Molecular BioSystems

Electronic Supplementary Material

A cross-platform metabolomics workflow for volume-restricted tissue samples: application to an animal model for polycystic kidney disease

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Materials and methods

Sample preparation

The development of the sample preparation protocol started with the addition of 100 µL of a cold solution of methanol in different proportions (25, 50, 75, and 100 %) to one solely 20 µm-thick tissue section. After vortex agitation for 30 s, samples were sonicated in an ultrasound bath at 4 ºC for 20 min to facilitate the extraction of metabolites. Afterwards, samples were centrifuged at 14000 x g for 10 min at 4 ºC, the supernatant was evaporated in a SpeedVac instrument (Eppendorf, model 5301) for 90 min at room temperature and dried samples were reconstituted in 40 µL of water for RPLC analysis or in 40 µL of 100 % acetonitrile for HILIC analysis, and then centrifuged at 14000 x g for 5 min at 4 ºC.

Optimized protocol for sample preparation is as follows. 100 µL of 75 % MeOH were added to a 20 µm-thick mice tissue section. After vortex agitation for 30 s, microtubes were sonicated in an ultrasound bath for 20 min at 4 ºC and then centrifuged at 14.000 x g for 10 min at 4 ºC. The supernatant was then taken and evaporated at room temperature for 90 min in a SpeedVac instrument (Eppendorf, model 5301). Dried samples were reconstituted with 40 µL of cold water, centrifuged at 14.000 x g for 5 min at 4 ºC and supernatant was transferred to glass inserts placed in glass vials. Two different QC pools were prepared at this point: i) "standard QC-pool": a QC pool resulting of taking 4 µL of each of the 60 samples; and ii) "group-QC": four individual QC pools by taking 2 µL of each sample within each of the four groups (Wt0, Wt14, MCK, and ESCK) to perform analysis in the auto MS/MS mode. After injecting the samples in a randomized order and the different QC pools in the RPLC platform (ESI+ mode) the remaining content (20 µL for samples and the so-called "group-QC", and 120 µL of the "standard QC-pool" (which was divided in 6 different microtubes portioning 20 µL in each of them) was taken and placed into microtubes, and 5 µL of methanol were added to aid in the evaporation, which was carried out at room temperature for 90 min in a SpeedVac instrument. The dried product was reconstituted with 20 µL of 80:20 (acetonitrile:water, v/v), centrifuged at 14.000 x g for 5 min at 4 ºC and the supernatant was transferred to glass inserts placed in glass vials to be then analyzed by the HILIC platform in the ESI- mode. The 6 reconstituted microtubes of the "standard QC-pool" were mixed in the same insert for analysis.

At the beginning of the metabolic profiling sequence, blanks and QC pools were injected in each platform to ensure good repeatability in the chromatographic systems.

Samples of PKD study were randomized for injection, where every fifteen samples a QC pool was injected twice to allow the QC to be injected in both columns. At the end of the analysis sequence the four different "group-QC" (one for each of the four groups) were injected once in the auto MS/MS mode to aid in the metabolite identification. Once samples were analyzed by RPLC-MS, according to **Figure 1**, they were analyzed in the HILIC-MS platform.

RPLC-MS conditions

In the MS mode, the instrument capillary voltage was set to 3500 V with and end plate voltage of 500 V, with a scan range of *m/z* 50-1500 with an acquisition rate of 1.0 Hz. The dry gas (N_2) was set at 9.0 L/min at 200 ºC, with a nebulizer pressure (N_2) of 2.1 bar. Regarding auto MS/MS analysis mode, the capillary voltage was set to 4500 V, with a scan range of *m/z* 20-1000, with an acquisition rate of 8.0 Hz. The dry gas and dry temperature were 7.0 L/min and 200 ºC, respectively, whereas the nebulizer pressure was 2.1 bar. A solution of water/isopropranol (50:50, v/v) which contained sodium formate clusters was infused at the beginning of each analysis to allow for mass recalibration. HyStar (version 3.2) was employed for MS and MS/MS control, data acquisition, and data handling.

HILIC-MS conditions

In the MS mode, the instrument capillary voltage operated at 4000 V, and end plate voltage was 500 V, with a scan range of *m/z* 50-1000 with an acquisition rate of 1.0 Hz. The dry gas (N_2) was set at 6.0 L/min at 200 ºC, with a nebulizer pressure (N_2) of 1.5 bar. Regarding auto MS/MS analysis mode, the capillary voltage was set to 4000 V, with a scan range of *m/z* 20-1000, with an acquisition rate of 8.0 Hz. The dry gas, and dry temperature and nebulizer pressure were as in the MS mode. As in RPLC-MS, a solution of water/isopropranol (50:50, v/v) which contained sodium formate clusters was infused at the beginning of each analysis to allow for mass recalibration. HyStar (version 3.2) was also employed for MS and MS/MS control, data acquisition, and data handling.

Data handling

Optimization of the extraction protocol was based on the number of molecular features obtained for each methanol proportion (from 25 to 100 %). To this end, the

Bruker Molecular Extraction tool was used with the following parameters: a S/N threshold of 10, a correlation coefficient threshold of 0.7, minimum compound length, 10 spectra; smoothing width, 1; and possible adducts, for RPLC: M+H+, M+Na+, $M+NH_4+$, M+K+, neutral loses of H2O, CO2, and NH_3, and dimers, 2M+H+, 2M+Na+, $2M+NH_4+$, and 2M+K+; for HILIC: M-H-, $M+CH_3COOH$-H-, neutral losses of H2O, and dimmers, 2M-H-, and $2M+CH_3COOH$-H-.

Peak picking was carried out using XCMS R-package (The Scripp Research Institute, La Jolla, CA, USA) based on the centWave algorithm using the following settings: maximum tolerated m/z deviation in consecutive scans, 15 ppm (RPLC and HILIC); chromatographic peak width, 5-30 s (RPLC) and 5-40 s (HILIC); scan range, 60-480 s (RPLC) and 60-420 s (HILIC), noise, 5000 (RPLC), and 10000 (HILIC); prefilter step, at least 3 peaks with intensity > 5000 (RPLC and HILIC); signal-to-noise ratio threshold, 10 (RPLC and HILIC). After peak picking, peak grouping was performed with the following parameters: bandwidth, 2; and *m/z* width, 0.25 *m/z*. Since the difference between the maximum and minimum retention time was lower than 15 s for RPLC and 20 s for HILIC, no peak alignment was necessary. After peak picking and grouping, features with RSD values above 30 % in the QC were excluded.

Figures

Fig. S1

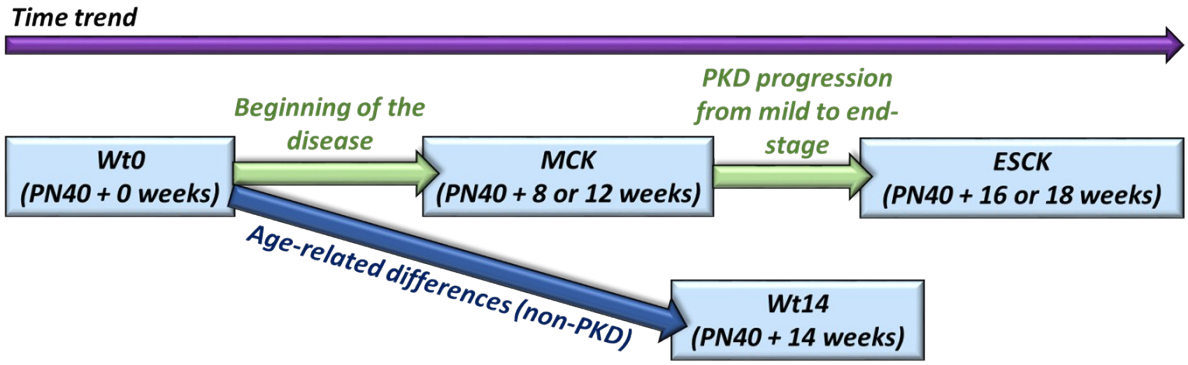


Fig. S2

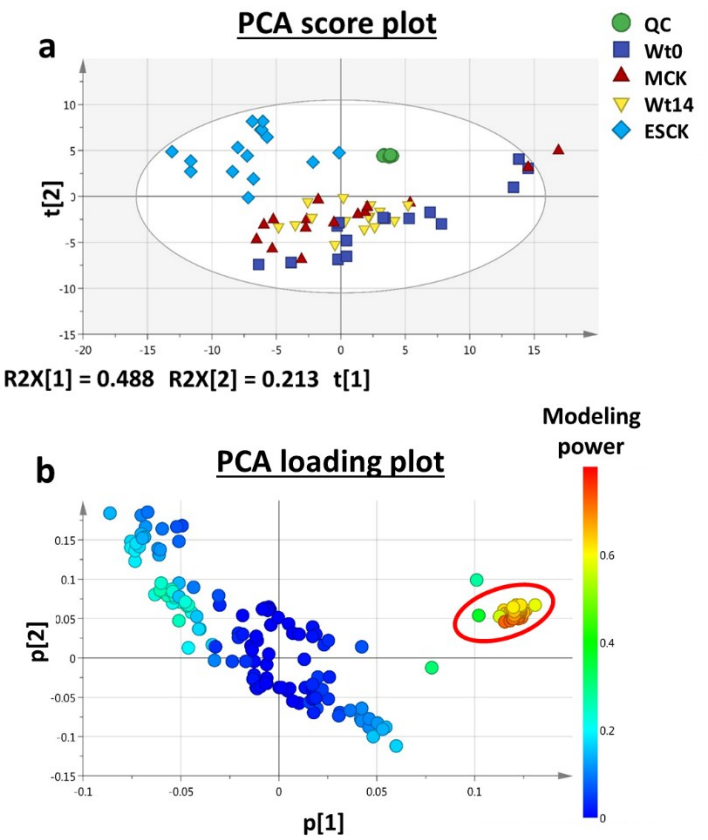

a
PCA score plot
QC
Wt0
MCK
Wt14
ESCK
t[2]
R2X[1] = 0.488 R2X[2] = 0.213 t[1]
b
PCA loading plot
Modeling power
p[2]
p[1]

Fig. S3

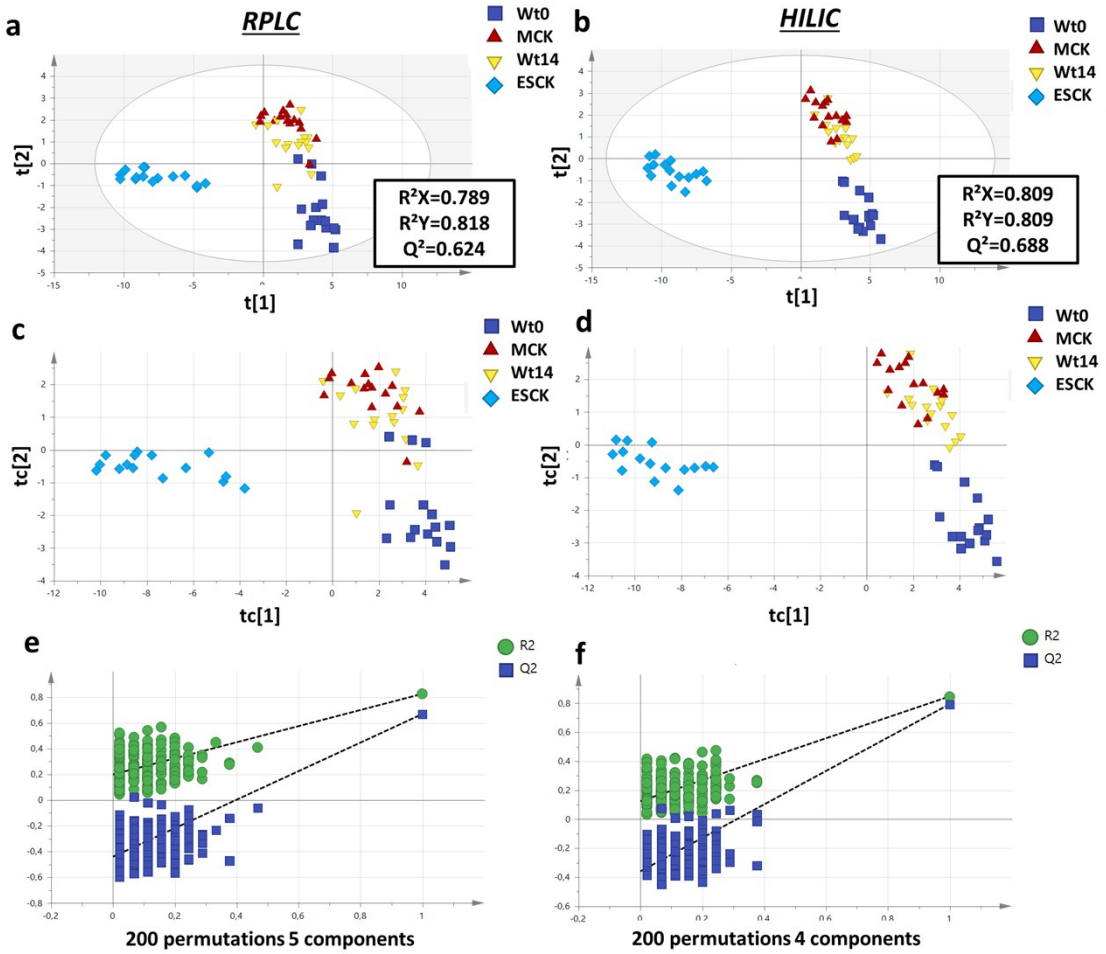
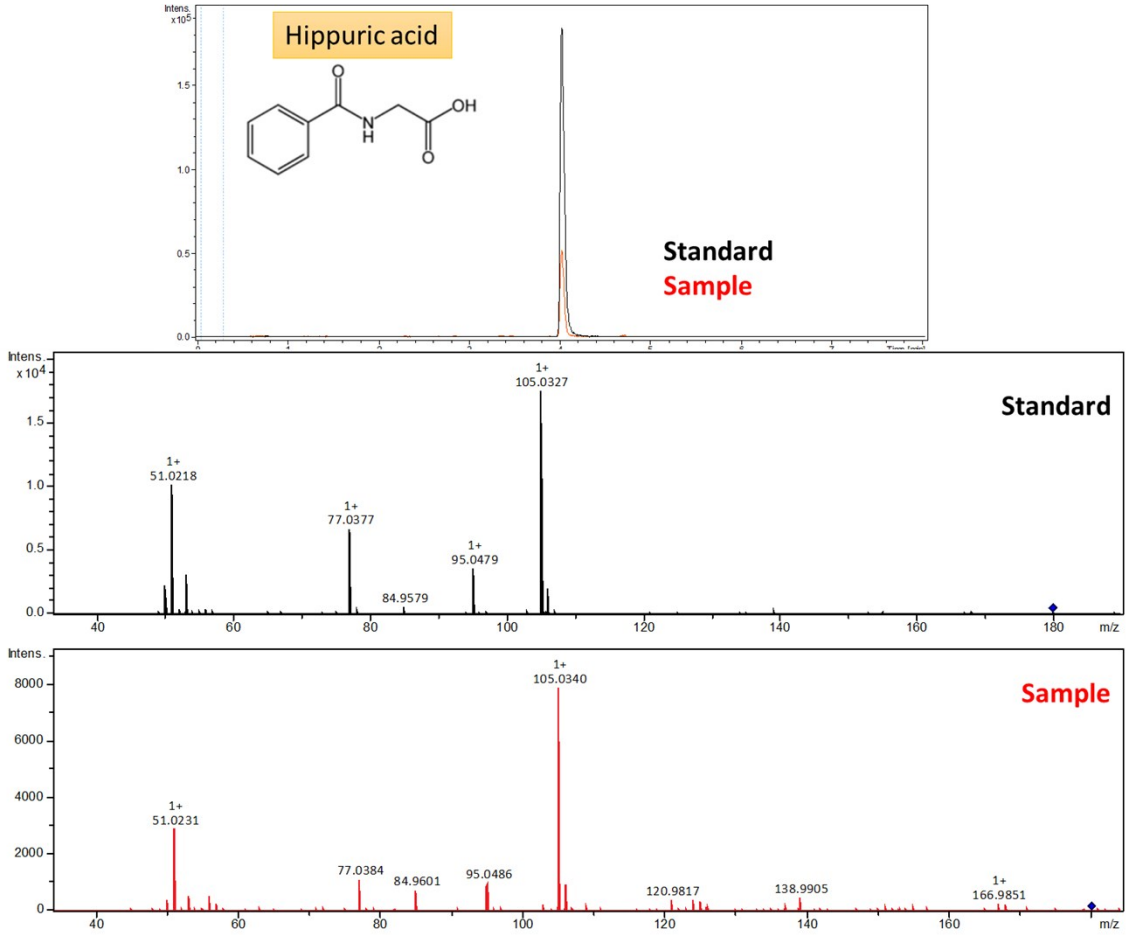

a
RPLC
Wt0
MCK
Wt14
ESCK
t[2]
t[1]
R²X=0.789
R²Y=0.818
Q²=0.624
b
HILIC
Wt0
MCK
Wt14
ESCK
t[2]
t[1]
R²X=0.809
R²Y=0.809
Q²=0.688
c
Wt0
MCK
Wt14
ESCK
tc[2]
tc[1]
d
Wt0
MCK
Wt14
ESCK
tc[2]
tc[1]
e
R2
Q2
200 permutations 5 components
f
R2
Q2
200 permutations 4 components

Fig. S4.


Hippuric acid
Standard
Sample
Standard
1+
105.0327
1+
51.0218
1+
77.0377
1+
95.0479
84.9579
Sample
1+
105.0340
1+
51.0231
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84.9601
95.0486
120.9817
138.9905
1+
166.9851
m/z

Fig. S4 (continuation)

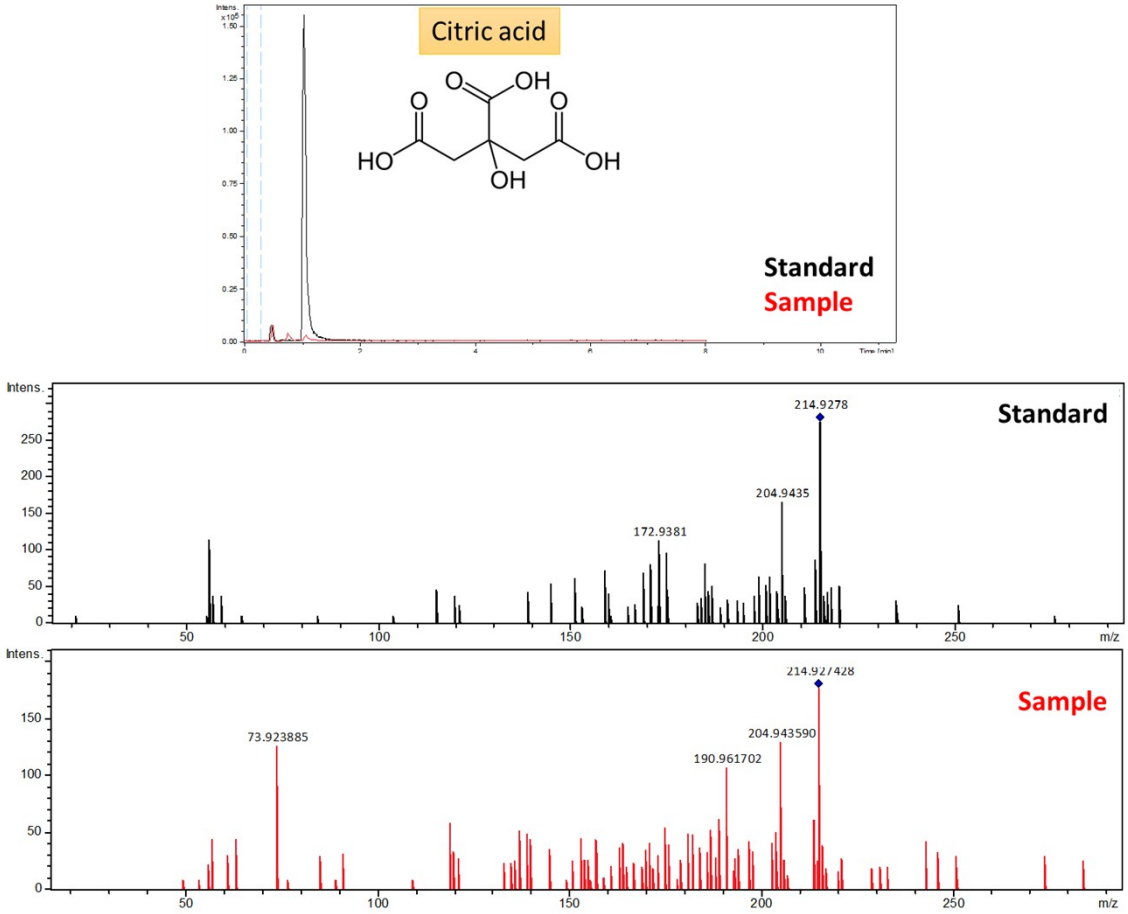


Fig. S4 (continuation)

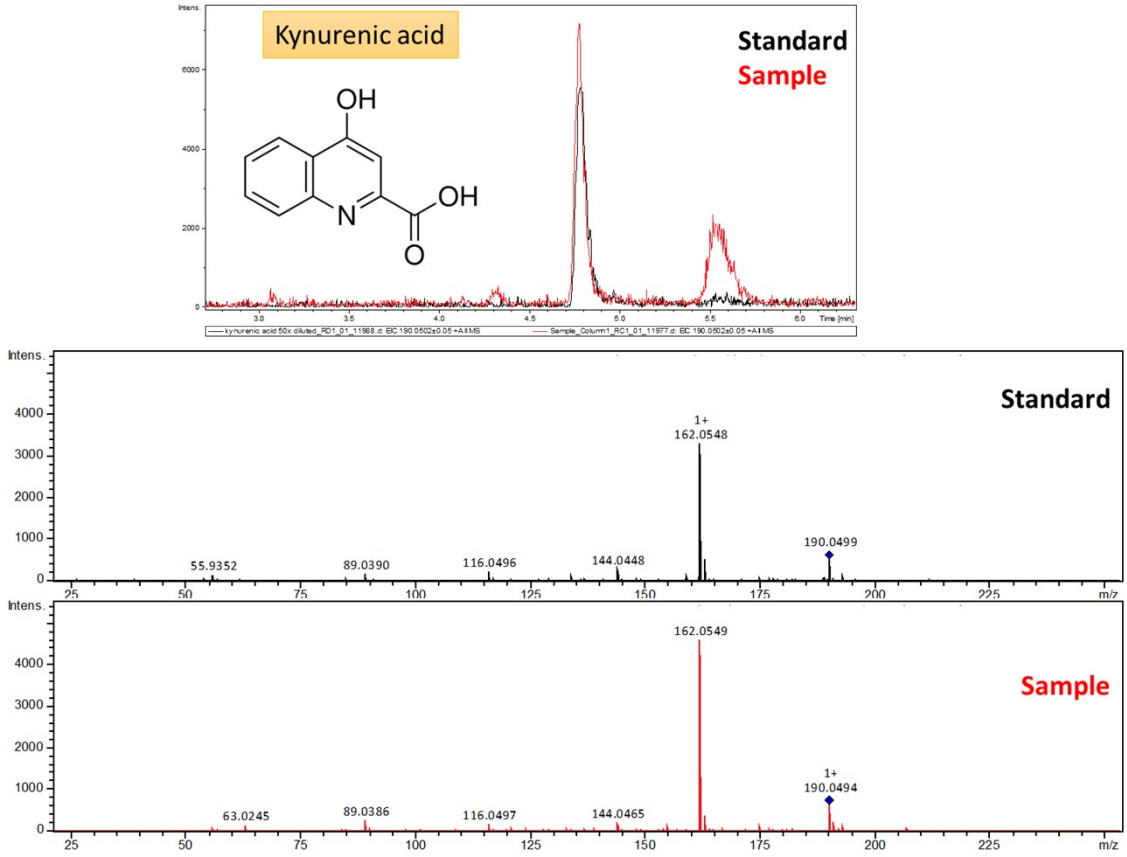


Fig. S5

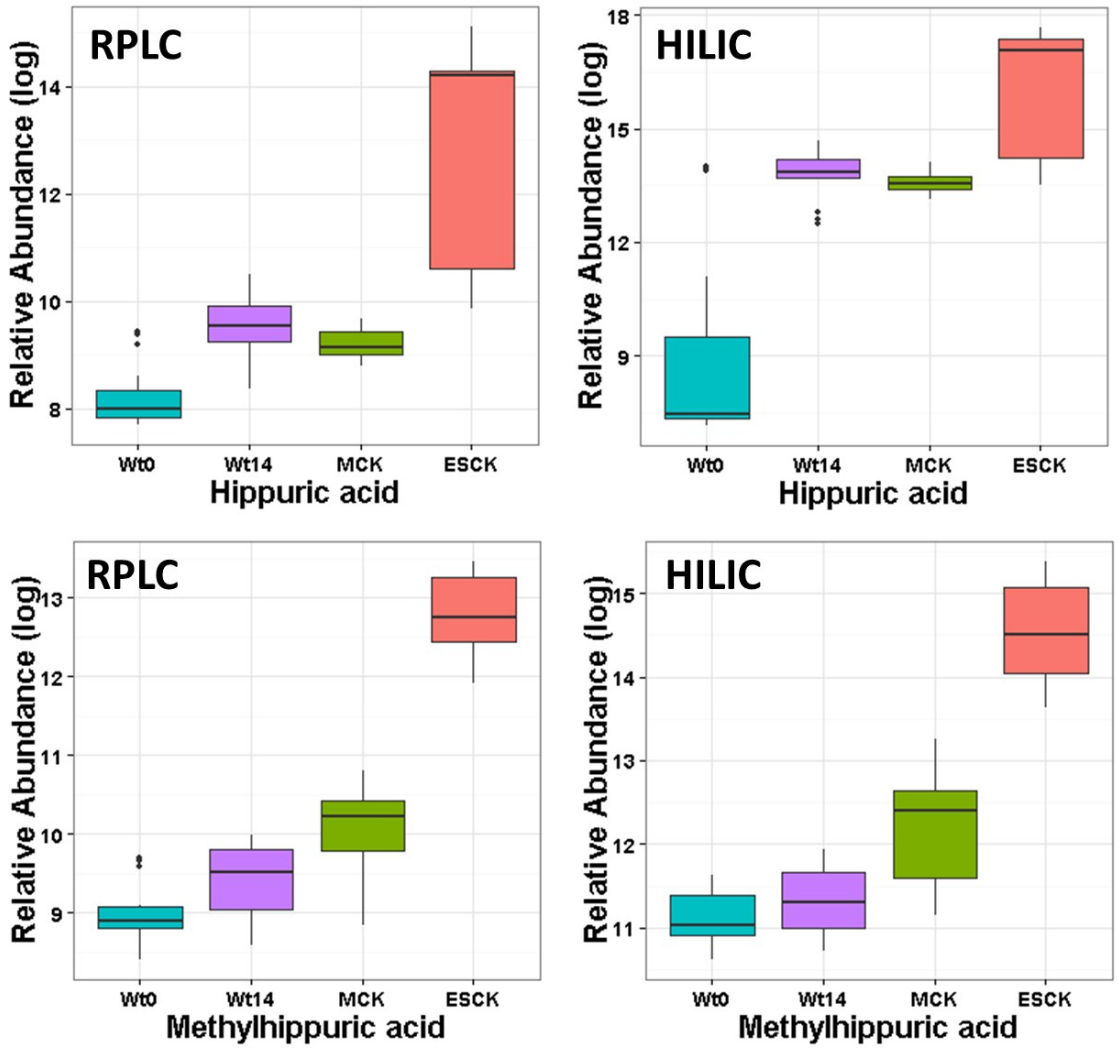
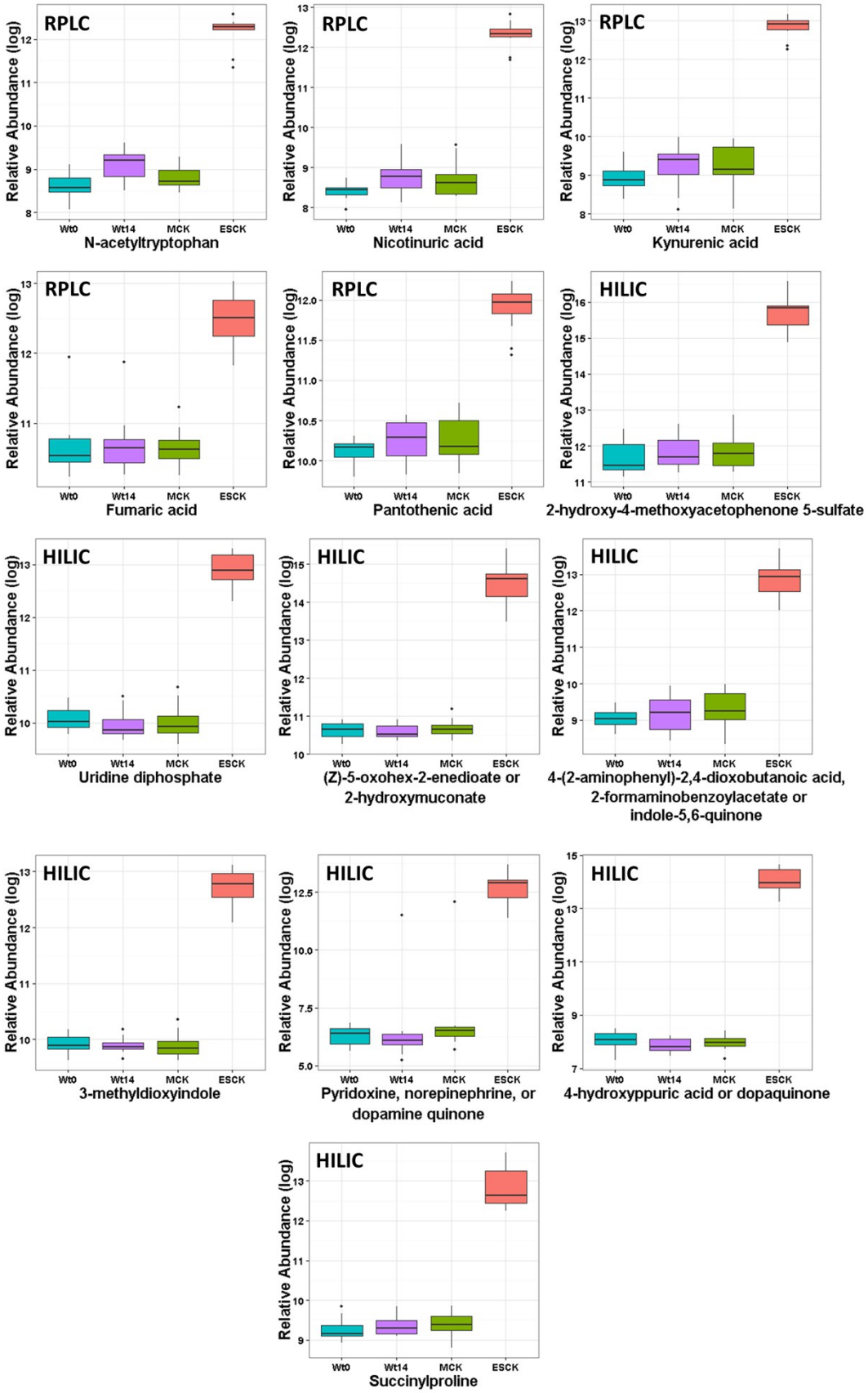

RPLC
Relative Abundance (log)
14
12
10
8
Wt0
Wt14
MCK
ESCK
Hippuric acid
HILIC
Relative Abundance (log)
18
15
12
9
Wt0
Wt14
MCK
ESCK
Hippuric acid
RPLC
Relative Abundance (log)
13
12
11
10
9
Wt0
Wt14
MCK
ESCK
Methylhippuric acid
HILIC
Relative Abundance (log)
15
14
13
12
11
Wt0
Wt14
MCK
ESCK
Methylhippuric acid

Fig. S6


RPLC
Relative Abundance (log)
Wt0
Wt14
MCK
ESCK
N-acetyltryptophan
Nicotinuric acid
Kynurenic acid
Fumaric acid
Pantothenic acid
HILIC
2-hydroxy-4-methoxyacetophenone 5-sulfate
Uridine diphosphate
(Z)-5-oxohex-2-enedioate or
2-hydroxymuconate
4-(2-aminophenyl)-2,4-dioxobutanoic acid,
2-formaminobenzoylacetate or
indole-5,6-quinone
3-methyldioxyindole
Pyridoxine, norepinephrine, or
dopamine quinone
4-hydroxyppuric acid or dopaquinone
Succinylproline

Tables

Table S1. Number of features obtained in the analysis of 20 micron mice kidney tissue samples by RPLC and HILIC after the metabolite extraction with different methanol proportions.

Platform	% Methanol content as extracting solvent			
	25	**50**	**75**	**100**
RPLC	1085	838	956	528
HILIC	203	186	545	679

Table S2. Number of latent variables, quality parameters and statistics values for the PLS-DA models built for the four different pairwise groups comparisons for the two analytical platforms used for PKD samples.

PLS-DA models	RPLC-MS ESI+					HILIC-MS ESI-				
	Number of latent components	R^2X	R^2Y	Q^2	F (and p-values) of CV-ANOVA	Number of latent components	R^2X	R^2Y	Q^2	F (and p-values) of CV-ANOVA
Wt0 *vs* Wt14	1	0.195	0.808	0.727	36.0 (2.4×10^{-8})	5	0.769	0.983	0.909	15.82 (3.5×10^{-7})
Wt0 vs MCK	1	0.264	0.809	0.744	39.1 (1.0×10^{-8})	3	0.644	0.963	0.913	35.3 (1.8×10^{-10})
MCK *vs* ESCK	3	0.771	0.991	0.980	161.0 (1.3×10^{-17})	2	0.743	0.987	0.978	274.04 (3.0×10^{-20})

Table S3. List of the influencing metabolites annotated in the RPLC-MS platform.

Tentative identification	Molecular formula	Level of assignment*	Metabolic pathway (according to KEGG)	Specie (as detected)	Retention time (min)	Experimental *m/z*	Theorical *m/z*	Δ*m/z* (mDa)	Δ*m/z* (ppm)	CV % in QC	VIP values of the pairwise comparison PLS-DA models		
											Wt0 *vs* Wt14	Wt0 vs MCK	MCK *vs* ESCK
Hippuric acid	$C_9H_9NO_3$	5	Phenylalanine metabolism	$[M+H]^+$	4.07	180.0653	180.0655	0.2	1.1	3.3	2.18	1.69	1.91
				$[M+Na]^+$	4.08	202.0472	202.0475	0.3	1.5	3.4	2.68	2.27	1.80
				$[MH-75Da]^+$	4.08	105.0334	105.0340	0.6	5.7	3.4	2.11	1.52	1.69
2,8-dihydroxyquinoline-β-D-glucuronide	$C_{15}H_{15}NO_8$	4	-	$[M+H]^+$	4.57	338.0868	338.0870	0.2	0.6	3.3	2.98	2.51	2.31
5-(3'-Carboxy-3'-oxopropenyl)-4,6-dihydroxypicolinate	$C_{10}H_7NO_7$	3	Tryptophan metabolism	$[M+H]^+$	4.08	254.0331	254.0295	3.6	14.2	6.1	2.82	2.35	1.55
				$[M-H_2O+H]^+$	4.08	236.0226	236.0195	3.1	13.1	2.3	2.97	2.53	1.49
				$[M-2H_2O+H]^+$	4.08	218.0122	218.0095	2.7	12.4	3.5	2.01	1.51	1.45
5-methylthiopentanaldoxime	$C_6H_{13}NOS$	2	Glucosinolate biosynthesis. 2-Oxocarboxylic acid metabolism	$[M+Na]^+$	1.03	170.0653	170.0610	4.3	25.3	21.5	1.87	0.77	0.74
2-, 3-, or 4-methylhippuric acid	$C_{10}H_{11}NO_3$	4	-	$[M+Na]^+$	4.38	216.0627	216.0631	0,4	1.9	4.3	0.92	1.83	1.73
				$[M-C_3H_4NO_2+H]^+$	4.38	91.05422	91.0548	5.8	6.4	7.9	0.94	1.68	1.69
			Citrate cycle (TCA cycle). Alanine,										

			aspartate and glutamate metabolism, and other pathways										
N-acetyltryptophan	$C_{13}H_{14}N_2O_3$	2	-	$[M-H_2O+H]^+$	4.60	229.1043	229.0977	6.6	28.8	15.4	1.34	0.53	2.00
5-Hydroxyindoleacetylglycine	$C_{12}H_{12}N_2O_4$	3	Tryptophan metabolism	$[M-H_2O+H]^+$	1.15	231.0835	231.0770	6.5	28.1	12.3	1.03	1.65	1.17
Nicotinuric acid	$C_8H_8N_2O_3$	4	Nicotinate and nicotinamide metabolism	$[M+H]^+$	3.63	181.0605	181.0608	0.3	1.7	14.0	0.94	0.70	2.05
Kynurenic acid	$C_{10}H_7NO_3$	5	Tryptophan metabolism	$[M+H]^+$	4.87	190.0495	190.0499	0.4	2.1	7.6	0.76	0.73	2.04
				$[M+H-60Da]^+$	4.87	130.0649	130.0651	0.2	1.5	18.4	1.21	0.88	1.95
Fumaric acid	$C_4H_4O_4$	2	Citrate cycle (TCA cycle). Oxidative phosphorylation, and other pathways	$[M+Na]^+$	1.12	139.0024	139.0002	2.2	15.8	26.7	0.09	0.02	1.44
Pantothenic acid	$C_9H_{17}NO_5$	4	beta-Alanine metabolism. Pantothenate and CoA biosynthesis	$[M+H]^+$	3.02	220.1176	220.1179	0.3	1.4	4.7	0.76	0.57	1.40
				$[M+Na]^+$	3.02	242.0995	242.0999	0.4	1.7	6.9	0.47	0.46	1.35

*See section “Identification of the variables” from *Materials and methods* for interpretation.

Table S4. List of the influencing metabolites annotated in the HILIC-MS platform

Tentative identification	Molecular formula	KEGG ID	Level of assignment*	Metabolic pathway	Specie (as detected)	Retention time (min)	Experimental *m/z*	Theorical *m/z*	Δ*m/z* (mDa)	Δ*m/z* (ppm)	CV % in QC	VIP values of the pairwise comparison PLS-DA models		
												Wt0 *vs* Wt14	Wt0 vs MCK	MCK *vs* ESCK
2-hydroxy-4-methoxyacetophenone 5-sulfate	$C_9H_{10}O_7S$	-	3	-	$[M-H]^-$	1.10	261.0081	261.0074	0.7	2.7	12.3	0.43	0.38	1.77
					$[M-SO_3-H]^-$	1.10	181.0507	181.0506	0.1	0.6	10.1	0.36	0.47	1.64
					$[M-SO_3-CO_2-H]^-$	1.08	137.0610	-	-	-	12.2	0.15	0.04	1.75
Uridine diphosphate	$C_9H_{14}N_2O_{12}P_2$	C00015	2	Pyrimidine metabolism	$[M+CH_3COO]^-$	1.12	463.0154	463.0160	0.6	1.3	22.1	0.36	0.21	1.55
3,4-dihydroxyphenylglycol-O-sulfate	$C_8H_{10}O_7S$	-	4	-	$[M-H_2O-H]^-$	1.18	230.9968	230.9963	0.5	2.2	5.0	1.06	1.91	1.60
					$[M-H_2O-SO_3-H]^-$	1.18	151.0401	151.0400	0.1	0.7	7.3	0.44	1.65	1.43
(Z)-5-oxohex-2-enedioate	$C_6H_6O_5$	C03453	2	Benzoate degradation. Tryptophan metabolism. Dioxin degradation	$[M+CH_3COO]^-$	1.30	137.0246 (which comes from fragmentation of 217.0341)	217.0354	1.3	6.0	22.2	0.10	0.10	1.79
2-hydroxymuconate	$C_6H_6O_5$	C02501		Benzoate degradation. Dioxin degradation										
4-(2-aminophenyl)-2,4-dioxobutanoic acid	$C_{10}H_9NO_4$	C01252	2	Trp metabolism	$[M-H]^-$	1.50	206.0458	206.0459	0.1	0.5	11.6	0.29	0.45	1.71
2-formaminobenzoylacetate	$C_{10}H_9NO_4$	C05835		Trp metabolism	$[M-H]^-$									

Indole-5,6-quinone	$C_8H_5NO_2$	C05579		Tyr metabolism	$[M+CH_3COO]^-$									
3-methyldioxyindole	$C_9H_9NO_2$	C05834	2	Trp metabolism	$[M-H_2O-H]^-$	1.80	144.0454	144.0449	0.5	3.5	25.3	0.09	0.10	1.54
2-,3-, or 4-methylhippuric acid	$C_{10}H_{11}NO_3$	-	2	-	$[M-H]^-$	2.02	192.0666	192.0666	0	0	7.0	0.54	1.59	1.32
3-methoxytyrosine	$C_{10}H_{13}NO_4$	-	2	-	$[M-H_2O-H]^-$			192.0661	0.5	2.6				
2-phenylacetamide	C_8H_9NO	C02505	2	Phe metabolism	$[M-H]^-$	2.12	134.0612	134.0611	0.1	0.7	6.7	3.83	3.07	1.18
octopamine	$C_8H_{11}NO_2$	C04227	2	Neurotransmitter metabolism	$[M-H_2O-H]^-$									
dopamine	$C_8H_{11}NO_2$	C03758	2	Neurotransmitter metabolism	$[M-H_2O-H]^-$									
hippuric acid	$C_9H_9NO_3$	C01586	2	Phe metabolism	$[M-H]^-$	2.12	178.0508	178.0510	0.2	1.1	6.3	4.24	3.36	1.18
indolelactic acid,	$C_{11}H_{11}NO_3$	C02043	2	Trp metabolism	$[M-H]^-$	2.33	204.0666	204.0666	0	0	5.5	1.59	1.93	0.26
5-methoxyindoleacetate	$C_{11}H_{11}NO_3$	C05660	2	Trp metabolism	$[M-H]^-$			204.0666	0	0				
acetyl-l-tyrosine	$C_{11}H_{13}NO_4$	-	2	-	$[M-H_2O-H]^-$			204.0661	0.5	2.5				
pyridoxine	$C_8H_{11}NO_3$	C00314	2	vitamin B6 metabolism	$[M-H_2O-H]^-$	2.58	150.0556	150.0555	0.1	0.7	12.4	0.19	0.61	2.17
Norepinephrine	$C_8H_{11}NO_3$	C00314	2	Phe/Tyr metabolism	$[M-H_2O-H]^-$			150.0555	0.1	0.7				
dopamine quinone	$C_8H_9NO_2$	C17756	2	betalain biosynthesis	$[M-H]^-$			150.0561	0.5	3.3				
4-hydroxyppuric acid	$C_9H_9NO_4$	-	2	-	$[M-H]^-$	2.58	194.0470	194.0459	1.1	5.7	9.4	0.58	0.25	2.26
dopaquinone		-	2	tyr and metabolism										
Succinylproline	$C_9H_{13}NO_5$	C11711	2	Renin-angiotensin system inhibitors	$[M-H]^-$	2.63	214.0720	214.0721	0.1	0.5	8.4	0.36	0.36	1.69

*See section “Identification of the variables” from *Materials and methods* for interpretation.