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Simultaneous analysis of primary metabolites by triple quadrupole LC/MS/MS using pentafluorophenylpropyl column

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Introduction

Various metabolic pathways are controlled to keep a biological function in the cell and to monitor the rapid and slight changes of these metabolism, a simple simultaneous analysis is required for quantification of primary metabolites. A typical LC/MS system with an ODS column is not effective to measure primary metabolites because of low affinity of ODS column to hydrophilic metabolites. Here we report the simultaneous measurement of 97 metabolites by triple quadrupole LC/MS/MS using pentafluorophenylpropyl column. In this experiment, MRM transitions of these metabolites were optimized and this method was applied to biological samples. Furthermore, to evaluate the accuracy of developed method for quantification, simultaneous analysis by PFPP column was compared to measurement of ion-paring chromatography.

Methods and materials

Commercially available compounds were used as standards to optimize MRM transition and LC condition for separation. Mixed standard solutions were diluted to a range of 10 nM~10000 nM for a calibration curve and an aliquot of 3 µL was subjected to LC/MS/MS measurement.
Mice were sacrificed under anesthesia and the isolated heart/liver tissues were rapidly frozen in liquid nitrogen. Frozen liver or heart tissues (>50 mg) from mice were homogenized in 0.5 mL methanol including L-methionine sulfone and 2-morpholinoethanesulfonic acid (MES) as internal standards. After a general chloroform/methanol extraction, upper aqueous layer filtered through 5-kDa cutoff filter. The filtrate was dried up and dissolved in 0.1 mL purified water. Further, the solution was diluted to 20-100 folds in purified water. An aliquot of 3 µL was analyzed to measure primary metabolites by LC/MS instrument, Nexera UHPLC system and LCMS-8030/LCMS-8040 triple quadrupole mass spectrometer. The following is detailed conditions of LC/MS mesurement.

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UHPLC conditions (Nexera system using a PFPP column)

Column	: Discovery HS F5 150 mm×2.1 mm, 3.0 µm
Mobile phase A	: 0.1% Formate/water
B	: 0.1% Formate/acetonitrile
Flow rate	: 0.25 mL/min
Time program	: B conc.0%(0-2.0 min) - 25%(5.0 min) - 35%(11.0 min) - 95%(15.0.-20.0 min) - 0%(20.1-25.0 min)
Injection vol.	: 3 µL
Column temperature	: 40°C

MS conditions (LCMS-8030/LCMS-8040)

Ionization	: Positive/Negative, MRM mode
DL Temp.	: 250°C
HB Temp	: 400°C
Drying Gas	: 10 L/min
Nebulizing Gas	: 2.0 L/min

Result

Optimization of MRM transition

The MRM transitions for 97 standard compounds were optimized on both positive and negative mode by flow injection analysis (FIA). The MRM transitions of the 97 metabolites were determined as described in Table 1. Subsequently, LC condition was investigated to separate the 97 metabolites with a good resolution. As a consequence, the 97 metabolites were eluted from a PFPP column with a gradient of acetonitrile for <15 min in the condition described in Figure 1. The linearity of this method was also confirmed by the simultaneous analysis of a serial of diluted calibration curve.

Figure 1 shows the MRM chromatogram of 97 metabolites at a concentration of 5 µM. In this figure, we can see the peak from all metabolites with a good separation.

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Table 1 MRM transition of 97 metabolites

No.	Name	Product ion	Precursor ion	Polarity	Linearity (R^2)
1	2-Aminobutyrate	104.10	58.05	+	0.99
2	Acetylcarnitine	204.10	85.05	+	0.99
3	Acetylcholine	147.10	87.05	+	0.99
4	Adenine	136.00	119.05	+	0.98
5	Adenosine	268.10	136.05	+	0.99
6	Adenylsuccinate	464.10	252.10	+	0.99
7	ADMA	203.10	70.10	+	0.99
8	Ala	89.90	44.10	+	0.99
9	AMP	348.00	136.05	+	0.99
10	Arg	175.10	70.10	+	0.99
11	Argininosuccinate	291.00	70.10	+	0.99
12	Asn	133.10	87.15	+	0.99
13	Asp	134.00	74.05	+	0.99*
14	cAMP	330.00	136.05	+	0.99
15	Carnitine	162.10	103.05	+	0.99
16	Carnosine	227.10	110.05	+	0.99*
17	cCMP	306.00	112.10	+	0.99
18	cGMP	346.00	152.05	+	0.99
19	Choline	104.10	60.05	+	0.99
20	Citicoline	489.10	184.10	+	0.99*
21	Citrulline	176.10	70.05	+	0.99
22	CMP	324.00	112.05	+	0.99
23	Creatine	132.10	44.05	+	0.99
24	Creatinine	114.10	44.05	+	0.99
25	Cys	122.00	76.05	+	0.99*
26	Cystathionine	223.00	88.05	+	0.99
27	Cysteamine	78.10	61.05	+	0.98*
28	Cystine	241.00	151.95	+	0.99
29	Cytidine	244.10	112.05	+	0.99
30	Cytosine	112.00	95.10	+	0.99
31	Dimethylglycine	104.10	58.05	+	0.99
32	DOPA	198.10	152.10	+	0.99*
33	Dopamine	154.10	91.05	+	0.99*
34	Epinephrine	184.10	166.10	+	0.99
35	FAD	786.15	136.10	+	0.99*
36	GABA	104.10	87.05	+	0.99
37	gamma-Glu-Cys	251.10	84.10	+	0.99*
38	Gln	147.10	84.15	+	0.99
39	Glu	147.90	84.10	+	0.99*
40	Gly	75.90	30.15	+	0.99*
41	GMP	364.00	152.05	+	0.99
42	GSH	308.00	179.10	+	0.99*
43	Guanosine	284.00	152.00	+	0.99
44	His	155.90	110.10	+	0.99
45	Histamine	112.10	95.05	+	0.99*
46	Homocysteine	136.00	90.10	+	0.99*
47	Homocystine	269.00	136.05	+	0.99
48	Hydroxyproline	132.10	86.05	+	0.99
49	Hypoxanthine	137.00	55.05	+	0.98*
50	Ile	132.10	86.20	+	0.99
51	Inosine	269.10	137.05	+	0.99
52	Kynurenine	209.10	192.05	+	0.99
53	Leu	132.10	86.05	+	0.99
54	L-Norepinephrine	170.10	152.15	+	0.99
55	Lys	147.10	84.10	+	0.99
56	Met	149.90	56.10	+	0.99
57	Methionine-sulfoxide	166.00	74.10	+	0.99
58	Nicotinamide	123.10	80.05	+	0.99
59	Nicotinic acid	124.05	80.05	+	0.99
60	Ophthalmic acid	290.10	58.10	+	0.99
61	Ornitine	133.10	70.10	+	0.99
62	Pantothenate	220.10	90.15	+	0.99
63	Phe	166.10	120.10	+	0.99
64	Pro	115.90	70.10	+	0.99
65	SAH	385.10	134.00	+	0.98
66	SAM	399.10	250.05	+	0.99*
67	SDMA	203.10	70.15	+	0.99
68	Ser	105.90	60.10	+	0.99*
69	Serotonin	177.10	160.10	+	0.99
70	Thr/Homoserine	120.10	74.15	+	0.99
71	Thymidine	243.10	127.10	+	0.99
72	Thymine	127.10	54.05	+	0.99*
73	TMP	322.90	81.10	+	0.99*
74	Trp	205.10	188.15	+	0.99
75	Tyr	182.10	136.10	+	0.99
76	Uracil	113.00	70.00	+	0.99*
77	Uridine	245.00	113.05	+	0.99
78	Val	118.10	72.15	+	0.99
79	2-Oxoglutarate	144.90	101.10	-	0.98*
80	Allantoin	157.00	97.10	-	0.98*
81	Cholate	407.20	343.15	-	0.99**
82	cis-Aconitate	172.90	85.05	-	0.99
83	Citrate	191.20	111.10	-	0.99*
84	FMN	455.00	97.00	-	0.99
85	Fumarate	115.10	71.00	-	0.99**
86	GSSG	611.10	306.00	-	0.99*
87	Guanine	150.00	133.00	-	0.99*
88	Isocitrate	191.20	111.10	-	0.99*
89	Lactate	89.30	89.05	-	0.97*
90	Malate	133.10	114.95	-	0.99*
91	NAD	663.10	541.05	-	0.99*
92	Orotic acid	155.00	111.10	-	0.99
93	Pyruvate	86.90	87.05	-	0.99*
94	Succinate	117.30	73.00	-	0.99*
95	Taurocholate	514.20	107.10	-	0.99*
96	Uric acid	167.10	123.95	-	0.99*
97	Xanthine	151.00	108.00	-	0.99*

Calibration curve was obtained at a range of concentration from 10 nM to 10000 nM.
* Calibration curve was obtained at a range of concentration from 100 nM to 10000 nM.
** Calibration curve was obtained at a range of concentration from 1000 nM to 10000 nM.

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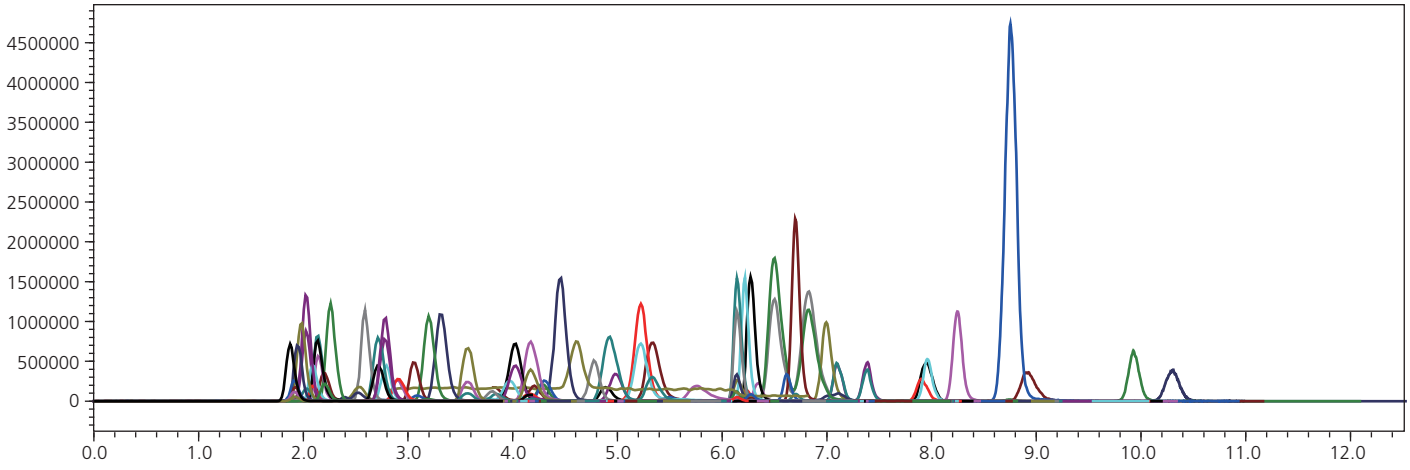

Figure 1 MRM chromatogram of 97 compounds

Application to tissue extracts as biological samples

Simultaneous analysis of 99 compounds was performed for heart / liver tissue extracts as biological samples. Figure 2 shows MRM chromatograms of 99 compounds from tissue extracts (liver/heart). In this measurement, 83/97 metabolites were detected from liver tissue extracts and 88/97 metabolites were confirmed from heart tissue extracts. These results show this method is also effective to simultaneous analysis of biological samples. As shown in the resulting MRM chromatogram, some major peaks were derived from the metabolites which were known to be characteristic to each tissue. Furthermore, this characteristic difference in each tissue was also confirmed in some faint peaks (e.g., cholate, cystine and homocysteine).

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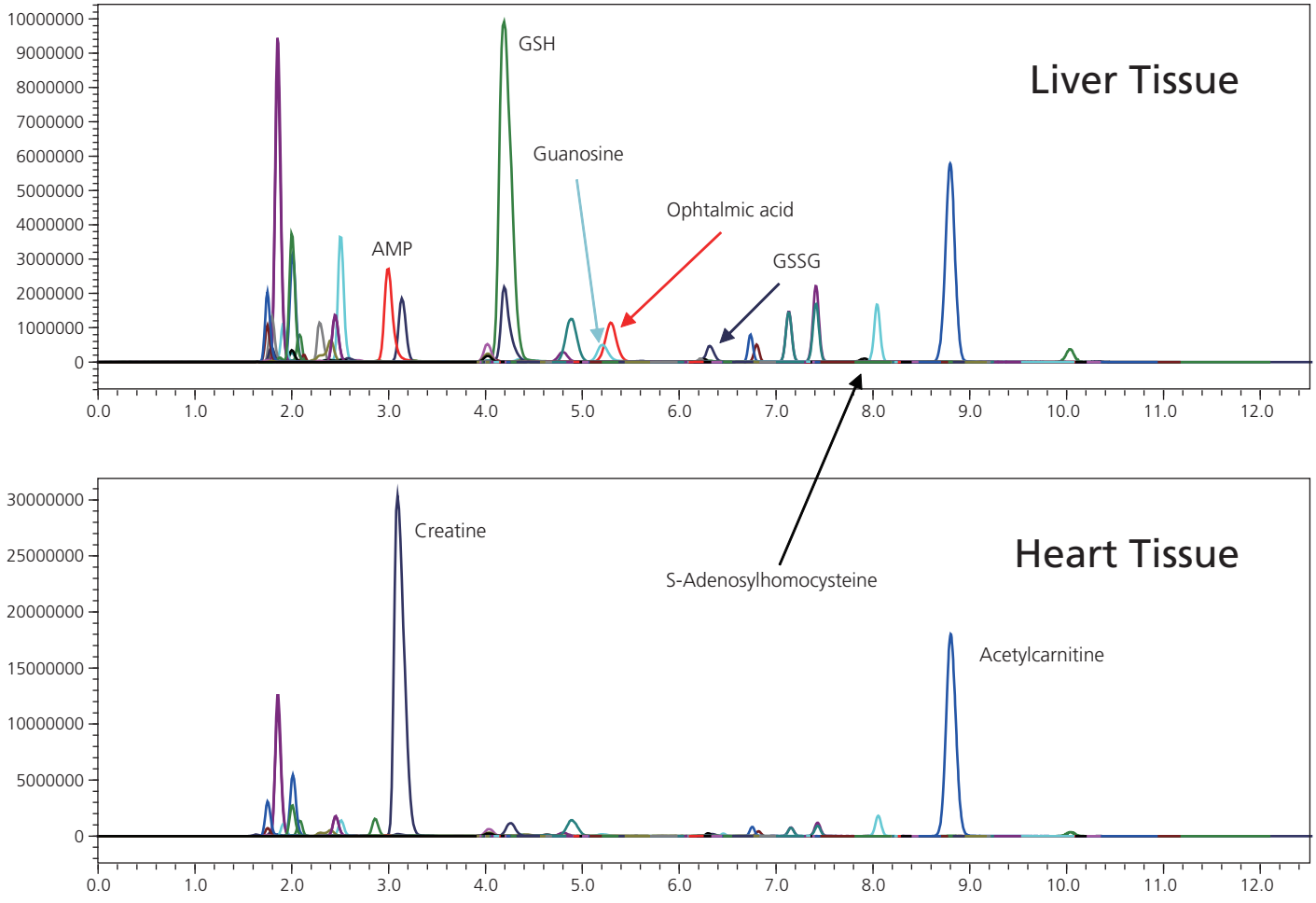

Figure 2 MRM chromatogram of liver/heart tissue extracts

Correlation between PFPP and ion pairing Methods

We have previously reported simultaneous analysis of 55 metabolites which were related to central carbon metabolic pathway by using ion pairing chromatography at ASMS conference 2013. To evaluate the accuracy of this simultaneous method using PFPP column, we compared the resulting peak area of 25 metabolites, which were covered as targets in both methods. The 25 metabolites are Lysine, Arginine, Histidine, Glycine, Serine, Asparagine, Alanine, Glutamine, Threonine, Methionine, Tyrosine, Glutamate, Aspartae, Phenylalanine, Tryptophan, Cysteine, CMP, NAD, GMP, TMP, AMP, cGMP, cAMP, MES and L-Methionine sulfone as internal standards. Heart tissue extracts were prepared from mice (n=9) according to the method described above and the aliquots were measured by the simultaneous method using either ion pairing chromatography or PFPP separation system. As a result, we could see the similar trend of elevation/decrease of peak area in metabolites of 20/25 between nine samples. The peak areas between 9 samples of representative metabolites are shown in Figure 3. This result shows that a ratio of areas between 9 samples is kept in both methods. The four metabolites (TMP, cGMP, cAMP and Cysteine) could be hardly detected on simultaneous analysis by alternately ion-paring chromatography or PFPP column. Tryptophan had a faint peak in this experiment and led to the low similarity.

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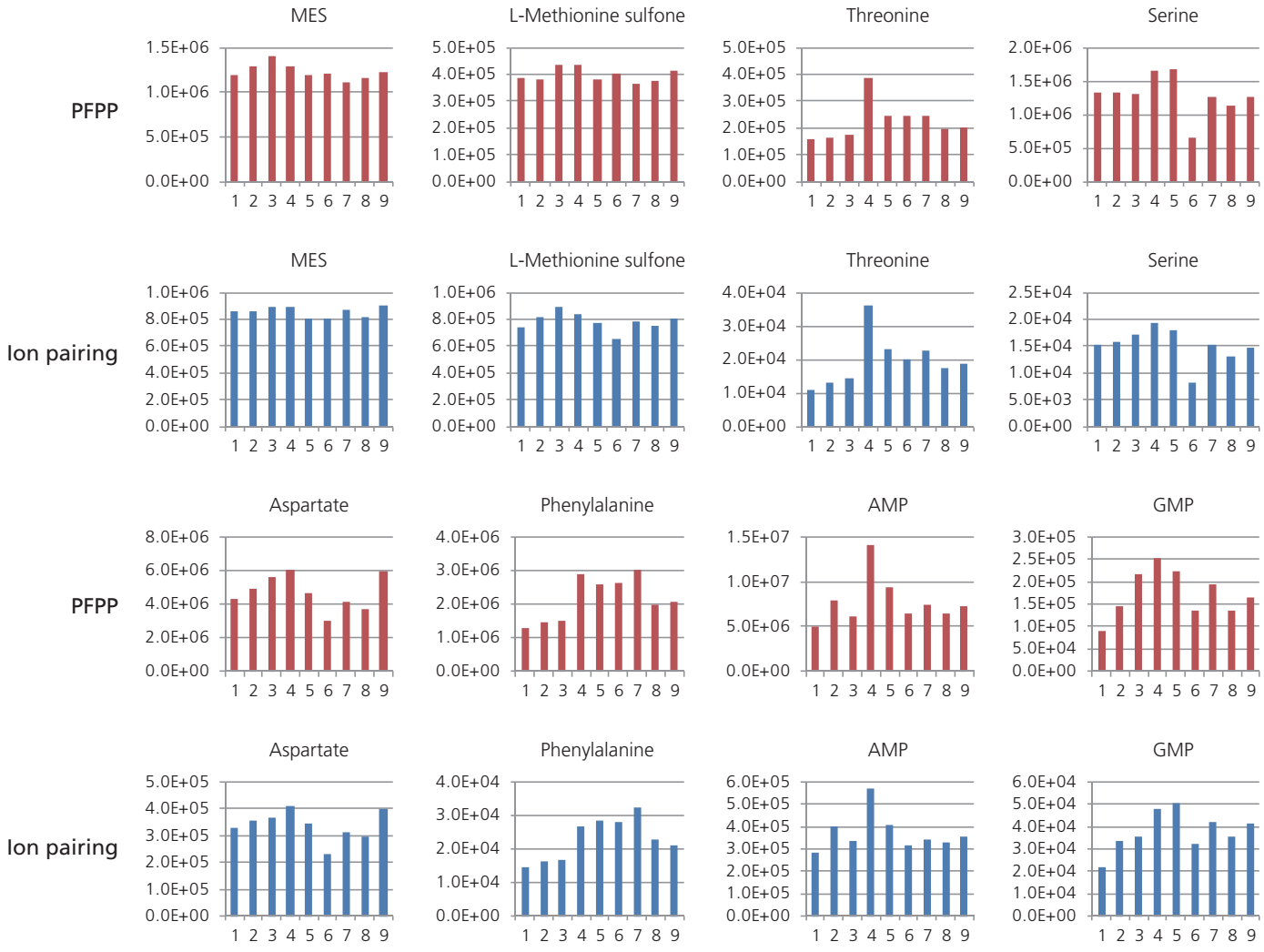

Figure 3 Correlation of peak areas between PFPP and ion-pairing method

Conclusions

- The 97 metabolites were separated by PFPP column with high resolution and this method was applied to biological samples.
- The utility of this simultaneous analysis using PFPP column was confirmed by comparing between PFPP and ion paring chromatography.

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