SUPPLEMENTARY INFORMATION

An optimization and evaluation strategy of esophageal tissue preparation protocols for metabolomics by LC-MS

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METHODS

Sample collection. The metabolism study was approved by the Animal Care & Welfare Committee, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, and esophageal tissue was collected from the New Drug Safety Evaluation Center, Institute of Materia Medica, Peking Union Medical College. The esophageal tissue was washed with saline after surgical resection to avoid the impact of blood, placed directly into liquid nitrogen, and stored at -80°C prior to analysis. Before metabolite extraction, esophageal tissue was cut into small pieces before homogenization to avoid heterogeneity homology.

Chemicals and Reagents. HPLC-grade acetonitrile (ACN) and formic acid were purchased from Merck (Darmstadt, Germany), and other chemicals were of analytical grade. Pure water was obtained from the Wahaha Group Co., Ltd., Hangzhou, China. L-tyrosine, proline, valine, phenylalanine, tryptophan, kynurenic acid, arachidonic acid, nicotinic acid, hippuric acid, linoleic acid, oleic acid, L-carnitine, lysoPC 16:0, acetylcarnitine, octanoylcarnitine, decanoylcarnitine, cytidine, inosine, and methyltestosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation. Methods for homogenization and metabolite extraction were as follows.

Three tissue disruption methods were used: *a.HW* (Homogenized Wet): 50 mg of wet tissue was accurately weighed, cut into small pieces, and extracted. *b.GW* (Ground Wet): tissue was cut into pieces, ground in a liquid N_2–cooled mortar and pestle, and placed into the freeze dryer to be lyophilized. Ten milligrams of accurately weighed lyophilized powder was then extracted (the weight of lyophilized tissue was 1/5 that of wet tissue). *c.GD* (Ground Dry): the opposite of *b.GW*; the sample was lyophilized first, and then ground into powder.

Four kinds of solvent extraction methods were used. Method 1 comprised methanol/water, dichloromethane/methanol (*TS*: two-step addition): corresponding tissue samples were accurately weighed into a 2 mL homogenization tube containing four ceramic beads (3.0 mm diameter). To the tube, 1.5 mL of pre-cooled extraction solvent (1:1 methanol/water) was added, and the tissue was homogenized three times for 30 s at a shock velocity of 4.0 m/s using a high-throughput MasterPrep^{TM}-24 tissue homogenizer; the 30 s intervals were used to ensure maintenance of freezing temperatures in sample vials between homogenization steps. Homogenate was centrifuged at 15,000 rpm for 5 min at 4°C, and then 1.6 mL of pre-cooled 3:1 dichloromethane/methanol was added to the solid precipitate at lower homogenate after centrifuge, followed by another round of homogenization using the same parameters described above. The supernatants were collected and dried using a Savant SPD121P-230 SpeedVac concentrator with RVT-4104 refrigerated vapor traps (Thermo Fisher Scientific Electron Corporation, Milford, MA, USA). Dried polar and nonpolar extracts were stored at -80°C prior to LC-MS analysis.

Method 2: dichloromethane/methanol/water (*SW*: stepwise addition) was the optimized protocol described in the article.

Method 3: dichloromethane/methanol/water (*AO*: all-in one addition) was similar to method 2. In this method, 1.5 mL of pre-cooled extraction solvent (2:2:1.8

dichloromethane/methanol/water) was added at one time. The rest of the operation was the same as in method 2.

Method 4: methanol/water (4:1, *SP*: single phase); 1.5 mL of pre-cooled extraction solvent (4:1 methanol/water) was added at one time, and the rest of the operation was the same as in method 3.

The optimal protocol is detailed in the following steps. 1). Frozen tissue (wet weight: 50 mg) was accurately weighed into a 2 mL homogenization tube containing four ceramic beads (3.0 mm diameter). 2). Pre-cooled extraction solvents (410 μL of methanol, 210 μL of water) were added, and the tissue was homogenized three times for 30 s at a shock velocity of 4.0 m/s using a high-throughput MasterPrep^{TM}-24 tissue homogenizer. 3). After homogenization, 140 μL of dichloromethane was added, and the sample was vortexed for 2 min. 4). Then 140 μL of dichloromethane and 210 μL of water were added, and the sample was vortexed for 2 min followed by incubation at 4° C for 20 min. 5). Next, the sample was centrifuged at 15,000 rpm for 4 min at 4° C. Centrifugation produced a biphasic mixture. 6). The upper (polar) and lower (nonpolar) layers were collected separately and dried using a Savant SPD121P-230 SpeedVac concentrator with RVT-4104 refrigerated vapor traps. 7). Before analysis, polar extracts were resuspended in 1.2 mL of 80:20 ACN/water, and nonpolar extracts were resuspended in 120 μL of 40:60 ACN/water.

HPLC-MS conditions. Separation of analytes from the tissue extract was carried out using a Dionex UltiMate 3000 HPLC system, which consisted of an UltiMate 3000 RS pump, an UltiMate 3000 RS auto-sampler, and an UltiMate 3000 RS column compartment (Dionex, Olten, Switzerland). Gradient elution was performed as follows:

(1) HILIC-LC-MS analysis of polar extracts. A Phenomenex Kinetex HILIC column (2.6 μm, 2.1 × 150 mm) was used. Column temperature was 45°C, and the flow rate was 0.3 mL/min. Mobile phase A was ACN and mobile phase B was 5 mM ammonium acetate in water. Injection volumes of 10 μL and 20 μL were used for positive and negative ionization modes, respectively. The auto-sampler was set at 4°C. The elution gradient was as follows:

Retention Time(min)	A%	B%
0	95	5
25	60	40
30	95	5

(2) RP-LC-MS analysis of nonpolar extracts. A Waters ACQUITY UPLC CSH C_{18} column (1.7 μm, 2.1 × 100 mm) was used. Column temperature was 30°C, and the flow rate was 0.2 mL/min. Mobile phase A was ACN and mobile phase B was 0.1% formic acid in water. An injection volume of 10 μL was used for both positive and negative electrospray ionization (ESI) modes. The auto-sampler was set at 4°C. The elution gradient was set as follows:

Retention Time(min)	A%	B%
0	2	98
3	60	40
10	60	40
12	81	19
20	81	19
22	90	10
32	90	10
33	100	0
35	100	0

The HPLC was coupled to a Q-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific) via a heated electrospray source (HESI) using the following operation parameters: sheath gas flow rate, 40 PSI; aux gas flow rate, 11 Arb; sweep gas flow rate, 0; spray voltage, 3.5 kV for positive mode and 3.2 kV for negative mode; capillary temperature, 350°C; s-lens RF level, 55; aux gas heater temperature, 220°C. High-purity nitrogen (N_2) was used as the nebulizing gas and the collision gas for higher energy collisional dissociation.

LC-MS raw data files were converted to mzXML format using the Mass Matrix MS Data File Conversion Tools (http://www.massmatrix.net/). Peak finding, filtering, alignment, and scaling were performed using the open-source XCMS software (http://masspec.Scripps.edu/xcms/xcms.php) operated within the R statistical environment (version 2.15.2). The detailed parameters were as follows.

Data Preprocessing.

The program used for peak discrimination, filtering, alignment and CAMERA analysis of the LC-(+)ESI-MS data:

```
rm(list=ls(all=TRUE)）
library(Biobase)
library(xcms)
library(multtest)
library(CAMERA)
sessionInfo()
xs<-xcmsSet(profmethod="binlin",method="centWave",ppm=2.5,  peakwidth=c(5,35),
snthresh =30,prefilter=c(10,10000),integrate=1, mzdiff =0.005)
xs <-group(xs,bw=2,minfrac=0.3,mzwid=0.015)
save(xs,file="xs.Rda")
ret.xs.obiwarp <-retcor(xs,method="obiwarp",plottype="deviation")
ret.xs.obiwarp<-group(ret.xs.obiwarp, bw=2,minfrac=0.3,mzwid=0.015)
ret.xs.obiwarp
fill.ret.xs.obiwarp<-fillPeaks(ret.xs.obiwarp)
fill.ret.xs.obiwarp
save(fill.ret.xs.obiwarp, file="fill.ret.xs.obiwarp.Rda")
an.1<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="positive")
peaklist.1<-getPeaklist(an.1)
```

```
write.csv(peaklist.1,file='annotated.1.csv')
an.2<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="positive")
peaklist.2<-getPeaklist(an.2)
write.csv(peaklist.2,file='annotated.2.csv')
an.3<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="positive")
peaklist.3<-getPeaklist(an.3)
write.csv(peaklist.3,file='annotated.3.csv')
an.4<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="positive")
peaklist.4<-getPeaklist(an.4)
write.csv(peaklist.4,file='annotated.4.csv')
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","2",eicmax=8000,file="shi
guan pos12")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","3",eicmax=8000,file="shi
guan pos13")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","4",eicmax=8000,file="shi
guan pos14")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"2","3",eicmax=8000,file="shi
guan pos23")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"2","4",eicmax=8000,file="shi
guan pos24")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"3","4",eicmax=8000,file="shi
guan pos34")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
```

The program used for the LC-(-)ESI-MS data:

```
rm(list=ls(all=TRUE)）
library(Biobase)
library(xcms)
library(multtest)
library(CAMERA)
sessionInfo()
xs<-xcmsSet(profmethod="binlin",method="centWave",ppm=2.5,  peakwidth=c(5,35),
snthresh =30,prefilter=c(10,10000),integrate=1, mzdiff =0.005)
xs <-group(xs,bw=2,minfrac=0.3,mzwid=0.015)
save(xs,file="xs.Rda")
ret.xs.obiwarp <-retcor(xs,method="obiwarp",plottype="deviation")
ret.xs.obiwarp<-group(ret.xs.obiwarp, bw=2,minfrac=0.3,mzwid=0.015)
```

```
ret.xs.obiwarp
fill.ret.xs.obiwarp<-fillPeaks(ret.xs.obiwarp)
fill.ret.xs.obiwarp
save(fill.ret.xs.obiwarp, file="fill.ret.xs.obiwarp.Rda")
an.1<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="negative")
peaklist.1<-getPeaklist(an.1)
write.csv(peaklist.1,file='annotated.1.csv')
an.2<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="negative")
peaklist.2<-getPeaklist(an.2)
write.csv(peaklist.2,file='annotated.2.csv')
an.3<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="negative")
peaklist.3<-getPeaklist(an.3)
write.csv(peaklist.3,file='annotated.3.csv')
an.4<-annotate(fill.ret.xs.obiwarp,sigma=6,perfwhm=0.3,cor_eic_th=0.75,maxcharge
=3,maxiso=3,mzabs=0.03,multiplier=3,polarity="negative")
peaklist.4<-getPeaklist(an.4)
write.csv(peaklist.4,file='annotated.4.csv')
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","2",eicmax=8000,file="shi
guan neg12")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","3",eicmax=8000,file="shi
guan neg13")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"1","4",eicmax=8000,file="shi
guan neg14")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"2","3",eicmax=8000,file="shi
guan neg23")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"2","4",eicmax=8000,file="shi
guan neg24")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
report.fill.ret.xs.obiwarp<-diffreport(fill.ret.xs.obiwarp,"3","4",eicmax=8000,file="shi
guan neg34")
save(report.fill.ret.xs.obiwarp,file="report.fill.ret.xs.obiwarp.Rad")
```

The resultant two-dimensional matrices, including observations (sample names) in columns, variables (*m/z*-retention time pairs) in rows, and peak areas, were further imported into the SIMCA-P 12.0 software package (Umetrics AB, Umeå, Sweden) for multivariate statistical data analysis (MVDA). Mean-centering and Pareto-scaling were applied to all data prior to MVDA to reduce noise and artifacts in the models. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to

evaluate the reproducibility of different methods. To test the validity of the model against overfitting, the cross-validation parameter Q2 was computed as in partial least-squares discriminant analysis (PLS-DA) by permutation testing using 100 random permutations.

Method validation. For preparation of endogenous standard mixture solution *(MIX)*, the following 19 endogenous standards were accurately weighed (5 mg each) into a 1 mL tube (containing methanol or methanol/water as a solvent): L-tyrosine, proline, valine, phenylalanine, tryptophan, kynurenic acid, arachidonic acid, nicotinic acid, hippuric acid, linoleic acid, oleic acid, L-carnitine, lysoPC 16:0, acetylcarnitine, octanoylcarnitine, decanoylcarnitine, cytidine, inosine, and methyltestosterone. The standard solutions were saved as stock solutions. Different volumes of these standard solutions were collected, mixed together, and diluted into 2 mL of water, followed by vortexing for 30 seconds to ensure complete mixing. The concentration of each analyte in *MIX* was 10 times its concentration in normal tissues. This standard solution was used for the method validation study.

Sensitivity. The *MIX* solution was separately diluted 1-, 2-, 5-, 10-, 50-, or 100-fold, each with two replicates. Thus, the concentrations of analytes in the diluted solutions were 10, 5, 2, 1, 1/5, and 1/10 times their concentrations in normal tissues[1] (concentrations in the normal mouse tissues can be found in database of MMDB); these solutions were labeled *mix1*, *mix2*, *mix3*, *mix4*, *mix5*, and *mix6*, respectively. Established LC-MS methods were used to calculate the minimum detectable concentration of each compound. When the actual minimum detectable concentration of the sample was less than $1/10^{th}$ of that in normal tissue, $1/10^{th}$ of the normal tissue concentration was defined as the minimum detectable concentration, and the signal-to-noise ratio (S/N) was recorded under that condition.

Precision. Instrument precision was characterized by relative standard deviation (RSD) of the extracted ion chromatogram peak area from six continuous injections of the same sample. Method precision: Intra-day precision was evaluated by freshly preparing six independent replicates (n = 6) of tissue samples; *mix3* (2 times normal tissue concentration) was added into the tissues. The inter-day precision was monitored using six replicates of the samples described above on three different days. As shown in Table S3, the RSD of metabolite extracted peak area was 1.9–16.2%. Results of the method precision study are shown in Table S4; the inter- and intra-day precisions were 4.4–23.2% and 3.5–20.9%, respectively. These results indicate that our protocol is reproducible and reliable for esophageal tissue metabolomics research.

Recovery. *mix2*, *mix3*, and *mix4* (5, 2, and 1 times the normal tissue concentration respectively) were added to the blank tissues using the tissue preparation protocol described above; these were defined as high, middle, and low concentration levels, respectively (replicates: n =3). Standard solutions at the corresponding concentrations were analyzed in parallel. Molecular recovery was calculated as ([area of spiked tissue samples – area of blank tissue samples]/[area of spiked after preparation tissue samples – area of blank tissue samples])*100%. The extraction recoveries of the 19 compounds at three different concentrations are summarized in Table S5. Extraction recoveries were 50.7–128.6%, and mean recovery

was 89.6%. Most of the compounds are present in both polar and nonpolar extracts, but the proportion differed according to the polarity. Recovery was the sum of the two parts. Thus, extraction recoveries of our method meet the acceptance criteria.

Stability. To study metabolite stability under analysis conditions, sets of samples were prepared and either incubated in the auto-sampler at 4°C for 1, 2, 3, or 7 days, or subjected to three freeze-thaw cycles (-20°C–20°C). Stability was characterized as RSD of the extracted ion chromatogram peak areas of endogenous standard compounds.

SUPPLEMENTARY TABLES AND FIGURES

Table S1. Endogenous metabolites used to evaluate different methods.

Polar-Pos	N0.	Compound	Formula	$[M+H]^+$	Retention Time
	1	Valine [a]	$C_5H_{11}NO_2$	118.0863	11.62
	2	L-isoleucine [a]	$C_6H_{13}NO_2$	132.1019	12.57
	3	L-leucine [a]	$C_6H_{13}NO_2$	132.1019	12.57
	4	L-methionine [a]	$C_5H_{11}NO_2S$	150.0583	11.41
	5	Phenylalanine [a]	$C_9H_{11}NO_2$	166.0863	10.99
	6	Proline [a]	$C_5H_9NO_2$	116.0706	13.69
	7	Hydroxy-L-proline [a]	$C_5H_9NO_3$	132.0655	13.44
Polar extracts	8	Tryptophan [a]	$C_{11}H_{12}N_2O_2$	205.0972	10.61
in positive ion mode of	9	L-Tyrosine [a]	$C_9H_{11}NO_3$	182.0812	12.78
ESI	10	L-glutamine [a]	$C_5H_{10}N_2O_3$	147.0764	17.38
Amino Acids	11	Phenylacetylglutamine [a]	$C_{13}H_{16}N_2O_4$	265.1183	6.79
	12	L-cystine [b]	$C_6H_{12}N_2O_4S_2$	241.0311	4.03
	13	S-carboxymethylcysteine [b]	$C_5H_9NO_4S$	180.0325	2.96
	14	L-glutamic acid [a]	$C_5H_9NO_4$	148.0604	3.12
	15	Taurine [a]	$C_2H_7NO_3S$	126.0219	6.99
	16	L-citrulline [a]	$C_6H_{13}N_3O_3$	176.103	15.92
	17	(R)-(+)-2-Pyrrolidone-5-carboxylic acid [a]	$C_5H_7NO_3$	130.0499	18.45
	18	Creatine [a]	$C_4H_9N_3O_2$	132.0768	14.08
	1	L-Carnitine [a]	$C_7H_{15}NO_3$	162.1125	19.56
	2	γ-butyrobetaine [b]	$C_7H_{15}NO_2$	146.1176	23.66
	3	L-Acetylcarnitine [a]	$C_9H_{17}NO_4$	204.123	17.92
	4	Hexanoylcarnitine(C6:0) [b]	$C_{13}H_{25}NO_4$	260.1856	14.93
Polar extracts	5	Heptanoylcarnitine(C7:0) [b]	$C_{14}H_{27}NO_4$	274.2013	17.71
in positive ion mode of	6	Octanoylcarnitine(C8:0) [a]	$C_{15}H_{29}NO_4$	288.2169	13.98
ESI	7	Nonanoylcarnitine(C9:0) [b]	$C_{16}H_{31}NO_4$	302.2326	13.60
Carnitines	8	Decanoylcarnitine(C10:0) [b]	$C_{17}H_{33}NO_4$	316.2482	13.52
	9	Undecanoylcarnitine(11:0) [b]	$C_{18}H_{35}NO_4$	330.2639	1.42
	10	Octenoylcarnitine(C8:1) [b]	$C_{15}H_{27}NO_4$	286.2013	14.20
	11	Decenoylcarnitine(C10:1) [b]	$C_{17}H_{31}NO_4$	314.2326	13.52
	12	Tetradecadiennoylcarnitine(14:2) [b]	$C_{21}H_{37}NO_4$	368.2795	13.02

Continued

	N0.	Compound	Formula	$[M+H]^+$	Retention Time
	13	3-OH-octanoylcarnitine(C8:0-OH) [b]	$C_{15}H_{29}NO_5$	304.2118	15.23
	14	Hydroxydecanoylcarnitine(C10:0-OH) [b]	$C_{17}H_{33}NO_5$	332.2432	14.96
	15	Carnitine C12:0-OH [b]	$C_{19}H_{37}NO_5$	360.2745	14.29
	16	Carnitine C14:0-OH [b]	$C_{21}H_{41}NO_5$	388.3058	13.74
	17	Carnitine C14:2-OH [b]	$C_{21}H_{37}NO_5$	384.2745	13.71
	18	Carnitine C16:2-OH [b]	$C_{23}H_{41}NO_5$	412.3058	13.58
	19	Carnitine C18:2-OH [b]	$C_{25}H_{45}NO_5$	440.3371	13.22
	1	LPC(14:0) [b]	$C_{22}H_{46}NO_7P$	468.3085	13.25
	2	LPC(15:0) [a]	$C_{23}H_{48}NO_7P$	482.3241	13.29
	3	LPC(16:0) [a]	$C_{24}H_{50}NO_7P$	496.3398	13.29
	4	LPC(17:0) [b]	$C_{25}H_{52}NO_7P$	510.3554	13.10
	5	LPC(18:0) [b]	$C_{26}H_{54}NO_7P$	524.3711	15.73
	6	LPC(21:0) [b]	$C_{30}H_{50}NO_7P$	568.3398	11.71
Polar extracts	7	LPC(16:1) [b]	$C_{24}H_{48}NO_7P$	494.3241	16.1
in positive ion mode	8	LPC(17:1) [b]	$C_{25}H_{50}NO_7P$	508.3398	15.95
of ESI	9	LPC(18:1) [a]	$C_{26}H_{52}NO_7P$	522.3554	13.18
Cholines	10	LPC(18:2) [b]	$C_{26}H_{50}NO_7P$	520.3398	13.04
	11	LPC(18:3) [b]	$C_{26}H_{48}NO_7P$	518.3241	13.10
	12	LPC(20:3) [b]	$C_{28}H_{52}NO_7P$	546.3554	12.86
	13	LPC(20:4) [b]	$C_{28}H_{50}NO_7P$	544.3398	12.96
	14	LPC(20:5) [b]	$C_{28}H_{48}NO_7P$	542.3241	15.94
	15	LPE(22:6) [b]	$C_{27}H_{44}NO_7P$	526.2928	12.59
	16	LPE(20:4) [b]	$C_{25}H_{44}NO_7P$	502.2928	12.82
Polar-POS	1	1-methylguanine [a]	$C_6H_7N_5O$	166.0723	4.93
Nucleosides	2	Hypoxanthine [a]	$C_5H_4N_4O$	137.0458	3.61
	3	3-Methylxanthine [a]	$C_6H_6N_4O_2$	167.0564	2.06
	4	7-Methylxanthine [a]	$C_6H_6N_4O_2$	167.0564	5.95
	5	Dimethylguanosine [c]	$C_{12}H_{17}N_5O_5$	312.1302	13.06
	6	5-Methylthioadenosine [a]	$C_{11}H_{15}N_5O_3S$	298.0968	2.75
	7	Cytidine [a]	$C_9H_{13}N_3O_5$	244.0928	5.15
	8	2′-Deoxycytidine [a]	$C_9H_{13}N_3O_4$	228.0979	5.44
	9	cGMP [b]	$C_{10}H_{12}N_5O_7P$	346.0547	5.85
	1	Pregnenolone [b]	$C_{21}H_{32}O_2$	317.2475	4.06
Polar extracts	2	Androsterone sulfate [b]	$C_{19}H_{30}O_5S$	371.1887	1.83
in positive ion mode of	3	DHEA sulfate [b]	$C_{19}H_{28}O_5S$	369.173	1.96
ESI	4	L-Fucose [b]	$C_6H_{12}O_5$	165.0758	1.90
Low-level metabolites	5	D-sorbitol [b]	$C_6H_{14}O_6$	183.0863	6.87

Polar-Neg	N0.	Compound	Formula	[M-H]$^-$	Retention Time
Polar extracts in negative ion mode of ESI Fatty acids	1	Linoleic acid [b]	$C_{18}H_{32}O_2$	279.233	1.37
	2	Palmitoleic acid [b]	$C_{16}H_{30}O_2$	253.2173	0.42
	3	Oleic acid [b]	$C_{18}H_{34}O_2$	281.2486	1.38
	4	Linolenic acid [a]	$C_{18}H_{30}O_2$	277.2173	1.35
	5	Arachidonic acid [a]	$C_{20}H_{32}O_2$	303.233	1.41
	6	FAC 14：1-OH [b]	$C_{14}H_{26}O_3$	241.1809	0.75
	7	Shikimic acid [b]	$C_7H_{10}O_5$	173.0455	2.77
	8	Kynuranic acid [a]	$C_{10}H_7NO_3$	188.0353	3.18
	9	Nicotinic acid [b]	$C_6H_5NO_2$	122.0248	5.23
	10	N-methyl nicotinate [b]	$C_7H_7NO_2$	136.0404	3.59
	11	Niacinamide [a]	$C_6H_6N_2O$	121.0407	1.66
	12	Allantoin [a]	$C_4H_6N_4O_3$	157.0367	2.53
	13	Stachydrine [b]	$C_7H_{13}NO_2$	142.0874	22.93
Polar extracts in negative ion mode of ESI Amino Acids	1	Proline [a]	$C_5H_9NO_2$	114.0561	12.88
	2	Proline betaine [b]	$C_7H_{13}NO_2$	142.0874	22.93
	3	Taurine [a]	$C_2H_7NO_3S$	124.0074	6.71
	4	Pyroglutamic acid [a]	$C_5H_7NO_3$	128.0353	6.98
Polar extracts in negative ion mode of ESI Low-level metabolites	1	Pregnenolone [b]	$C_{21}H_{32}O_2$	315.233	1.37
	2	2-Methoxyestrone [b]	$C_{19}H_{24}O_3$	299.1653	1.42
	3	3-β-hydroxyandrost-5-en-17-one sulfate [b]	$C_{19}H_{28}O_5S$	367.1585	0.82
	4	Aldosterone [b]	$C_{21}H_{28}O_5$	359.1864	13.77
	5	Cholic acid [b]	$C_{24}H_{40}O_5$	407.2803	4.27
	6	Digoxin [b]	$C_{41}H_{64}O_{14}$	779.4223	0.55
	7	D-Talose [a]	$C_6H_{12}O_6$	179.0561	1.80
	8	Galactose [a]	$C_6H_{12}O_6$	179.0561	1.80
	9	1-methylguanine [a]	$C_6H_7N_5O$	164.0578	4.31
	10	Hypoxanthine [a]	$C_5H_4N_4O$	135.0312	2.98
	11	5,6-Dihydrouridine [b]	$C_9H_{14}N_2O_6$	245.0779	6.39
	12	Cytidine [a]	$C_9H_{13}N_3O_5$	242.0782	19.66
	13	2-aminophenol [b]	C_6H_7NO	108.0455	5.08
	14	Glucose [a]	$C_6H_{12}O_6$	179.0561	1.80

Nonpolar-Pos	N0.	Compound	Formula	$[M+H]^+$	Retention Time
Nonolar extracts in positive ion mode of ESI Amino Acids	1	Valine [a]	$C_5H_{11}NO_2$	118.0863	1.36
	2	Proline [a]	$C_5H_9NO_2$	116.0706	1.43
	3	Hydroxy-L-proline [a]	$C_5H_9NO_3$	132.0655	1.37
	4	Tryptophan [a]	$C_{11}H_{12}N_2O_2$	205.0972	4.08
	5	Serine [a]	$C_3H_7NO_3$	106.0499	1.32
	6	Threonine [b]	$C_4H_9NO_3$	120.0655	1.08
	7	L-asparagine [a]	$C_4H_8N_2O_3$	133.0608	1.32
	8	L-glutamine [a]	$C_5H_{10}N_2O_3$	147.0764	1.32
	9	S-carboxymethylcysteine [b]	$C_5H_9NO_4S$	180.0325	1.81
	10	L-lysine [a]	$C_6H_{14}N_2O_2$	147.1128	1.02
	11	L-arginine [a]	$C_6H_{14}N_4O_2$	175.119	1.12
	12	Dimethylarginine [a]	$C_8H_{18}N_4O_2$	203.1503	1.09
	13	L-histidine [a]	$C_6H_9N_3O_2$	156.0768	1.12
	14	1-methyl-L-histidine [a]	$C_7H_{11}N_3O_2$	170.0924	1.12
	15	Taurine [a]	$C_2H_7NO_3S$	126.0219	1.40
	16	L-citrulline [a]	$C_6H_{13}N_3O_3$	176.103	1.35
	17	L-carnosine [a]	$C_9H_{14}N_4O_3$	227.1139	1.12
	18	L-anserine [b]	$C_{10}H_{16}N_4O_3$	241.1295	1.04
	19	(R)-(+)-2-Pyrrolidone-5-carboxylic acid [a]	$C_5H_7NO_3$	130.0499	1.37
	20	Creatine [a]	$C_4H_9N_3O_2$	132.0768	1.29
	21	L-glutamic acid [a]	$C_5H_9NO_4$	148.0604	1.38
	22	3-methyl-L-histidine [a]	$C_7H_{11}N_3O_2$	170.0924	1.12
Nonolar extracts in positive ion mode of ESI Carnitines	1	L-Carnitine [a]	$C_7H_{15}NO_3$	162.1125	1.17
	2	L-Acetylcarnitine [a]	$C_9H_{17}NO_4$	204.123	1.44
	3	Heptanoylcarnitine(C7:0) [b]	$C_{14}H_{27}NO_4$	274.2013	3.95
	4	Octanoylcarnitine(C8:0) [a]	$C_{15}H_{29}NO_4$	288.2169	4.96
	5	Undecanoylcarnitine(11:0) [b]	$C_{18}H_{35}NO_4$	330.2639	6.68
	6	Nonenoylcarnitine(C9:1) [b]	$C_{16}H_{29}NO_4$	300.2169	16.23
	7	Decenoylcarnitine(C10:1) [b]	$C_{17}H_{31}NO_4$	314.2326	18.85
	8	Tetradecenoylcarnitine(14:1) [b]	$C_{21}H_{39}NO_4$	370.2952	14.92
	9	Hydroxydecanoylcarnitine(C10:0-OH) [b]	$C_{17}H_{33}NO_5$	332.2432	4.09
	10	Carnitine C16:2-OH [b]	$C_{23}H_{41}NO_5$	412.3058	5.69
	11	Carnitine C18:2-OH [b]	$C_{25}H_{45}NO_5$	440.3371	6.23
Nonolar extracts in positive ion mode of ESI Cholines	1	LysoPC(14:0) [b]	$C_{22}H_{46}NO_7P$	468.3085	7.96
	2	LPC(15:0) [a]	$C_{23}H_{48}NO_7P$	482.3241	9.20
	3	LPC(16:0) [a]	$C_{24}H_{50}NO_7P$	496.3398	11.18
	4	LPC(17:0) [b]	$C_{25}H_{52}NO_7P$	510.3554	13.75
	5	LPC(18:0) [b]	$C_{26}H_{54}NO_7P$	524.3711	15.19
	6	LPC(21:0) [b]	$C_{30}H_{50}NO_7P$	568.3398	9.61
	7	LPC(16:1) [b]	$C_{24}H_{48}NO_7P$	494.3241	8.65

Continued

	N0.	Compound	Formula	$[M+H]^+$	Retention Time
	8	LPC(17:1) [b]	$C_{25}H_{50}NO_7P$	508.3398	14.75
	9	LPC(18:1) [a]	$C_{26}H_{52}NO_7P$	522.3554	12.51
	10	LPC(18:2) [b]	$C_{26}H_{50}NO_7P$	520.3398	9.16
	11	LPC(18:3) [b]	$C_{26}H_{48}NO_7P$	518.3241	11.28
	12	LPC(20:3) [b]	$C_{28}H_{52}NO_7P$	546.3554	11.24
	13	LPC(20:4) [b]	$C_{28}H_{50}NO_7P$	544.3398	9.88
	14	LPC(20:5) [b]	$C_{28}H_{48}NO_7P$	542.3241	9.69
	15	LPC(16:1)-NH2 [b]	$C_{24}H_{49}N_2O_7P$	509.335	14.76
	16	LPE(22:6) [b]	$C_{27}H_{44}NO_7P$	526.2928	9.14
	17	LPE(20:4) [b]	$C_{25}H_{44}NO_7P$	502.2928	9.19
	1	Theobromine [a]	$C_7H_8N_4O_2$	181.0720	4.07
	2	5-Methylthioadenosine [a]	$C_{11}H_{15}N_5O_3S$	298.0968	3.94
Nonolar extracts in	**3**	5,6-Dihydrouridine [c]	$C_9H_{14}N_2O_6$	247.0925	1.50
ESI+ mode	**4**	Cytidine [a]	$C_9H_{13}N_3O_5$	244.0928	1.31
Nucleosides	**5**	cGMP [b]	$C_{10}H_{12}N_5O_7P$	346.0547	4.4
	1	16α-hydroxyestrone [b]	$C_{18}H_{22}O_3$	287.1642	8.04
	2	Testosterone [a]	$C_{19}H_{28}O_2$	289.2162	6.86
	3	Corticosterone [a]	$C_{21}H_{30}O_4$	347.2217	6.04
Nonolar extracts in	**4**	Lathosterol [b]	$C_{27}H_{46}O$	387.3621	14.10
ESI+ mode Low-level	**5**	Myoinositol [a]	$C_6H_{12}O_6$	181.0707	1.63
metabolites	**6**	Lyxose [b]	$C_5H_{10}O_5$	151.0601	0.96
	7	L-Fucose [b]	$C_6H_{12}O_5$	165.0758	5.27

Nonpolar-Neg	N0.	Compound	Formula	[M-H]$^-$	Retention Time
Nonolar extracts in negative ion mode of ESI Amino Acids	1	L-isoleucine [a]	$C_6H_{13}NO_2$	130.0874	1.26
	2	Hydroxy-L-proline [a]	$C_5H_9NO_3$	130.051	1.38
	3	Serine [a]	$C_3H_7NO_3$	104.0353	1.32
	4	Threonine [a]	$C_4H_9NO_3$	118.051	1.34
	5	L-asparagine [a]	$C_4H_8N_2O_3$	131.0462	1.34
	6	L-glutamine [a]	$C_5H_{10}N_2O_3$	145.0619	1.34
	7	L-lysine [a]	$C_6H_{14}N_2O_2$	145.0983	1.21
	8	γ-hydroxylysine [b]	$C_6H_{14}N_2O_3$	161.0932	1.43
	9	Dimethylarginine [a]	$C_8H_{18}N_4O_2$	201.1357	1.15
	10	L-histidine [a]	$C_6H_9N_3O_2$	154.0622	1.13
	11	O-phospho-ethanolamine [a]	$C_2H_8NO_4P$	140.0118	1.38
	12	Taurine [a]	$C_2H_7NO_3S$	124.0074	1.37
	13	L-citrulline [a]	$C_6H_{13}N_3O_3$	174.0884	1.34
	14	Creatine [a]	$C_4H_9N_3O_2$	130.0622	1.28
Nonolar extracts in negative ion mode of ESI Fatty acids	1	Linoleic acid [b]	$C_{18}H_{32}O_2$	279.233	20.64
	2	palmitoleic acid [b]	$C_{16}H_{30}O_2$	253.2173	19.24
	3	Oleic acid [b]	$C_{18}H_{34}O_2$	281.2486	24.78
	4	Arachidonic acid [a]	$C_{20}H_{32}O_2$	303.233	19.67
	5	FAC 14：1-OH [b]	$C_{14}H_{26}O_3$	241.1809	7.69
	6	Niacinamide [a]	$C_6H_6N_2O$	121.0407	5.05
Nonolar extracts in negative ion mode of ESI Low-level metabolites	1	Galactose [a]	$C_{12}H_{22}O_{11}$	341.1089	1.42
	2	Melezitose [b]	$C_{18}H_{32}O_{16}$	503.1618	1.43
	3	alpha-Tocopherol [b]	$C_{29}H_{50}O_2$	429.3738	31.49

[a]Metabolites confirmed using standard compounds. [b]Metabolites identified by database searches and MS fragmentation. [c]As target ions with its exact mass and retention time.

Table S2. Method sensitivity.

Analytes	Normal Tissue Value (nmol/g)	C18					HILIC				
			(+)ESI		(-)ESI			(+)ESI		(-)ESI	
		RT (min)	LOD (nmol/g)	S/N	LOD (nmol/g)	S/N	RT (min)	LOD (nmol/g)	S/N	LOD (nmol/g)	S/N
L-Tyrosine	102.685 (33.22-172.15)	1.64	10.3	2904	—	—	10.76	10.3	8	—	—
Proline	250.475 (80.54-420.41)	1.41	25.0	4591	—	—	12.47	25.0	72	—	—
Valine	415.13 (78.54-751.72)	1.44	41.5	1693	—	—	11.52	41.5	31	—	—
Phenylalanine	142.355 (41.89-242.82)	3.73	14.2	313	—	—	11.65	14.2	21	—	—
Tryptophan	47.23 (13.17-81.29)	4.09	4.7	1813	4.7	688	10.03	4.7	13	4.7	7
Kynurenic acid	304.998	5.08	30.5	2259	30.5	4102	1.35	30.5	153	—	—
Arachidonic acid	304.998	19.62	30.5	220	30.5	3495	1.32	30.5	223	30.5	142
Nicotinic acid	304.998	2.09	30.5	190	—	—	5.62	30.5	169	—	—
Hippuric acid	5.535 (0.50-10.57)	4.78	0.6	205	—	—	4.74	0.6	10	—	—
Linoleic acid	304.998	20.53	30.5	54	30.5	612	1.31	30.5	96	30.5	112
Oleic acid	304.998	24.80	30.5	53	30.5	581	1.29	30.5	314	30.5	108
L-Carnitine	604.46 (105.08-1103.84)	4.05	60.4	4798	—	—	19.04	60.4	243	—	—
LysoPC 16:0	304.998	11.38	30.5	5130	30.5	74	13.30	30.5	518	—	—
Acetylcarnitine	143.065 (3.47-282.66)	1.30	14.3	6661	14.3	49	18.21	14.3	98	14.3	3213
Octanoylcarnitine	304.998	4.99	30.5	5140	30.5	84	14.29	30.5	692	30.5	475
Decanoylcarnitine	304.998	5.43	30.5	9171	30.5	411	13.72	30.5	432	30.5	2857
Cytidine	60.74 (5.39-116.09)	1.25	6.1	1169	6.1	215	5.29	6.1	451	6.1	148
Inosine	225.175 (8.58-441.77)	3.64	22.5	132	22.5	48	3.87	22.5	158	22.5	11
Methyltestosterone	304.998	7.28	30.5	1531	30.5	438	1.31	30.5	57	30.5	136

Table S3. Instrument precision.

Analytes	RPLC-MS				HILIC-MS			
	Positive ion mode		Negative ion mode		Positive ion mode		Negative ion mode	
	Average Area (counts)	RSD (%)	Average Area (counts)	RSD (%)	Average Area (counts)	RSD (%)	Average Area (counts)	RSD (%)
L-Tyrosine	1.07E+09	3.7	—	—	3.45E+09	10.9	—	—
Proline	8.64E+09	5.9	—	—	2.51E+10	9.4	—	—
Valine	1.04E+10	6.3	—	—	1.63E+10	12.7	—	—
Phenylalanine	2.02E+10	3.2	—	—	4.15E+10	16.2	—	—
Tryptophan	4.92E+09	4.4	7.12E+08	7.7	4.21E+09	12.6	1.05E+10	11.7
Kynurenic acid	3.58E+10	5.2	1.30E+10	14.2	3.85E+10	14.9	—	—
Arachidonic acid	1.66E+09	3.6	2.65E+10	3.8	7.65E+06	7.6	2.29E+10	9.5
Nicotinic acid	5.23E+10	4.7	—	—	7.22E+10	2.9	—	—
Hippuric acid	7.38E+08	7.9	—	—	3.41E+07	7.9	—	—
Linoleic acid	2.35E+08	4.7	1.71E+10	4.4	3.01E+06	6.5	3.42E+10	8.2
Oleic acid	1.01E+11	5.4	2.33E+10	3.4	4.33E+05	8.6	3.38E+10	8.0
L-Carnitine	1.02E+11	6.2	—	—	2.09E+11	4.2	—	—
LysoPC 16:0	7.89E+10	3.4	5.39E+06	5.2	9.15E+10	7.9	—	—
Acetylcarnitine	4.02E+10	4.7	1.44E+06	3.3	9.40E+10	3.6	2.36E+08	2.5
Octanoylcarnitine	2.24E+11	4.1	1.29E+07	11.4	2.72E+11	9.3	1.21E+09	3.7
Decanoylcarnitine	2.39E+11	4.3	5.02E+07	1.9	2.23E+11	6.5	9.71E+08	3.1
Cytidine	1.16E+09	6.2	4.54E+08	2.9	1.77E+10	8.7	5.63E+09	2.9
Inosine	1.19E+09	7.1	5.91E+08	7.3	1.54E+10	10.2	6.35E+10	2.6
Methyltestosterone	1.32E+11	4.1	6.91E+07	3.7	7.29E+09	7.7	1.29E+08	7.9

Table S4. Method precision.

Analytes	RPLC-MS				HILIC-MS			
	Positive ion mode RSD(%)		Negative ion mode RSD(%)		Positive ion mode RSD(%)		Negative ion mode RSD(%)	
	Intra-batch (n=6)	Inter-batch (n=3)	Intra-batch	Inter-batch	Intra-batch	Inter-batch	Intra-batch	Inter-batch
L-Tyrosine	9.8	12.4	—	—	10.8	12.9	—	—
Proline	14.6	16.2	—	—	16.4	23.2	—	—
Valine	12.9	14.2	—	—	13.0	17.0	—	—
Phenylalanine	12.3	12.2	—	—	16.6	17.9	—	—
Tryptophan	9.4	9.8	14.6	17.5	18.2	21.2	13.0	12.9
Kynurenic acid	8.6	10.7	17.9	19.8	15.0	14.7		
Arachidonic acid	5.5	6.6	5.5	5.8	16.0	18.7	12.3	17.7
Nicotinic acid	7.0	10.2	—	—	14.0	13.6	—	—
Hippuric acid	10.1	18.2	—	—	—	—	—	—
Linoleic acid	3.5	4.4	7.9	7.7	15.8	15.8	12.9	16.6
Oleic acid	7.6	8.0	12.8	12.2	18.8	23.1	10.1	10.4
L-Carnitine	14.8	17.1	—	—	10.8	10.5	—	—
LysoPC 16:0	5.9	8.8	10.8	13.7	14.6	14.2	—	—
Acetylcarnitine	10.4	12.1	9.3	9.4	14.6	14.1	15.7	16.0
Octanoylcarnitine	7.5	9.2	11.1	10.8	7.0	7.2	6.8	7.5
Decanoylcarnitine	4.9	5.8	7.0	8.5	7.8	7.3	13.0	13.2
Cytidine	12.5	15.1	20.9	20.1	14.1	14.8	8.6	9.3
Inosine	10.9	12.0	—	—	11.1	11.2	8.8	10.1
Methyltestosterone	3.7	4.5	9.5	9.4	12.7	12.7	10.7	12.0

Table S5. Extraction recoveries of endogenous metabolites.

Analytes	Spiked level	Mean area of RPLC-MS (sample/reference)	Mean area of HILIC-MS (sample/reference)	Recovery rate	
				(C18/HILIC)	SUM
L-Tyrosine	blank tissue	8.27E+06	4.38E+07	—	—
	low	2.97E+07/6.38E+08	4.95E+07/5.37E+07	3.4 /57.5	60.9
	middle	3.35E+07/8.90E+08	7.41E+08/8.74E+08	2.9/84.0	86.9
	high	9.38E+07/1.03E+09	4.46E+08/6.94E+08	8.4/61.8	70.2
Proline	blank tissue	3.24E+07	1.39E+09	—	—
	low	1.11E+08/3.07E+09	2.53E+09/2.62E+09	2.6/92.4	95.0
	middle	2.00E+08/4.63E+09	1.06E+10/1.23E+10	3.6/84.1	87.7
	high	5.13E+08/8.53E+09	9.57E+09/1.37E+10	5.7/66.6	72.3
Valine	blank tissue	3.85E+07	4.21E+09	—	—
	low	1.81E+08/2.16E+09	4.66E+09/1.06E+10	6.7/44.0	50.7
	middle	4.25E+08/3.32E+09	1.02E+10/1.01E+10	11.8/101.5	113.3
	high	7.28E+08/5.84E+09	7.91E+09/8.38E+09	11.9/88.8	100.7
Phenylalanine	blank tissue	6.39E+06	5.64E+08	—	—
	low	2.47E+07/6.80E+08	7.77E+08/8.05E+08	2.7/88.3	91.0
	middle	3.12E+07/1.29E+09	5.21E+09/5.95E+09	1.9/86.2	88.1
	high	8.16E+07/2.55E+09	5.25E+09/5.65E+09	3.0/92.2	95.2
Tryptophan	blank tissue	9.87E+05	2.50E+07	—	—
	low	3.45E+06/1.23E+08	2.60E+07/2.62E+07	2.0/81.4	83.4
	middle	4.49E+06/2.51E+08	3.30E+08/4.79E+08	1.4/67.1	68.5
	high	1.23E+07/5.45E+08	3.29E+08/4.93E+08	2.1/65.0	67.1
Kynurenic acid	blank tissue	1.71E+07	4.06E+07	—	—
	low	2.10E+08/1.61E+10	4.64E+09/6.12E+09	1.2/75.6	76.8
	middle	3.57E+08/2.35E+10	8.77E+09/7.89E+09	1.5/111.2	112.7
	high	1.88E+09/3.58E+10	9.72E+09/1.15E+10	5.2/84.8	90.0
Arachidonic acid	blank tissue	1.33E+08	7.79E+05	—	—
	low	4.51E+08/4.20E+08	8.75E+05/3.39E+06	110.8/3.7	114.5
	middle	4.67E+08/4.75E+08	1.30E+06/3.58E+06	97.6/18.6	116.2
	high	7.87E+08/8.24E+08	1.75E+06/5.81E+06	94.6/19.3	113.9
Nicotinic acid	blank tissue	1.31E+07	7.63E+08	—	—
	low	2.64E+07/9.95E+09	8.44E+09/1.37E+10	4.2/59.4	63.6
	middle	3.61E+07/1.62E+10	2.60E+10/3.05E+10	3.8/85.1	88.9
	high	7.45E+07/2.87E+10	2.96E+10/4.33E+10	5.3/67.8	73.1
Hippuric acid	blank tissue	3.76E+06	—	—	—
	low	9.21E+06/7.86E+07	—	7.3/—	7.3
	middle	1.49E+07/1.45E+08	—	7.9/—	7.9
	high	2.56E+07/3.22E+08	—	6.9/—	6.9
Linoleic acid	blank tissue	1.31E+07	2.33E+05	—	—
	low	4.71E+07/4.81E+07	1.01E+06/2.71E+06	97.3/31.3	128.6
	middle	3.61E+07/5.65E+07	1.22E+06/4.12E+06	53.1/25.5	78.6
	high	7.45E+07/9.54E+07	5.85E+05/4.67E+06	74.7/8.0	82.7

Continued

Analytes	Spiked level	Mean area of RPLC-MS (sample/reference)	Mean area of HILIC-MS (sample/reference)	Recovery rate	
				(C18/HILIC)	SUM
Oleic acid	blank tissue	3.76E+06	9.53E+04	—	—
	low	1.61E+07/1.52E+07	9.83E+04/1.13E+05	108.3/17.6	125.9
	middle	1.49E+07/1.71E+07	1.19E+05/1.73E+05	83.5/30.3	113.8
	high	2.56E+07/3.11E+07	1.22E+05/1.60E+05	79.8/41.6	121.4
L-Carnitine	blank tissue	2.30E+08	2.10E+10	—	—
	low	7.12E+08/1.91E+10	3.73E+10/3.99E+10	2.6/86.4	89.0
	middle	8.78E+08/3.10E+10	8.84E+10/1.01E+11	2.1/84.0	86.1
	high	2.80E+09/5.64E+10	9.76E+10/1.35E+11	4.6/67.4	72.0
LysoPC 16:0	blank tissue	1.49E+09	5.31E+08	—	—
	low	6.25E+09/7.86E+09	1.28E+09/5.08E+09	74.7/16.6	91.3
	middle	9.34E+09/1.22E+10	1.49E+09/9.26E+09	73.4/11.0	84.4
	high	1.67E+10/2.24E+10	9.76E+10/1.90E+10	72.7/15.4	88.1
Acetylcarnitine	blank tissue	6.60E+07	8.16E+09	—	—
	low	2.98E+08/7.14E+09	1.27E+10/1.27E+10	3.3/99.9	103.2
	middle	3.63E+08/1.15E+10	3.94E+10/4.54E+10	2.6/84.1	86.7
	high	1.26E+09/2.16E+10	3.91E+10/6.12E+10	5.6/58.4	64.0
Octanoylcarnitine	blank tissue	6.70E+07	2.66E+08	—	—
	low	5.80E+09/5.00E+10	1.76E+10/2.78E+10	11.5/63.0	74.5
	middle	1.06E+10/7.23E+10	5.90E+10/6.38E+10	14.5/92.4	106.9
	high	2.49E+10/1.14E+11	7.74E+10/1.15E+11	21.8/67.1	88.9
Decanoylcarnitine	blank tissue	5.81E+07	2.89E+08	—	—
	low	1.39E+10/4.80E+10	1.53E+10/3.11E+10	28.8/48.5	77.3
	middle	2.43E+10/7.09E+10	1.85E+10/4.70E+10	34.3/39.0	73.3
	high	5.01E+10/1.13E+11	5.71E+10/7.57E+10	44.4/75.3	119.7
Cytidine	blank tissue	5.33E+05	6.38E+08	—	—
	low	1.56E+06/1.32E+08	8.88E+08/9.51E+08	0.8/79.8	80.6
	middle	2.41E+06/2.27E+08	3.86E+09/3.95E+09	0.8/97.3	98.1
	high	1.24E+07/4.31E+08	4.49E+09/6.82E+09	2.8/62.3	65.1
Inosine	blank tissue	4.07E+06	2.43E+09	—	—
	low	1.58E+07/2.16E+08	3.95E+09/4.63E+09	5.6/69.0	74.6
	middle	1.52E+07/3.74E+08	8.24E+09/8.75E+09	3.0/92.0	95.0
	high	4.88E+07/7.78E+08	6.68E+09/9.03E+09	5.8/64.3	70.1
Methyltestosterone	blank tissue	5.30E+07	1.49E+07	—	—
	low	3.53E+10/2.39E+10	1.05E+08/2.31E+09	114.1/3.9	118.0
	middle	3.51E+10/3.78E+10	1.23E+08/2.81E+09	92.9/3.9	96.8
	high	7.40E+10/6.54E+10	2.17E+08/2.90E+09	113.0/7.0	120.0

Table S6. Freeze-thaw stability.

Analytes	RPLC-MS				HILIC-MS			
	Positive ion mode		Negative ion mode		Positive ion mode		Negative ion mode	
	Average area of 3 cycles (counts)	RSD(%)	Average area of 3 cycles (counts)	RSD(%)	Average area of 3 cycles (counts)	RSD (%)	Average area of 3 cycles (counts)	RSD (%)
L-Tyrosine	4.19E+07	12.1	—	—	1.71E+08	14.6	—	—
Proline	2.53E+08	7.1	—	—	9.25E+09	6.0	—	—
Valine	1.67E+08	9.8	—	—	3.52E+09	3.7	—	—
Phenylalanine	5.44E+07	9.4	—	—	2.34E+09	1.9	—	—
Tryptophan	8.27E+06	16.9	1.31E+06	21.5	3.49E+07	13.7	2.64E+07	23.2
Kynurenic acid	8.13E+08	9.6	7.48E+07	22.4	1.33E+10	13.1	—	—
Arachidonic acid	1.12E+09	8.3	1.08E+10	4.6	5.32E+05	12.2	1.33E+07	21.8
Nicotinic acid	1.65E+09	11.6	—	—	2.87E+10	14.3	—	—
Hippuric acid	3.49E+06	27.1	—	—	—	—	—	—
Linoleic acid	1.17E+08	11.0	3.62E+09	3.4	5.12E+05	13.5	1.10E+08	7.2
Oleic acid	3.22E+07	10.2	1.68E+09	13.6	5.20E+04	14.1	1.10E+08	2.6
L-Carnitine	1.52E+09	15.0	—	—	8.40E+10	12.4	—	—
LysoPC 16:0	1.34E+10	23.6	1.09E+06	20.8	4.56E+09	4.8	—	—
Acetylcarnitine	6.20E+08	18.4	1.60E+06	12.0	3.19E+10	12.5	2.51E+07	18.1
Octanoylcarnitine	1.72E+10	18.7	3.09E+06	15.4	4.85E+10	13.8	4.40E+07	13.0
Decanoylcarnitine	3.83E+10	21.2	8.03E+06	12.0	3.78E+10	7.4	7.06E+07	16.8
Cytidine	4.86E+06	14.9	3.26E+06	9.6	2.66E+09	8.9	8.74E+08	6.3
Inosine	1.59E+07	21.9	—	—	4.27E+09	8.2	3.63E+10	8.2
Methyltestosterone	9.65E+10	9.2	1.59E+08	5.8	3.92E+08	2.1	3.12E+06	11.6

Tissue stability after three freeze-thaw cycles (-20°C–20°C).

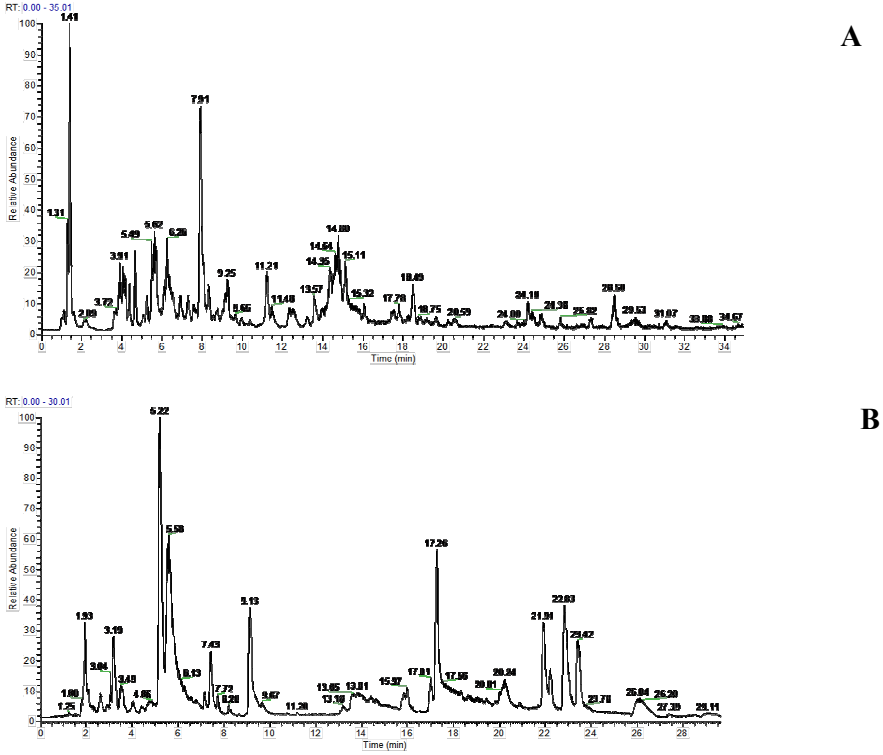

Figure S1. Typical TICs obtained from esophagus tissue by LC-(+)ESI-MS. A: organic extracts; B: aqueous extracts.

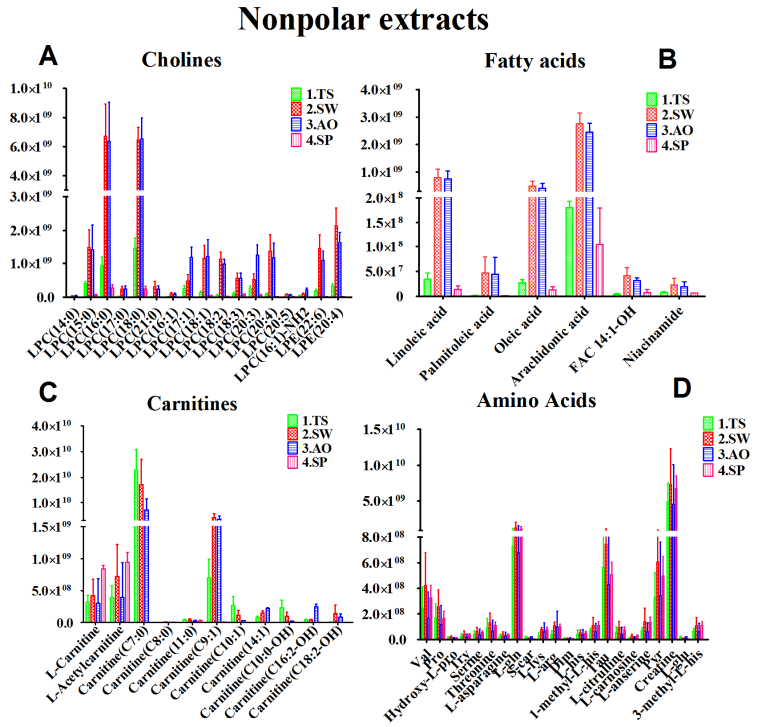

Figure S2. Comparison of extraction efficiency using different solvent extraction methods for cholines (A), fatty acids (B), carnitines (C), and amino acids (D) in nonpolar extracts. Data are shown as column charts.

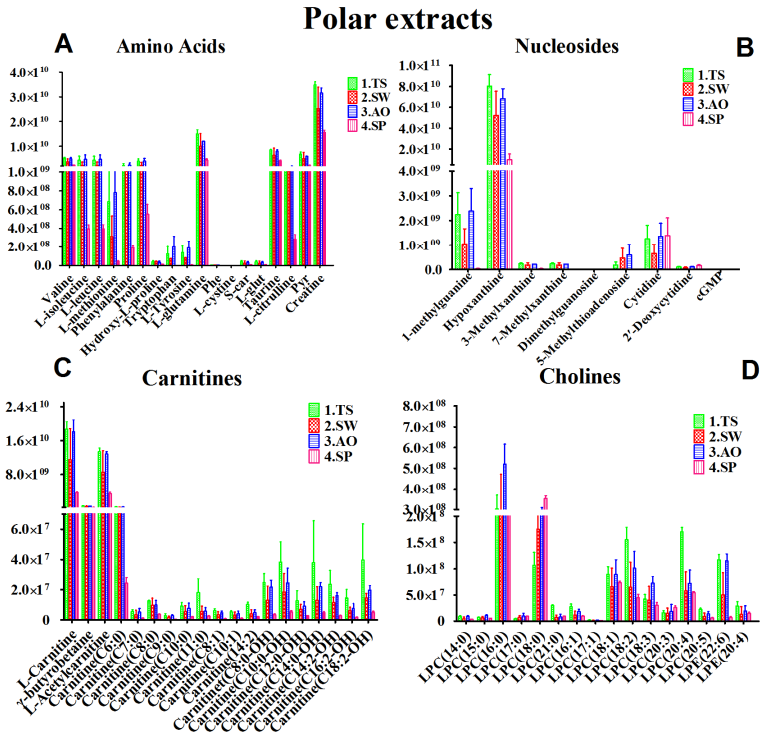

Figure S3. Comparison of extraction efficiency using different solvent extraction methods for amino acids (A), nucleosides (B), carnitines (C), and cholines (D) in polar extracts. Data are shown as column charts.

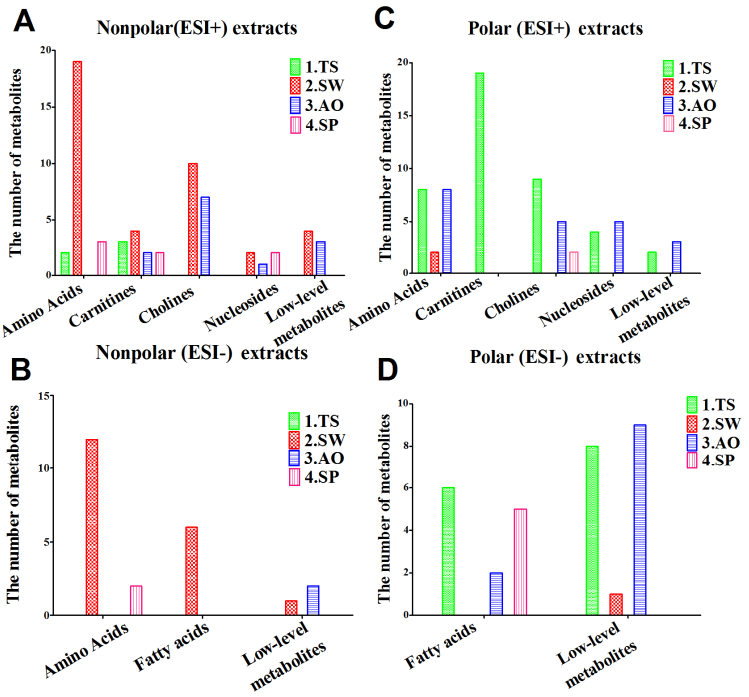

Figure S4. Comparison of extraction efficiency using different solvent extraction methods for positive and negative ion modes of ESI in nonpolar extracts (A, B) and polar extracts (C, D).

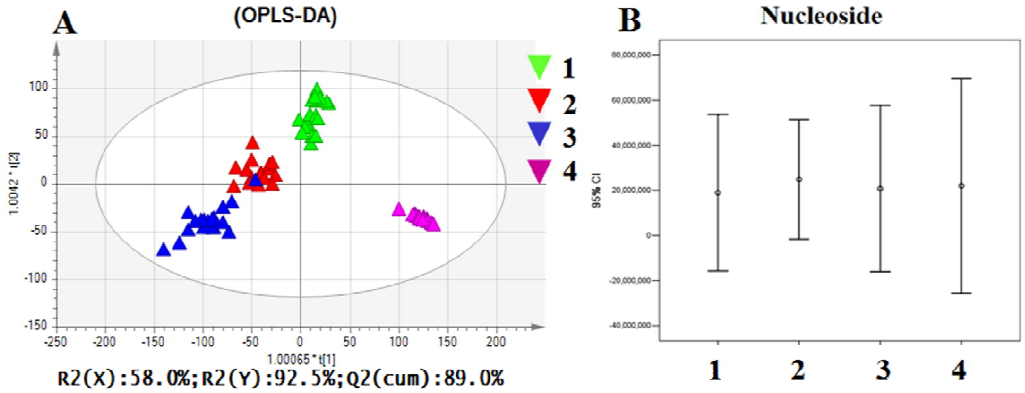

Figure S5. Comparison of method reproducibility, evaluated by OPLS-DA (A) and error bars (B), for positive ion mode of ESI in nonpolar extracts.

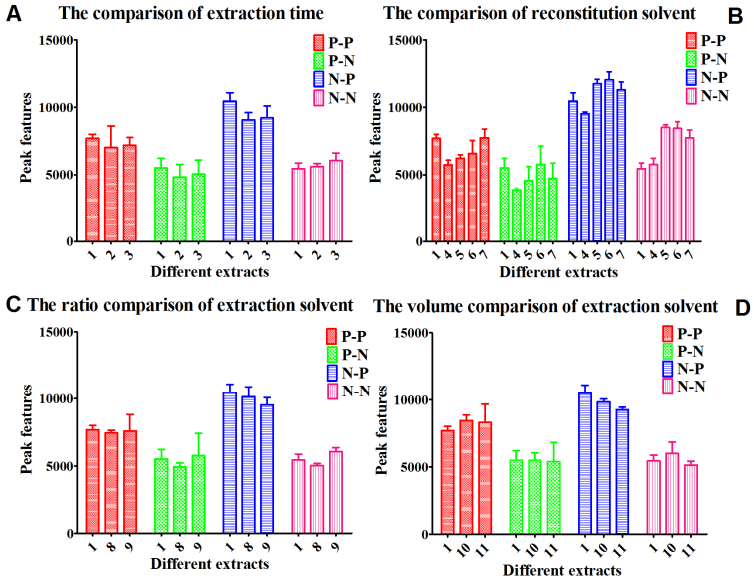

Figure S6. Peak features under different parameters in different extracts. **P-P**: polar extracts in positive ESI mode; **P-N**: polar extracts in negative ESI mode; **N-P**: nonpolar extracts in positive ESI mode; **N-N**: nonpolar extracts in negative ESI mode. The comparison of extraction time (**A**): **1** (20min), **2** (40min), **3** (60min). The comparison of reconstitution solvent (**B**): polar extracts (**4**: 50/50 acetonitrile/water; **5**: 60/40 acetonitrile/water; **6**: 80/20 acetonitrile/water; **7**: 95/5 acetonitrile/water); nonpolar extracts (**4**: acetonitrile; **5**: 70/30 acetonitrile/water; **6**: 40/60 acetonitrile/water; **7**: 2/98 acetonitrile/water). The ratio comparison of extraction solvent (**C**): **1**: methanol/water/dichloromethane (2:1.5:2); **8**: methanol/water/dichloromethane (2:1:2); **9**: methanol/water/dichloromethane (1.5:1.5:1). The volume comparison of extraction solvent (**D**): **1**: 1.6 mL; **10**: 1.0 mL; **11**: 2.0 mL. The parameters we finally chose were 1, 6, 9 and 10 after the systematic comparison of polar and nonpolar extracts.

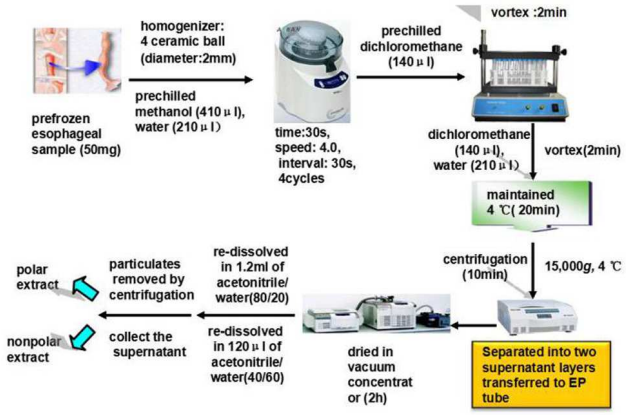

Figure S7. Workflow for esophageal tissue preparation.

SUPPLEMENTARY REFERENCES

(1) Sugimoto, M.; Ikeda, S.; Niigata, K.; Tomita, M.; Sato, H.; Soga, T. *Nucleic. Acids. Res*. 2012, 40(Database issue), D809-D814.