Supporting Information

Phosphate-containing Metabolite Enrichment with TiO_2 Micro-tips

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Experimental Procedure

Mass spectra were acquired with a 7 Tesla quadrupole-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker, Daltonics, MA) using acetonitrile (ACN)/H_2O (50/50, v/v) as spraying solvent for direct infusion. All data were obtained in negative ion mode. Mass spectra were acquired with XMASS software (version 6.1, Bruker Daltonics) in broadband mode from *m/z* 21 to 1000 with 256K data points and summed over 10 scans. Data processing was performed with the MIDAS analysis software (v 3.21). Two most abundant calculated masses of $[M - H]^-$ were used for internal calibration. Metabolites were bound to TiO_2 micro-tips (Glygen) in 10 μL acidic solution (formic or acetic acid, pH 2-3). The micro-tips were subsequently washed with 50 μl of ACN/H_2O (80/20, v/v) and bound metabolites were eluted with 50 μl of 0.5% piperidine (pH 11). Detailed optimization process will be discussed later in this supporting information. Signal enhancement shown in Table 1 was compared using the standard metabolite mixture shown in table S1.

Table S1. Metabolites and their concentrations in the standard metabolite mixture used for figure 1

	Metabolites	[M-H]-	Conc. (uM)
1	Aspartic acid	132.03	100
2	Glutamine	145.06	100
3	Glutamic acid	146.05	100
4	Glycerol-3-phosphate	171.01	2
5	3-phosphoglyceric acid	184.99	2
6	Citrate	191.09	5
7	Tryptophan	203.08	2
8	Glucose-6-phosphate	259.02	2
9	Trp- Gly-Gly	317.13	100
10	Thr-Ser-Lys	333.18	100
11	Fructose -1,6-diphosphate	338.99	2
12	Glu-Ala-Glu	346.13	100
13	ADP	426.02	2
14	ATP	505.99	2
15	NADH	664.12	2

Mass spectra of standard metabolite mixture before and after enrichment

Table S2. Signal intensities observed in figure 1 and signal enhancement (%). Signal enhancement was calculated by (signal intensity after TiO_2 treatment/signal intensity before TiO_2 treatment) ×100. For G6P and F1,6BP, signal enhancement could not be calculated due to the lack of signal before TiO_2 treatment. Instead, "Observed" was used to indicate that G6P and F1,6BP were observed only after TiO_2 treatment

	Metabolites	Signal intensity		Signal enhancement
		BEFORE	AFTER	(%)
1	Aspartic acid	7,991,126	Not observed	0
2	Glutamine	5,467,197	Not observed	0
3	Glutamic acid	7,541,567	Not observed	0
4	Glycerol-3-phosphate	4,595,603	1,505,411	33
5	3-phosphoglyceric acid	4,823,832	3,203,825	66
6	Citrate	11,043,173	1,376,171	12
7	Tryptophan	3,492,166	Not observed	0
8	Glucose-6-phosphate	Not observed	2,885,087	Observed
9	Trp-Gly-Gly	2,076,340,608	7,540,862	0
10	Thr-Ser-Lys	92,242,368	Not observed	0
11	Fructose-1,6-diphosphate	Not observed	29,974,670	Observed
12	Glu-Ala-Glu	83,076,120	Not observed	0
13	ADP	44,860,332	70,055,848	156
14	ATP	15,775,021	27,762,078	176
15	NADH	6,798,988	13,816,759	203

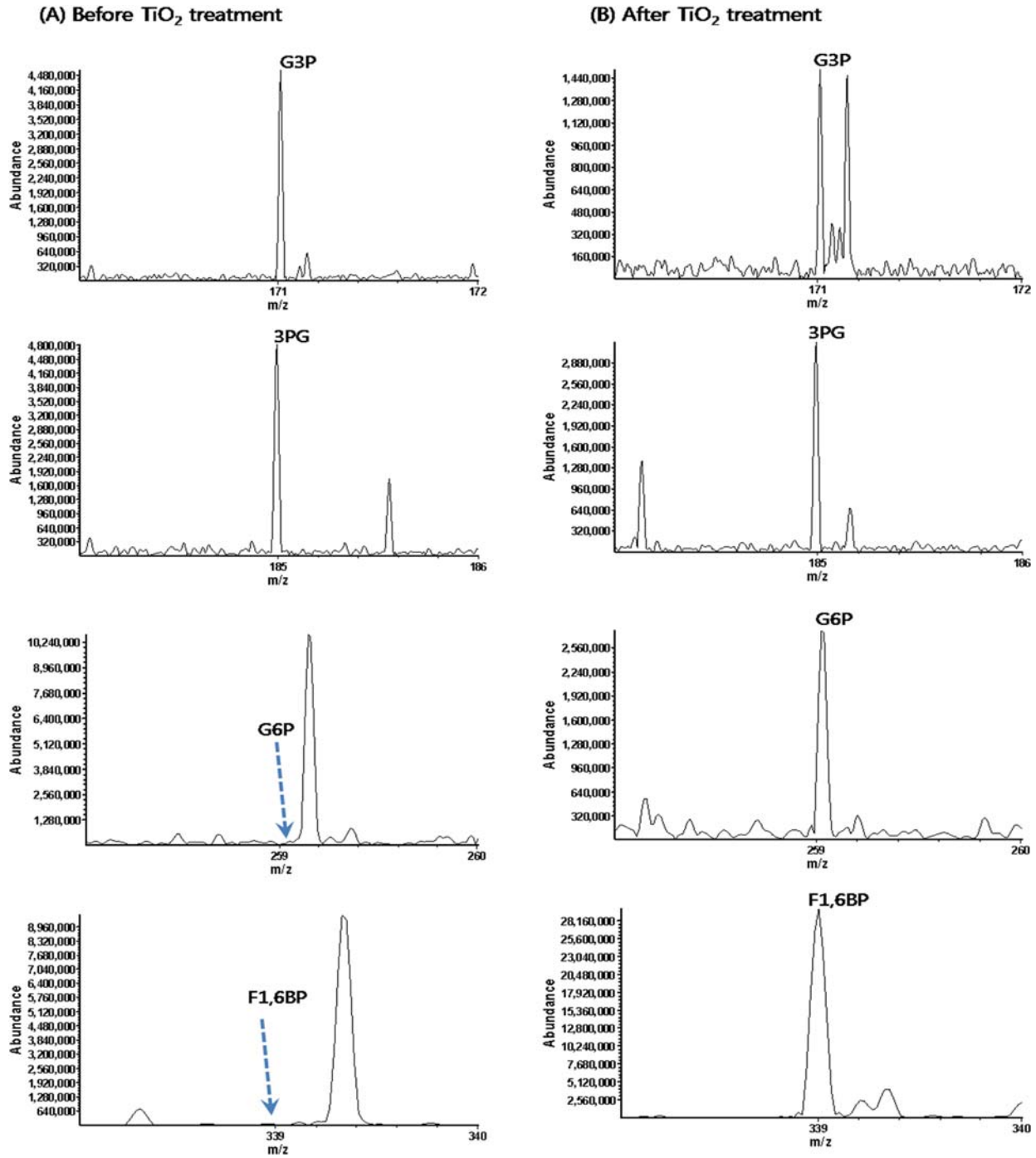

Figure S1. The detailed view of figure 1 (A) and (B), where G3P, 3PG, G6P, and F1,6BP were hard to be observed due to the complexity of the mass spectra.

Organic content in loading and washing solution

Another standard metabolite mixture was prepared as shown in table S3. Different sample solution should alter ion suppression, and ionization efficiency of each metabolite. It is reasonable to anticipate different level of signal enhancement of each phosphate-containing metabolite. Although metabolite composition has been changed, signal enhancement of phosphate-containing metabolites still follows the general tendency. In general, phosphate-containing metabolites with more than two phosphate groups showed significant signal enhancement after treated with TiO_2. Steric effect might play a role in poor signal enhancement of NADH.

Bar graph denoted by "Loading solution" in figure S2 indicates signal abundance of each metabolite remained in loading solution after pipetting with TiO_2 micro-tips. Bar graph denoted by "Washing solution" indicates signal intensity of each metabolite in washing solution after washing TiO_2 micro-tips with either H_2O or ACN/H_2O (80/20, v/v). As expected, signal enhancement of each phosphate-containing metabolite in figure S2 is different from that observed in figure 1 due to the different composition of sample solution.

As shown in figure S2, most metabolites, which do not contain phosphate groups, did not bind to TiO_2 and remained in loading solution. Some of phosphate-containing metabolites (*e.g.* G6P and ADP) were washed away with higher H_2O content. In addition, some of phosphate-containing metabolites (*e.g.* G6P and ADP) did not bind well with TiO_2 and remained in loading solution with higher H_2O content.

Best performance was observed with 50/50-80/20 ACN/H_2O (v/v) in loading and washing solutions. Acidic condition (pH 2-3) of loading solution using formic acid or acetic acid is necessary for the interaction between phosphate-containing metabolites and TiO_2 (data not shown).

Table S3. Metabolites and their concentrations in standard metabolite mixture used for figure S2

	Metabolites	[M-H]-	Conc. (uM)
A	Asparagine	131.05	237
B	Aspartic acid	132.03	237
C	Glutamine	145.06	237
D	Glutamic acid	146.05	237
E	Glycerol- 3-phosphate	171.01	2
F	3-phosphoglyceric acid	184.99	2
G	4-methylisophthalic acid	203.08	4
H	Tryptophan	259.02	2
I	Glucose 6-phosphate	317.13	6
J	Thr-Ser-Lys	333.18	4
K	Fructose-1,6-diphosphate	338.99	2
L	Glu-Ala-Glu	346.13	8
M	ADP	426.02	2
N	ATP	505.99	2
O	NADH	664.12	2

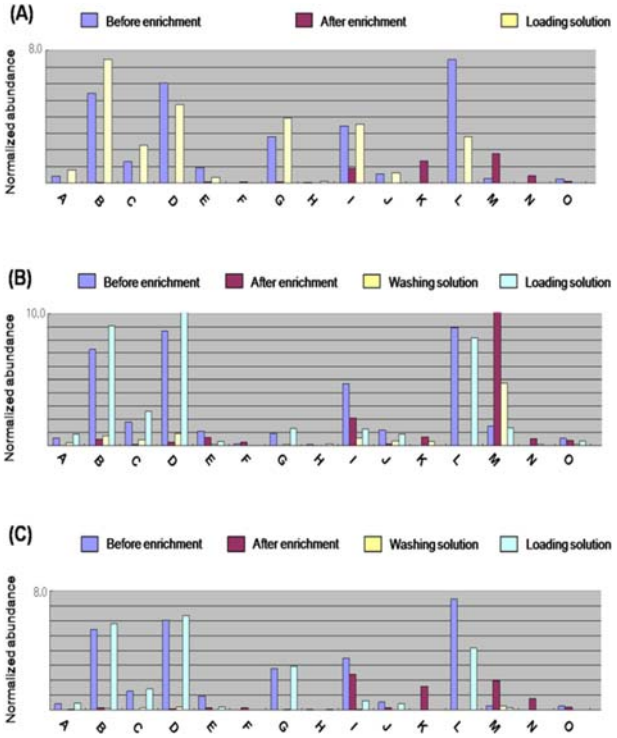

Figure S2. Normalized signal abundance of each metabolite in standard mixture shown in table S3. (A) 3.3% formic acid in H_2O, H_2O, and 0.5% piperidine were used as loading, washing, and eluting solution, respectively. (B) 3.3% formic acid in 80/20 ACN/H_2O, H_2O, and 0.5% piperidine were used as loading, washing, and eluting solution, respectively. (C) 3.3% formic acid in 80/20 ACN/H_2O, 80/20 ACN/H_2O, and 0.5% piperidine were used as loading, washing, and eluting solution, respectively. Letter A to letter O in the above follows the denotation shown in table S3. Signal intensity was normalized to the intensity of a certain unknown peak whose intensity is almost constant over times.