Supporting Information

A strategy for overcoming urine variability in LC-MS-based metabolomics studies

Yanhua Chen,[†] *Guoqing Shen,*[†] *Ruiping Zhang,*[†] *Jiuming He,*[†] *Yi Zhang,*[†] *Jing Xu,*[†] *Wei Yang,*[†] *Xiaoguang Chen,*[†] *Yongmei Song*[‡] *and Zeper Abliz* [*,†]

[†] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, P. R. China

[‡] Cancer Institute and Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, P. R. China

Experimental Section

2.1. Chemicals

HPLC grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Methanol (HPLC grade) was purchased from J&K Chemical Ltd.

2.2. Animal handling and sample collection

The animal study was approved by the Animal Care & Welfare Committee, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (No. 001669) and was performed at the Animal Experimental Centre, Institute of Materia Medica. Thirty male Wistar rats, 6–8 weeks old, 150 ± 10 g, were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, housed individually in stainless steel wire mesh cages, and provided with a certified standard rat chow and water *ad libitum*. Room temperature and relative humidity were regulated at 23 ± 5 °C and 55 ± 5%, respectively. A light cycle of 12 hours on and 12 hours off was set.

After 3 days of acclimatization in metabolic cages, the rats were randomly divided into two groups: Walker 256 tumor model group (n = 20, labeled M01–M20) and control group (n = 10, labeled C01–C10). Walker 256 tumor cells were obtained from the ascitic fluid in the Wistar rats after two cycles of 7 days cell passage by intraperitoneal injection of 10^7 Walker 256 carcinoma cells. After cell harvesting, a tumor cell suspension of 10^6 cells/100 μL was obtained by dilution with sterile saline. The rat model was established by subcutaneous injection of 200 μL of a suspension of the Walker 256 tumor cells into the right forelimb. An equal volume of sterile saline

was injected into the control group. After fasting for 12 h before each sampling, 24 h baseline urine samples from each animal were collected on day 0 (day before inoculation), and on days 4, 6, 9, 11, 14, and 16 after inoculation. In this study, the urine samples of day 0 and day 9 were chosen as the research objects for the comparison of calibration methods.

2.3. Sample preparation

All urine samples were centrifuged at 10000 rpm (7833 *g*) for 5 min at 4 ℃ to remove particle contaminants. The resultant supernatants were stored at –80 ℃ pending sample preparation.

Creatinine analysis was carried out at the Inspection Department of the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences (Beijing, China) using an enzymatic procedure.

2.4. LC-MS/MS analysis

For the LC-MS analysis, an Agilent 1200 series RRLC (rapid resolution liquid chromatography) system (Agilent Technologies, Waldbronn, Germany) was coupled to a Q-TOF RRLC–MS/MS system (QSTAR^{TM} Elite, AB Sciex, Canada).

RRLC conditions: Column: Zorbax SB-C_{18} (150 × 3.0 mm; 1.8 μm; Agilent Technologies, Santa Clara, CA, USA). Mobile phase A consisted of 0.01% formic acid; mobile phase B was acetonitrile. The gradient started at 0% B and was then linearly increased over 20 min to 100% B, which was maintained for 3 min. The flow rate was 250 μL/min.

Injection volume: identical volumes (10.0 μL) or volumes calculated according to

creatinine values. Injection volume calculation method: to ensure the accuracy of injection volumes, the sample with the highest creatinine value was injected in the smallest volume (1.0 μL); the injection volumes of other samples were calculated according to their creatinine values.

MS conditions: The mass spectrometer was operated in positive-ion mode. The scan mode was TOF and the scan range was *m*/*z* 100 to 1000. The source voltage was set at 5.5 kV, and the vaporizer temperature at 450 ℃. Gas settings: 50 arb for nebulizing gas, 50 arb for drying gas, 30 arb for curtain gas. The declustering potential was set at 50 V. Nitrogen was used as both nebulizing and drying gas. Data acquisition was performed using Analyst QS 2.0 (AB Sciex).

2.5. Data handling

Raw LC-MS data files (.wiff format) were converted into mzData format using a Wiff to mzData utility (AB Sciex, USA) and directly processed by means of an open-source XCMS package[1] under R statistical software (Version 2.10.0) for peak discrimination, filtering, and alignment. Two-dimensional matrices, including variable index (paired *m*/*z*–retention time), sample names (observations), and peak areas were thereby obtained. The peak areas of the matrices were then calibrated using common methods (see Section 2.6) and the calibrated matrices were introduced into SIMCA-P software 12.0 (Umetrics AB, Umeå, Sweden) for multivariate statistical analysis. The pareto variance scaled[2] data were analyzed by principal component analysis (PCA) to visualize general clustering effect trends among the observations. The advantages and disadvantages were evaluated by means of PCA score plots.

2.6. Normalization methods

Score plots derived from non-normalized data were used as controls to obtain a clearer picture of the normalization effects. The methods used were normalization to all MS signals, normalization to MSTUS, normalization to creatinine values, and normalization to creatinine peak areas.

A: No calibration.

B: Normalization to all MS signals: the peak area of each ion was divided by peak area sum of all ions in each sample.

C: Normalization to MSTUS ("total useful MS signals"):[3] the peak area of each ion was divided by peak area sum of all ions common to all samples.

D: Normalization to creatinine values: the peak area of each ion was divided by the creatinine value of the urine sample.

E: Normalization to creatinine peak areas: the peak area of each ion was divided by the creatinine peak area of each urine sample (area of extracted ion chromatographic peak at *m*/*z* 114.0–114.2 at 1.2 min).

Reference

(1) Smith, C. A.; Want, E. J.; O'Maille, G.; et al. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 2006, 78 (3), 779-787.

(2) van den Berg, R. A.; Hoefsloot, H. C.; Westerhuis, J. A.; et al. Centering, scaling, and transformations: improving the biological information content of metabolomics data[J]. *BMC genomics* 2006, 7, 142.

(3) Zhou, B.; Xiao, J. F.; Tuli, L.; Ressom, H. W., LC-MS-based metabolomics. *Mol Biosyst* 2012, 8 (2), 470-481.

Table S1. The creatinine values and the calculated injecting volumes of all samples.

Sample	1a	1/4a	1/8a	1/12a	1/16a	1/20a
Creatinine value (μmol/L)	9605	2401	1201	800	600	480
Injection volume (μL)	0.7	2.8	5.5	8.3	11.1	13.8

Sample	1b	1/4b	1/8b	1/12b	1/16b	1/20b
Creatinine value (μmol/L)	8160	2040	1020	680	510	408
Injection volume (μL)	0.8	3.3	6.5	9.8	13.0	16.3

Sample	1c	1/4c	1/8c	1/12c	1/16c	1/20c
Creatinine value (μmol/L)	7294	1824	912	608	456	365
Injection volume (μL)	0.9	3.6	7.3	10.9	14.6	18.2

Sample	1d	1/4d	1/8d	1/12d	1/16d	1/20d
Creatinine value (μmol/L)	6948	1737	869	579	434	347
Injection volume (μL)	1.0	3.8	7.6	11.5	15.3	19.1

Sample	1e	1/4e	1/8e	1/12e	1/16e	1/20e
Creatinine value (μmol/L)	6640	1660	830	553	415	332
Injection volume (μL)	1.0	4.0	8.0	12.0	16.0	20.0

Table S2. The creatinine values and the calculated injecting volumes of all samples.

Sample	M1-day0	M2-day0	M3-day0	M4-day0	M5-day0	M6-day0
Creatinine Value	1430	1094	1540	1652	1521	640
Injection Volume(μL)	8.9	11.6	8.2	7.7	8.3	19.8

Sample	C1-day0	C2-day0	C3-day0	C4-day0	C5-day0	C6-day0
Creatinine Value	2828	1512	1573	1085	1100	770
Injection Volume(μL)	4.5	8.4	8.1	11.7	11.5	16.5

Sample	M1-day9	M2-day9	M3-day9	M4-day9	M5-day9
Creatinine Value	2467	3421	1373	4301	2709
Injection Volume(μL)	5.1	3.7	9.2	2.9	4.7

Sample	M6-day9	M7-day9	M8-day9	M9-day9	M10-day9
Creatinine Value	2645	2970	1063	2653	1426
Injection Volume(μL)	4.8	4.3	11.9	4.8	8.9

Sample	C1-day9	C2-day9	C3-day9	C4-day9	C5-day9
Creatinine Value	3773	5468	3472	3215	1766
Injection Volume(μL)	3.4	2.3	3.7	3.9	7.2

Sample	C6-day9	C7-day9	C8-day9	C9-day9	C10-day9
Creatinine Value	6338	4035	5229	4339	1088
Injection Volume(μL)	2.0	3.1	2.4	2.9	11.7

Table S3. The peak areas of typical ions pre- and post-calibrations in sample 1a and its sequential diluted samples.

	1a	1a	1/4 a	1/4 a	1/8 a	1/8 a	1/12 a	1/12 a	1/16 a	1/16 a	1/20 a	1/20 a	RSD(%)
						***m/z* 194.06, Rt 6.65**							
A	4.41E+06	4.82E+06	2.52E+06	2.87E+06	1.67E+06	2.00E+06	1.28E+06	1.62E+06	1.04E+06	1.32E+06	1.01E+06	1.21E+06	**60.16**
B	1.94E-02	1.95E-02	2.43E-02	2.43E-02	2.35E-02	2.63E-02	2.51E-02	2.52E-02	2.34E-02	2.72E-02	2.47E-02	2.45E-02	**9.81**
C	4.14E-02	4.42E-02	4.69E-02	4.86E-02	4.43E-02	5.08E-02	4.84E-02	4.63E-02	4.46E-02	5.30E-02	4.88E-02	5.50E-02	**8.24**
D	4.59E+02	5.02E+02	1.05E+03	1.20E+03	1.39E+03	1.67E+03	1.60E+03	2.02E+03	1.73E+03	2.19E+03	2.10E+03	2.51E+03	**43.12**
E	1.47E+00	2.48E+00	1.53E+00	1.73E+00	1.39E+00	1.58E+00	1.25E+00	1.56E+00	1.20E+00	1.46E+00	1.01E+00	1.46E+00	**23.84**
F	1.96E+06	1.93E+06	1.93E+06	1.91E+06	1.95E+06	1.86E+06	1.90E+06	1.88E+06	1.94E+06	1.79E+06	2.20E+06	2.03E+06	**5.15**
G	2.42E-02	2.65E-02	2.76E-02	2.82E-02	2.75E-02	2.78E-02	2.67E-02	2.86E-02	2.68E-02	2.55E-02	3.10E-02	2.99E-02	**6.67**
H	3.71E-02	4.22E-02	4.25E-02	4.39E-02	4.17E-02	4.27E-02	3.97E-02	4.42E-02	3.92E-02	3.87E-02	4.81E-02	4.71E-02	**7.83**
						***m/z* 195.06, Rt 6.65**							
A	4.81E+05	6.35E+05	1.96E+05	3.06E+05	1.18E+05	1.84E+05	8.61E+04	1.35E+05	6.69E+04	1.04E+05	7.23E+04	9.08E+04	**87.47**
B	2.12E-03	2.57E-03	1.89E-03	2.59E-03	1.66E-03	2.41E-03	1.69E-03	2.10E-03	1.51E-03	2.16E-03	1.77E-03	1.84E-03	**17.78**
C	4.52E-03	5.81E-03	3.65E-03	5.18E-03	3.14E-03	4.67E-03	3.25E-03	3.86E-03	2.88E-03	4.21E-03	3.50E-03	4.14E-03	**21.44**
D	5.01E+01	6.61E+01	8.16E+01	1.27E+02	9.86E+01	1.53E+02	1.08E+02	1.68E+02	1.11E+02	1.74E+02	1.51E+02	1.89E+02	**36.27**
E	1.61E-01	3.27E-01	1.19E-01	1.84E-01	9.88E-02	1.46E-01	8.43E-02	1.30E-01	7.75E-02	1.16E-01	7.25E-02	1.10E-01	**36.27**
F	2.08E+05	1.95E+05	2.00E+05	1.85E+05	1.92E+05	2.22E+05	1.85E+05	1.81E+05	1.81E+05	1.72E+05	2.05E+05	1.96E+05	**7.16**
G	2.57E-03	2.68E-03	2.84E-03	2.73E-03	2.71E-03	3.33E-03	2.59E-03	2.75E-03	2.50E-03	2.45E-03	2.90E-03	2.89E-03	**8.51**
H	3.94E-03	4.26E-03	4.38E-03	4.27E-03	4.11E-03	5.11E-03	3.86E-03	4.25E-03	3.67E-03	3.71E-03	4.50E-03	4.55E-03	**9.57**
						***m/z* 196.06, Rt 6.65**							
A	2.85E+04	2.32E+04	1.58E+04	1.68E+04	1.07E+04	1.28E+04	8.68E+03	1.05E+04	7.31E+03	8.99E+03	6.95E+03	7.10E+03	**52.27**
B	1.25E-04	9.40E-05	1.52E-04	1.43E-04	1.50E-04	1.68E-04	1.70E-04	1.64E-04	1.65E-04	1.86E-04	1.70E-04	1.44E-04	**16.07**
C	2.67E-04	2.13E-04	2.94E-04	2.85E-04	2.83E-04	3.24E-04	3.28E-04	3.01E-04	3.14E-04	3.62E-04	3.37E-04	3.24E-04	**12.81**
D	2.97E+00	2.42E+00	6.59E+00	7.00E+00	8.89E+00	1.06E+01	1.09E+01	1.31E+01	1.22E+01	1.50E+01	1.45E+01	1.48E+01	**44.35**
E	9.51E-03	1.19E-02	9.60E-03	1.01E-02	8.91E-03	1.01E-02	8.50E-03	1.02E-02	8.46E-03	9.99E-03	6.97E-03	8.63E-03	**13.17**
F	1.23E+04	1.26E+04	1.23E+04	1.21E+04	1.32E+04	1.20E+04	1.26E+04	1.23E+04	1.18E+04	1.17E+04	1.38E+04	1.28E+04	**4.64**
G	1.52E-04	1.73E-04	1.76E-04	1.79E-04	1.87E-04	1.81E-04	1.77E-04	1.87E-04	1.63E-04	1.67E-04	1.94E-04	1.89E-04	**6.76**
H	2.33E-04	2.76E-04	2.71E-04	2.79E-04	2.83E-04	2.77E-04	2.64E-04	2.89E-04	2.39E-04	2.54E-04	3.02E-04	2.97E-04	**7.86**

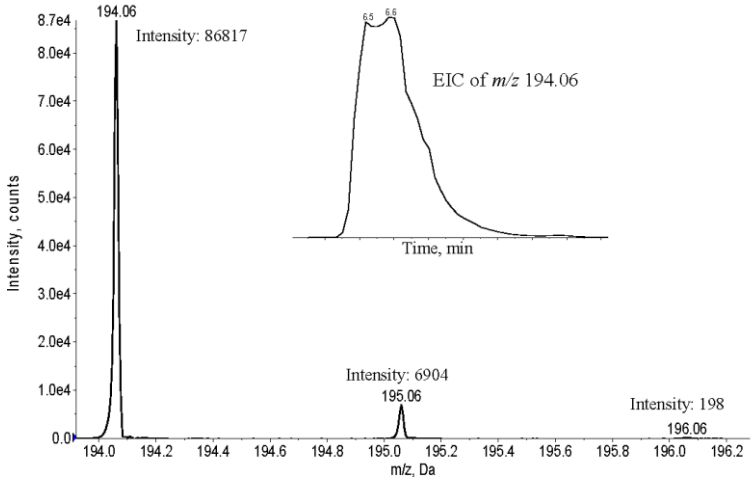

Figure S1. The intensity of *m/z* 194.06 and its isotope ions in sample 1a.