 78–84.

[13] Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wold, S., *Umetrics*, Umea, Sweden, 1999.

[14] Azmi, J., Connelly, J., Holmes, E., Nicholson, J. K. *et al., Biomarkers* 2005, *10*, 401–416.

[15] Brindle, J. T., Nicholson, J. K., Schofield, P. M., Grainger, D. J., Holmes, E., *Analyst* 2003, *128*, 32–36.

[16] Dumas, M. E., Maibaum, E. C., Teague, C., Ueshima, H. *et al., Anal. Chem.* 2006, *78*, 2199–2208.

[17] Espina, J. R., Shockcor, J. P., Herron, W. J., Car, B. D. *et al., Magn. Reson. Chem.* 2001, *39*, 559–565.

[18] Chen, M., Su, M., Zhao, L., Jiang, J. *et al., J. Proteome Res.* 2006, *5*, 995–1002.

[19] Plumb, R. S., Stumpf, C. L., Granger, J. H., Castro-Perez, J. *et al., Rapid Commun. Mass Spectrom.* 2003, *17*, 2632–2638.

[20] Wang, C., Kong, H., Guan, Y., Yang, J. *et al., Anal. Chem.* 2005, *77*, 4108–4116.

[21] Wilson, I. D., Plumb, R., Granger, J., Major, H. *et al., J. Chromatogr. B* 2005, *817*, 67–76.

[22] Yang, J., Xu, G., Zheng, Y., Kong, H. *et al., J. Chromatogr. A* 2005, *1084*, 214–221.

[23] Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H. *et al., J. Proteome Res.* 2003, *2*, 488–494.

[24] Ullsten, S., Danielsson, R., Bäckström, D., Sjöberg, P., Bergquist, J., *J. Chromatogr. A* 2006, *1117*, 87–93.

[25] Benavente, F., van der Heijden, R.b, Tjaden, U. R., van der Greef, J., Hankemeier, T., *Electrophoresis* 2006, *27*, 4580–4584.

[26] Fiehn, O., Kopka, J., Dormann, P., Altmann, T. *et al., Nat. Biotechnol.* 2000, *18*, 1157–1161.

[27] Fiehn, O., Kopka, J., Trethewey, R. N., Willmitzer, L., *Anal. Chem.* 2000, *72*, 3573–3580.

[28] Hušek, P., *J. Chromatogr. B* 1998, *717*, 57–71.

[29] Hušek, P., Matucha, P., Vrankova, A., Simek, P., *J. Chromatogr. B* 2003, *789*, 311–322.

[30] Namera, A., Yashiki, M., Nishida, M., Kojima, T., *J. Chromatogr. B* 2002, *776*, 49–55.

[31] Pretorius, V., Hopkins, B. J., Schielie, J. D., *J. Chromatogr.* 1974, *99*, 23–30.

[32] Xie, G. X., Qiu, Y. P, Qiu, M. F, Gao, X. F. *et al., J. Pharm. Biomed. Anal.* 2007, *43*, 920–925.

[33] Jiang, Z. J., Gao, Y. R., Zhou, Y. B., Zhang, Z. C. *et al., J. Microcol. Sep.* 2001, *13*, 191–196.

[34] Anthony, M. L., Rose, V. S., Nicholson, J. K., Lindon, J. C., *J. Pharm. Biomed. Anal.* 1995, *13*, 205–211.

[35] Beckonert, O., Bollard, M. E., Ebbels, T. M. D., Keun, H. C. *et al., Anal. Chim. Acta* 2003, *490*, 3–15.

[36] Yin, P., Zhao, X., Li, Q., Wang, J. *et al., J. Proteome Res.* 2006, *5*, 2135–43.

[37] Xie, G. X., *Metabonomic Study of Biological Samples Based on Different Chromatographic Analytical Methods*, Shanghai Jiao Tong University, Shanghai, 2007.

[38] Qiu, Y. P., Su, M. M., Liu, Y. M., Chen, M. J. *et al., Anal. Chim. Acta* 2007, *583*, 277–283.

[39] Liu, Z. C., Sun, F. M., Zhu, M. H., Wang, Y. Z. *et al., Acupunct. Res.* 2001, *26*, 122–126.

[40] Wang, Y. Z., Sun, F. M., Wei, Q. L., *Chin. J. Basic Med.Tradit. Chin. Med.* 2000, *6*, 52–55.

[41] Wang, Q. P., *Foreign Med. Sci. Sec. Pathophysiol. Clin. Med.* 2001, *21*, 451–453.

