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Organic solvent extraction and metabonomic profiling of the metabolites in erythrocytes

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ABSTRACT

We used erythrocytes as the model tissue to evaluate an optimal solution for the extraction of intracellular metabolites and time-dependent variation of the metabolome in living cells. Projection to latent structure (PLS) of the GC/MS and LC/MS data suggested that the most efficient solution for the extraction of metabolites from wet erythrocytes (50 mg) could be a methanol–chloroform–water mixture (950 μL, 700:200:50, v/v/v). PLS-discriminant analysis (DA) clearly profiled a time-dependent alternation of metabolic phenotype of erythrocytes. Identification of the metabolites showed that the process was characterized by accumulating of metabolic products and depleting of nutritious substances in erythrocytes during incubation.

1. Introduction

As metabonomics continues to develop and gain exciting achievements in biomedical science [1–9], increasing attention will be paid to the metabolites involved in diseases in specific tissues or organs. The direct measurement of metabolites in a tissue or organ can provide more relevant information than that is available from systemic fluids such as blood, plasma, or urine. Metabonomic studies focus on the analysis of as many low-molecular-weight compounds as possible. High-throughput metabonomic research based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques has been widely applied to the global analysis of metabolites. NMR has the advantages of directly analyzing tissues or organs and can thus minimize the extraction steps. As the high-throughput tools, MS coupled to, for example, gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE), ensure the inclusion of more endogenous compounds of diverse structures and physiochemical properties, and therefore provide more qualitative and quantitative information about biological samples.

MS techniques are increasingly used in metabonomic research. However, comprehensive extraction of the metabolites from tissue samples remains the most important challenge in MS based metabonomics. Because of the tight junction of the cells in tissues and organs by a solid connective tissue support, it is not as easy to comprehensively extract the metabolites from the cells of tissues as it is from body fluids. *Escherichia coli* has served as a valuable model and played a leading role in the development of cellular metabonomics [10–12], but the difference between *E. coli* and human cells is obvious. The erythrocyte is a typical living cell in human and animal body that is easy to collect and separate precisely into aliquots. Erythrocytes themselves are closely involved in biomedical sciences and sports medical sciences. The delicate membrane of the erythrocyte facilitates organic solvent extraction, circumventing the use of mechanical factors to break the cells during extraction, which inevitably affects extraction efficiency. These properties offer an ideal sample with which to quantitatively evaluate the extraction efficiency for targeting or non-targeting analysis [13–15]. Although previous reports have considered the comprehensive extraction of metabolites from plasma or serum [16–18], bacteria [10,11,19], cell cultures [17,20,21], and tissues [5,22,23], few of them have optimized the extraction strategy using different composition of extraction solution. Recently Sana et al. developed an extraction and chromatographic strategy for profiling the

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Table 1
Experimental design 1, investigating the extraction efficiencies of various combinations of organic solvents.

Exp name	Run order	Methanol (μL)	Ethanol (μL)	Isopropanol (μL)	Acetone (μL)	Chloroform (μL)	Erythrocyte (mg, wet)	H_2O (μL)
01	7	900	0	0	0	0	50	50
02	17	0	900	0	0	0	50	50
03	20	0	0	900	0	0	50	50
04	25	0	0	0	900	0	50	50
05	15	700	0	0	0	200	50	50
06	24	0	700	0	0	200	50	50
07	18	0	0	700	0	200	50	50
08	1	0	0	0	700	200	50	50
09	5	0	0	800	0	100	50	50
10	21	0	0	600	300	0	50	50
11	10	0	0	300	600	0	50	50
12	14	0	600	0	300	0	50	50
13	8	0	300	0	600	0	50	50
14	11	0	600	300	0	0	50	50
15	2	0	300	600	0	0	50	50
16	12	800	0	0	0	100	50	50
17	23	600	0	0	300	0	50	50
18	26	300	0	0	600	0	50	50
19	27	600	0	300	0	0	50	50
20	6	300	0	600	0	0	50	50
21	16	600	300	0	0	0	50	50
22	4	300	600	0	0	0	50	50
23	3	200	200	200	200	100	50	50
24	9	200	200	200	200	100	50	50
25	22	200	200	200	200	100	50	50
26	13	200	200	200	200	100	50	50
27	19	200	200	200	200	100	50	50

erythrocytes metabolome using LC/MS. This study used a two-step extraction with methanol and chloroform, respectively. But the metabolome was only profiled by LC/MS and the extraction efficiency was not assessed in comparison with other extraction solution [15]. In the present study, five commonly used organic solvents, methanol, ethanol, acetone, isopropanol, and chloroform, were set up by means of design of experiments (DOE) for the extraction of the metabolites. Based on the high-throughput GC/MS and LC/MS method developed [16,24], the samples were analyzed and the data were evaluated to approach an optimal solution that maximizes the extraction of the erythrocyte metabolome.

On the other hand, although immediate quenching of cell metabolism in tissue samples is important for metabonomic research, in practical work, tissue samples have to be prepared, washed, weighed and pretreated to undergo the process of biochemical metabolism. Besides tissue samples, the metabonomic variation of serum may be significant since the incubation of whole blood in tube will inevitably have influence on the metabolome of serum. Although evaluation of metabolic variation in living cells is of significant importance, this issue has never been investigated. Erythrocytes in whole blood presented the most suitable, natural system to approach dynamic modification of metabolome. Study on the dynamic modification time-dependent alternation of metabolites can help to make a standard operation procedure (SOP) for the extraction of tissue samples, which can minimize the metabonomic variation from sample treatment. Hence, erythrocytes were incubated and then quenched at different time points to profile dynamic modification of the metabolome.

2. Experimental

2.1. Materials and chemicals

All of the reference compounds were of analytical grade, purchased from Sigma (St Louis, MO, USA), Merck (Darmstadt, Germany), Aldrich (Steinhein, Germany), or Serva (Heidelberg, Germany). Methoxyamine hydrochloride and the stable-isotope-labeled internal standard compounds (IS), [$^{13}C_2$]-myristic acid and [$^{2}H_6$]-salicylic acid, were purchased from Sigma–Aldrich and Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), respectively. The alkane series (C_8–C_{40}) and pyridine (silylation grade) were obtained from Fluka (Buchs, Switzerland). N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and 1% trimethylchlorosilane (TMCS) were purchased from Pierce Chemical Company (IL, USA). Methanol and acetonitrile were of chromatography HPLC grade (>99.5%), and sodium ethylenediaminetetraacetate (EDTA), acetone, ethanol, and isopropanol were of analytical grade. Distilled water was produced with a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Erythrocytes

Fresh EDTA-anticoagulated blood was collected from six healthy young male volunteers (22–28 years old, all blood type O) in the morning before breakfast. A total of 30 mL of blood pool from the six healthy volunteers was collected for immediate experiments.

2.3. Experimental design 1: organic solvent extraction

Firstly, blood plasma was removed by centrifuging at 4000 rpm for 10 min at 4 °C. The leaving erythrocytes pellets were washed with 9 mL of 0.9% saline. Then the erythrocytes pellets were dispersed in 9 mL of 0.9% saline again. After removing the supernatant by centrifuging at 4000 rpm for 3 min at 4 °C, an aliquot of the erythrocytes pellets (50 mg) were obtained in each tube.

To determine the most efficient organic solvents with which to extract the metabolites from the erythrocytes, a D-optimal experimental design [25] was constructed with various combinations of organic solvents, resulting in a total of 27 experiments with five replicates of each for statistical analysis (Table 1). The total solution volume for each experiment was fixed at 1000 μL. To each of the Eppendorf tubes water was added and an organic solvent mixture stipulated by the experimental design. [$^{13}C_2$]-myristic acid (2 μg) and [$^{2}H_6$]-salicylic acid (2 μg) were added to each tube as the IS for normalization.

The same extraction procedure was applied as previously reported [16] with few modification. After vigorous extraction in an MM400 vibration mill (Retsch GmbH, Haan, Germany), an aliquot (100 μL) of the resulting supernatant was transferred to a GC or LC vial and evaporated to dryness. For GC/MS analysis, 35 μL of methoxyamine (10 μg/μL) in pyridine was added to each GC vial for methoximation was performed at room temperature for 16 h. And then 35 μL of MSTFA containing 1% TMCS was added for trimethylsilylation for 1 h at room temperature. Finally, 35 μL of heptane with methyl myristate as the reference standard (to check system variation alone, not including derivatization) at 30 μg/mL was added to each GC vial. For LC/MS analysis, the residue was dissolved in 100 μL of methanol–water (700:50, v/v) and centrifuged at 14,000 rpm for 10 min to obtain the supernatant.

2.4. Experimental design 2: incubation and extraction

Tissue or organ samples are generally not extracted immediately after sampling, although the metabolite composition of living cells is inevitably modified over time. To characterize the variation in the metabolites, fresh EDTA-anticoagulated blood was incubated for 1, 2, or 3 h, and then extracted with the two most promising organic solvents identified in the experiments described above, methanol alone and methanol–chloroform since neither of them showed obvious superiority over the other in the previous study. To analyze these two factors (i.e., incubation period and the organic solvent used), a simple experimental design was established with six different settings (six replicates each), resulting in 36 experiments (supplementary information, S Table-1). In each Eppendorf tube, 100 μL of blood was added and vibrated at 60 rpm in an incubator at 37 °C for 1, 2, or 3 h. The blood plasma was removed immediately after incubation by centrifugation at 4000 rpm for 10 min at 4 °C. The resulting erythrocytes were then gently vortexed and dispersed in 150 μL of 0.9% saline. After centrifugation at 4000 rpm for 3 min at 4 °C, the supernatant was removed. [$^{13}C_2$]-myristic acid and [$^{2}H_6$]-salicylic acid (2 μg) were added to each of the tubes as the IS. The erythrocytes were extracted with either methanol–water (900:50, v/v) or methanol–chloroform–water (700:200:50, v/v/v), as described above. The supernatants (100 μL) were dried and treated as described above for GC/MS and LC/MS analyses.

2.5. GC/time-of-flight (TOF)-MS analysis

To minimize systematic variations, all of the samples were randomly analyzed by both GC/TOF-MS and LC/TOF-MS. The derivatized sample (1.0 μL) was splitlessly injected with an Agilent 7683 Series autosampler (Agilent Technologies) into an Agilent 6980 GC system equipped with a fused-silica capillary column (10 m × 0.18 mm i.d.) chemically bonded with 0.18 μm DB-5 stationary phase (J&W Scientific, Folsom, CA, USA). The inlet temperature was set at 250 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min through the column. To achieve good separation, the column temperature was initially maintained at 70 °C for 2 min, and then increased at a rate of 35 °C/min from 70 °C to 305 °C, where it was held for 2 min. The column effluent was introduced into the ion source of a Pegasus III MS (Leco Corp., St Joseph, MI, USA). The transfer line temperature was set at 250 °C and the ion source temperature at 200 °C. Ions were generated by a 70 eV electron beam at a current of 3.2 mA. Masses were acquired with *m*/*z* 50–680 at a rate of 30 spectra/s, and the acceleration voltage was turned on after a solvent delay of 175 s.

Peaks (with signal-to-noise ratio > 20) were detected automatically. MS spectra were deconvoluted by using ChromaTOF™ software. To obtain accurate peak areas for the IS and specific peaks/compounds, the datum was processed in the manner reported previously [16]. Before compound identification, the retention index was calculated by comparing the retention time with those of the alkane series C_8–C_{40}. The compounds were identified by comparison of the mass spectra and retention indices of all the detected compounds with those of reference standards and those available in libraries: mainlibrary and publibrary, in the National Institute of Standards and Technology (NIST) library 2.0 (2005) and the in-house mass spectra library database established by Umeå Plant Science Center, Umeå University, and the Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University.

2.6. LC/MS analysis

An aliquot (2 μL) of the supernatant was injected into an Agilent 6210 RRLC/TOF MS system (Agilent). Chromatography was performed on a reverse-phase Zorbax SB-C18 Rapid Resolution HT column (2.1 mm × 5.0 mm, 1.8 μm; Agilent) maintained at 25 °C. Elution was performed by increasing mobile phase B (acetonitrile with 0.1% formic acid) in a linear gradient from 5 to 95% in 10 min, while mobile phase A (aqueous 0.1% formic acid solution, pH 3.5–4.0) decreased from 95 to 5%. The final composition of the mobile phase was held at 95% B for 5 min more, and then reduced in a linear gradient from 95 to 5% B within 5 min. The system was conditioned with 5% B for 3 min before next analysis. The mobile phase flow rate was kept constant at 0.2 mL/min. The eluent was introduced with an electrospray ionization source into the Agilent TOF MS system. The source temperature was set at 250 °C, with the dry gas temperature at 320 °C, a flow rate of 9.0 L/min, and a nebulization gas pressure of 35 psi. The capillary voltage was set at 3000 V with the fragmenter at 150 V. Because of the extreme complexity of the metabolites, both positive ions $[M+H]^+$, $[M+Na]^+$ and negative ions $[M-H]^-$, $[M+HCOO]^-$ were acquired at 50–1500 *m*/*z* to include as many metabolites as possible. The peak areas of the quasi-molecular ions were acquired with ChemStation (MassHunter Workstation B.01.02), and analytes with a S/N ratio < 20 were excluded from further analysis. Both positive and negative ion were processed first to combine the features with accurate mass less than 5 ppm and retention time less than 0.3 min using an developed algorithm in Excel. Then the adducts $[M+H]^+$, $[M+Na]^+$ (isotopes and dimers) in positive-ion mode, and adducts $[M-H]^-$, $[M+HCOO]^-$ (isotopes or dimers) in negative-ion mode were treated as a single compound or feature, respectively, and their peaks areas were summarized as long as the retention time less than 0.3 min. Peak area ratios relative to that of the internal standard ([$^{13}C_2$]-myristic acid and [$^{2}H_6$]-salicylic acid) were computed to minimize systematic variation.

2.7. Multivariate data analysis and DOE

All multivariate statistical analysis (MSA) and modeling were carried out with SIMCA-P 11 software (Umetrics, Umeå, Sweden). Modde 7.0 (Umetrics) was used to generate design matrices for the DOE. Principle components analysis (PCA), partial least-squares projection to latent structures (PLS), and discriminant analysis (DA) were used to calculate the models. PLS was used to relate the design matrix X (the different solvent mixtures) to the peak areas (Y matrix) normalized to the IS. After deconvolution GC/TOF-MS and LC/TOF-MS data (peaks areas) were extracted, normalized by IS and combined together in a single Excel file for data processing. The data matrix was constructed by the peak areas with the sample names/IDs as observations and the retention times as the response variables. Samples from the same group were classified into one in the PLS-DA model. In the experimental design of organic solvents, the data matrix was constructed in an extended way, as shown in Table 1. Columns 3–7 of the added solvents served as the factor variables/X variables: methanol, ethanol, isopropanol, ace-

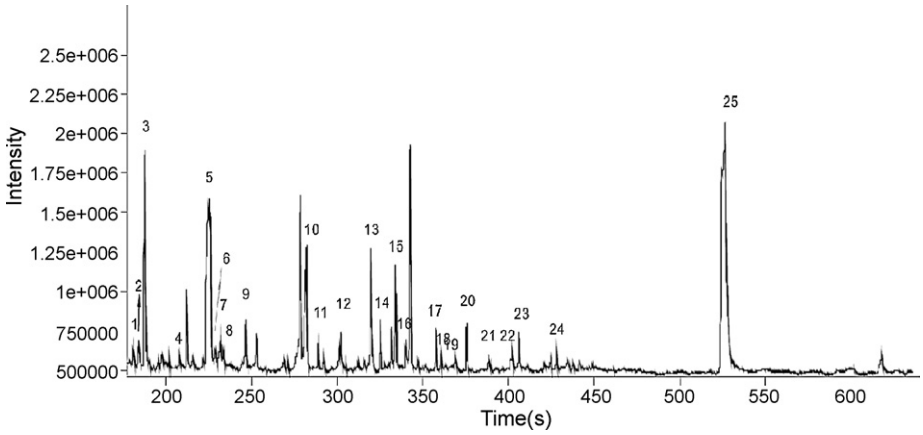

Fig. 1. GC/TOF-MS profile of the metabolites in erythrocytes extracted by methanol–chloroform–water mixture (950 μL, 700:200:50, v/v/v) for wet erythrocytes (50 mg). 1, glycine; 2, propanedioic acid; 3, lactic acid; 4, valine; 5, phosphoric acid; 6, proline; 7, glycine; 8, succinic acid; 9, serine; 10, aspartic acid; 11, creatinine; 12, glutamic acid; 13, methyl tetradecanoate; 14, glutamine; 15, azelaic acid; 16, ornithine; 17, lysine; 18, tyrosine; 19, gluconic acid; 20, hexadecanoic acid; 21, inositol; 22, oleic acid; 23, octadecanoic acid; 24, arachidonic acid; 25, cholesterol.

tone, and chloroform. The columns representing the peak areas, from both GC/TOF-MS and LC/TOF-MS, were aligned afterwards at various retention times and served as the response variables/Y variables. All of the peak areas were normalized to the stable isotope IS, [$^{13}C_2$]-myristic acid or [$^2H_6$]-salicylic acid, before MSA. Cross-validation [26] with seven cross-validation groups was used throughout to determine the number of principal components. All of the compounds contributing most to the PLS-DA model were statistically calculated (one-way ANOVA, $p < 0.05$, with confidence interval of 95%). The following statistics for the regression models are discussed throughout this paper. R^2X is the cumulative modeled variation in X, R^2Y is the cumulative modeled variation in Y, and Q^2Y is the cumulative predicted variation in Y, according to cross-validation. The ranges of these parameters are 0–1, where 1 indicates a perfect fit.

3. Results

3.1. Experimental design 1: extraction of the metabolites with organic solvent mixtures

To find the optimal extraction solution with which to extract the metabolites from erythrocytes, the five most commonly used organic solvent (methanol, ethanol, acetone, isopropanol, and chloroform) were formulated with a D-optimal design. The extracts were analyzed with both GC/TOF-MS and LC/TOF-MS. Direct inspection found obvious differences in the GC/TOF-MS chromatograms (TIC) extracted by methanol, ethanol, acetone, isopropanol (Supplementary Information, S Figure-3A–D), methanol:chloroform (7:2, v/v,) (Fig. 1), respectively. Since the compounds of very low level in biological samples often presented very high variation, and these compounds were usually easier to be dissolved and extracted due to their very low level, the compounds of rather low MS response (peak areas) was neglected for further data processing. As a consequence, 243 peaks were deconvoluted with GC/TOF-MS, 292 peaks were selected from 1326 features detected by LC/TOF-MS. Altogether, 85 metabolites were identified and presented in Supplementary Information, S Table-2.

To determine the appropriate IS for normalization, a PLS model was calculated first between the design matrix X and matrix Y, the peak areas (GC/TOF-MS data) of the two internal standards, and the reference standard. This was quite an invalid model according to cross validation (R^2X, 92.3%; R^2Y, 10.3%, Q^2Y 2.6%), suggesting that the two IS, [$^{13}C_2$]-myristic acid (RSD 17.2%, 27 samples) and [$^2H_6$]-salicylic acid (RSD 25.0%, 27 samples), and the reference standard methyl myristate (RSD 21.9%, 27 samples), were not systematically affected by the different experimental conditions, indicating in turn that they were independent of the experimental design and could be used for normalization. But in LC/MS analysis, [$^{13}C_2$]-myristic acid showed good retention and reproducibility (RSD 17.9%, 27 samples), while [$^2H_6$]-salicylic acid was not detected in the chromatographic system. Therefore, [$^{13}C_2$]-myristic acid was chosen for normalization both in GC/TOF-MS and LC/TOF-MS analysis.

After normalization, the GC/TOF-MS and LC/TOF-MS data were aligned, merged in one Excel file, and then imported into SIMCA, where the data matrix was constructed as the design matrix X and the combined response variables/peaks (Y matrix) of the 27 samples. An unsupervised PCA model of the resolved peaks showed no outliers. A two-component PLS model was then generated between design matrix X and the resolved peak areas (matrix Y). The model explained 32.1% and predicted 18.1% of the variation in the resolved peak areas, according to the R^2X, R^2Y, and Q^2 parameters (0.335, 0.321, and 0.181, respectively).

The PLS scores plot (t1–u1) can show the intrinsic relationship between the design variables, X matrix (the experimental conditions/solvents; plots labeled by run order; see Table 1), and the response variables, Y matrix (the peak areas obtained with GC/TOF-MS and LC/TOF-MS analyses) (Fig. 2). The lower left quadrant of the plots includes the experimental settings (Table 1) that produced the lowest overall peak areas. Analogously, the upper right quadrant includes the experimental settings that yielded the highest responses in terms of peak areas. Experimental settings of 01 and 16 maximized the GC/TOF-MS and LC/TOF-MS responses; 05, 17, 19, and 21 also had positive effects. These settings were promising for the extraction of metabolites from erythrocytes. All of these experimental settings (01, 16, 05, 17, 19, and 21) predominantly contained methanol, indicating that methanol-dominated solvents were most efficient.

To clarify the factors preferred for extraction efficiency, a PLS loading plot was constructed (Fig. 3). Based on the theory that underlies the PLS model, the relative positioning of the factor variables (plotted on the large squares) and the response variables (plotted on the small squares) are positively correlated. This means, for example, that all of the response variables located on the far right can be extracted more efficiently with methanol, which occurs on the right. These compounds include valine, phosphate, glycine, uracil, serine, threonine, pyroglutamate, aspartate, threonate, ornithine, glutamate, asparagine, glycerol-2-phosphate,

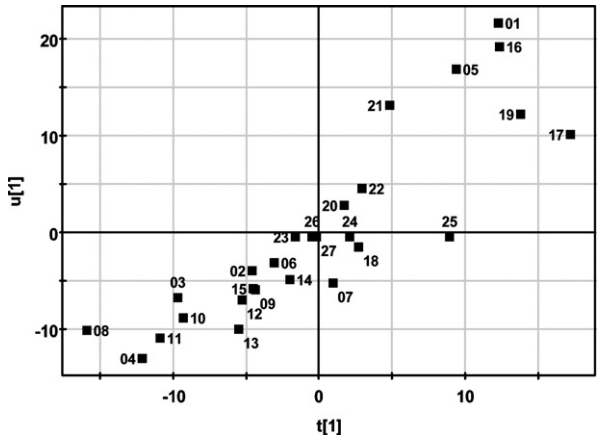

Fig. 2. PLS score plot (t1–u1) showing the best experimental design for the extraction of metabolites from erythrocytes. The lower left quadrant of the plot represents the experiments that produced the overall lowest peak areas, whereas the upper right quadrant represents those experiments that yielded the best results in terms of peak areas, here N01 and N16.

glutamine, fructose, tyrosine, myo-inositol, urate, myo-inositol-1-phosphate, and adenosine-5-monophosphate. In contrast, those response variables on the far left are not extracted well with methanol, such as glyceric acid-2,3-di-phosphate. Most of the response variables are clustered in the center of the figure, indicating that they are not strongly influenced by the different kinds of organic solvents.

As shown in Fig. 3, the addition of chloroform can enhance the extraction efficiency of some response variables in the upper part of the plot, including proline and cholesterol. This is consistent with the result shown in Fig. 2, in which the methanol–chloroform mixture yielded good extraction efficiency. Acetone and isopropanol were not effective in the extraction of most compounds, and were only appropriate for a small number of response variables, but none of which were tentatively identified. Ethanol is located very near the center of the plot, suggesting its balanced position in the extraction of most of the response variables. It was not chosen because it is not a good solvent for many of the variables in the far right, upper part of the plot. In general, methanol or methanol-dominated solvents resulted in the best extraction efficiency; chloroform was also a good solvent to include.

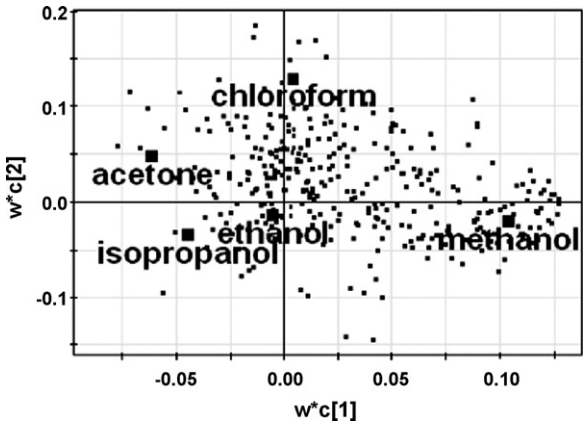

Fig. 3. PLS loading plot indicating the most important factors in maximizing the extraction of metabolites from erythrocytes. The PLS loadings plot summarizes the effects and correlation structure between variables in both the X matrix (large squares, design variables) and Y matrix (small squares, peak areas). The results of this experimental study suggest that methanol and chloroform are positive in maximizing the extraction of metabolites from erythrocytes.

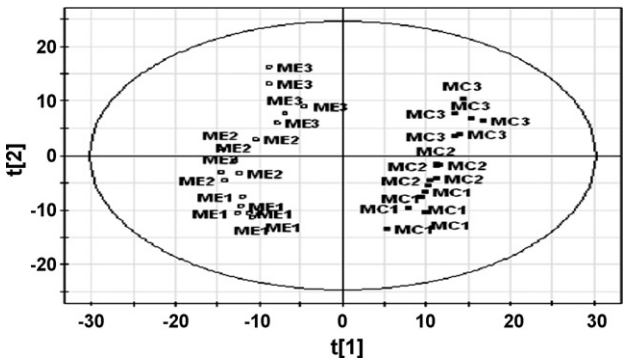

Fig. 4. Scores plot from design 2 (Table S-1) of erythrocytes samples extracted with methanol or methanol–chloroform for 1, 2, or 3 h. The observation samples were clearly separated into two clusters, on the right (methanol–chloroform [MC], solid squares) and on the left (methanol [ME], blank squares), suggesting different extraction efficiencies of ME and MC. It is interesting to note that the score plots move up gradually as the incubation time increases from 1 to 3 h, with both methanol–chloroform and methanol extractions. This indicates that the incubation of erythrocytes has a marked impact on the metabolites and that both methanol alone and methanol–chloroform extraction can describe the dynamics of these changes.

3.2. Incubation of erythrocytes

GC/TOF-MS and LC/TOF-MS analysis resulted in a total of 496 peaks. To minimize systematic variations, the data were normalized to the stable-isotope IS, [$^{13}C_2$]-myristic acid. An unsupervised PCA model of the data matrix revealed a single pronounced outlier in one sample (data not shown). The outlier was removed from further modeling because its inclusion reduced the validity of the model. A new PCA model was computed using the data from the remaining samples, and no additional outliers were found. A PLS-DA model was then calculated with six groups of samples incubated for 1, 2, or 3 h, and each extracted with methanol or methanol–chloroform (see Fig. 4). This is a good three-component PLS-DA model, explaining 39% and predicting 30% of the variation in the resolved peaks, according to the R^2X, R^2Y, and Q^2Y parameters (0.47, 0.39, and 0.30, respectively).

In the score plot in Fig. 4, the observed samples are clearly separated into two clusters: one on the right and the other on the left. The solid squares that plot in the right-hand part of the figure represent the samples extracted with methanol–chloroform (MC), whereas the open squares to the left represent samples extracted with methanol (ME). The clear separation of the two groups suggests different extraction efficiencies for the metabolites in erythrocytes.

It is interesting to note that the score plots move up gradually as the incubation time increases from 1 to 3 h for all samples extracted with either methanol–chloroform or methanol. This finding suggests that the incubation of the erythrocytes has a remarkable impact on the metabolites levels, and that both methanol alone and methanol–chloroform extraction can describe these dynamic changes. From the two-dimensional figure, the extraction solvents primarily contributed to the first component on the X-axis, and incubation time dominated the second component of the model on the Y-axis. The metabolite information can be obtained if we investigate the corresponding loadings plot (shown in supplementary information, S Figure-4). Most of the resolved peaks are located in the center of the figure, indicating that they are insensitive to both extraction solvent and the incubation time. In other words, these metabolites are not affected by extraction with methanol alone or methanol–chloroform, or by incubation for shorter or longer periods.

However, some peaks correlated strongly, either positively or negatively, with these two factors. Their identification revealed that

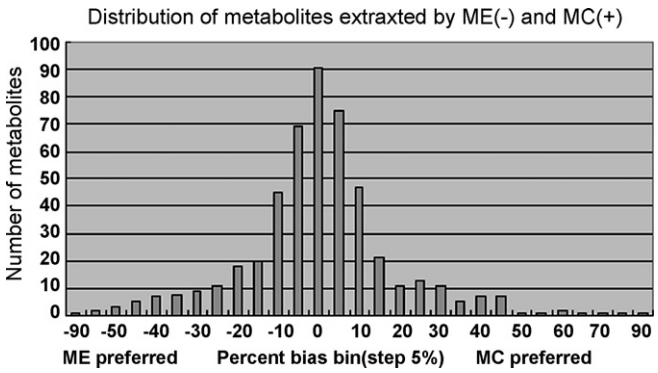

Fig. 5. The distribution profile of the detected metabolites showed the number of metabolites in each percent bias bin (5%) preferable either for MC or ME extraction. Generally, more peaks have a positive value so that they shifted to right a bit, suggesting methanol–chloroform was slightly better than methanol in terms of extraction efficiency.

urate, glucose, glutamate, and phosphate (on the far left, *S Figure-4*) correlated positively with methanol extraction but negatively with methanol–chloroform extraction, whereas glyceric acid-2,3-phosphate, ornithine, lysine, 5′-adenylic acid, pyrophosphate, and so on (on the far right, *S Figure-4*), were more efficiently extracted with methanol–chloroform.

For an overview of the extraction efficiency of ME and MC for the whole peaks, percent changes of all the metabolites in term of MC/ME ratio (peak area, percent) were calculated. Distribution profile of the 496 metabolites showed the number of metabolites in each percent bias bin (5%, Fig. 5). Generally, all of the peaks shifted to right a bit, suggesting methanol–chloroform was slightly better than methanol in terms of extraction efficiency. The most significantly affected metabolites were identified and their peak areas were compared between methanol–chloroform and methanol extraction. Altogether, 122 peaks were significant different between the two groups (ANOVA, $p < 0.05$), while there was not statistical difference for the other 374 peaks. Amongst the 122 peaks, some were identified; their percent changes were showed in the supplementary information, S Figure-5.

The incubation-dependent peaks were investigated and identified further. Levels of glucose-6-phosphate, glucose, tryptophan, ornithine, and many other amino acids were determined higher after shorter incubations (1 h, in the lower part of the plot), whereas the levels were higher for metabolic products, such as glycerate, succinate, (in the upper part of the plot) in erythrocytes after longer incubation for 3 h. Averagely, after incubation for 3 h peak areas declined by 65.0, 43.8, 38.1, 22.1, 15.1, 14.6% for glucose-6-phosphate, glucose, tryptophan, ornithine, leucine, glutamate, respectively, relative to those incubated for 1 h; while peaks areas increased by 38.5, 38.4, 17.5% for glycerate, succinate, and adenosine-5-monophosphate, respectively.

4. Discussion

Immediate quenching of metabolism in tissue samples is important to approach metabonomic snapshot, but in practice, usually samples have to be washed and weighed for necessary preparation before extraction. Therefore metabonomic profile of the dynamic modification provided the basic data to stipulate a standard operation procedure. To check the metabonomic variation over time, fresh whole blood with plasma was incubated such that the erythrocytes could survive by taking in nutrient substances from the plasma and discharging waste freely. To some extent, due to the influx of nutritious components into erythrocytes and outflow of waste the incubation of whole blood reduced the variation of the metabolites inside the erythrocytes compared with the incubation of the erythrocytes alone, but it provided the optimal environment for the erythrocytes and made it easier to perform the experiment. Because of the properties of living cells in tissues and organs, the levels of metabolites in fresh tissue samples will inevitably change with time, almost all the life functions of erythrocytes are supported by energy from glycolysis, and glucose is used to produce some intermediates (e.g., saccharide phosphates) that are subsequently converted to ultimate metabolites such as lactate and ethanol [27]. Ethanol was not detected because it is volatile and easily removed in a SpeedVac. Amino acids are consumed as important elementary materials in living cells; consequently, their concentrations decreased with longer incubation. Therefore, there were more saccharide phosphates and less glucose and amino acids in the erythrocytes after incubation for 3 h.

The above results provide direct evidence that nutrient compounds are consumed in living erythrocytes for the supply of energy and in normal metabolism, while the intermediate and final metabolic substances accumulate progressively. The result strongly supported the proposal that metabolism of cells should be quenched as soon as possible to prevent modification of the metabolome. But for the tissue samples subject to necessary pretreatment steps, identical pretreatment time would be useful to minimize sample variation from biochemical metabolism, especially for serum. The PCA score plot clearly showed a dynamic profile of the biochemical processes of the erythrocytes, suggesting that the extraction and analysis methods presented here are feasible for the metabonomic investigation of living cells or tissues samples.

Although methanol–chloroform has been widely utilized for extraction of substance from tissues, bacteria and cells cultures [5,10,11,13,14,22,23,28–35], optimization of the extraction strategy was weak and extraction evaluation was usually focused on small amount of target compounds, especially for lipids or fat, few were comprehensively endogenous compounds targeted and evaluated. And most of these studies applied high percent of chloroform (methanol–chloroform from 1:2 to 2:1) for extraction. Higher portion of chloroform brought about partition of multi-phase and inevitably resulted in high variation and thus not preferred [13]. Our previous study suggested that small amount of chloroform resulted in a homogenous solution, in this experimental design, a maximum of 20% chloroform were included in the experimental design. According to our result small amount of water favored the extraction, which is consistent with a previous report [14]. In previous studies, methanol alone has been suggested as the optimal solvent with which to extract metabolites from plasma or serum [16–18], bacteria [10,19], cell cultures [17,20,21] and tissues, whereas a methanol–chloroform mixture has been utilized for the extraction of metabolites from plant tissues as well as human solid tissue [5,23]. The present study again demonstrates the potential of methanol–chloroform in the extraction of erythrocytes.

5. Conclusion

The solution of methanol–chloroform–water (950 μL, 700:200:50, v/v/v), showed the best efficiency in extracting metabolites from wet erythrocytes (50 mg). The clearly time-dependent alternation of metabolic phenotype of erythrocytes was characterized by depleting of nutritious substances and accumulating of metabolic products during incubation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.04.041.

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