

NON-TARGET PROFILING OF THE WASTEWATER METABOLOME USING A SUITE OF HRMS TOOLS: A STUDY ACROSS DIVERSE TREATMENT PLANTS

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The internal standards specified in Tables S1 to S5 were not used to claim any MSI Level. However, the availability of >100 internal standards across platforms enabled partial validation of the analytical workflow by comparing retention behavior, ionization mode, and characteristic fragment ions. This supports the robustness and reproducibility of the analytical pipeline, even when standards for endogenous metabolites are unavailable.

Table S1. Internal standards used in the compounds' extraction.

CAR 10:0-d3
CAR 18:0-d3
CE 22:1
Cer 16:1 d18:1-d7/16:1
Cer 20:1 d18:1-d7/20:1
Cer 24:1 d18:1-d7/24:1
CUDA
DG 17:0/22:4-d5
FA 16:0-d3 (palmitic acid)
FA 18:1 (oleic acid)
FA 20:4-d11 (arachidonic acid)
LPC 17:0-d5
LPC 19:0-d5
LPE 17:0-d5
LPE 19:0-d5
PC 17:0/14:1-d5
PC 17:0/16:1-d5
PC 17:0/18:1-d5
PC 17:0/20:3-d5
PE 17:0/14:1-d5
PG 17:0/14:1-d5
PI 17:0/14:1-d5
SM 16:1 d18:1/16:1-d9
SM 22:1-d9 d18:1/22:1
SM 24:1-d9 d18:1/24:1
Sphingosine d17:1
TG 14:0/13:0/14:0-d5
TG 14:0/17:1/14:0-d5
TG 16:0/17:1/16:0-d5

Table S2. Internal standards used in RPLC-MS for the retention times correction.

<u>Positive</u>	<u>Negative</u>
CAR 10:0-d3 [M+]	CUDA [M-H]-
CAR 18:0-d3 [M+]	LPE 17:0-d5 [M-H]-
CE 22:1 [M+Na]+	FA 16:0-d3 (palmitic acid) [M-H]-
CE 22:1 [M+NH4]+	FA 18:1 (oleic acid) [M-H]-
Cer 16:1 d18:1-d7/16:1 [M+H]+	FA 20:4-d11 (arachidonic acid) [M-H]-
Cer 20:1 d18:1-d7/20:1 [M+H]+	LPC 19:0-d5 [M+Hac-H]-
Cer 24:1 d18:1-d7/24:1 [M+H]+	PI 17:0/14:1-d5 [M-H]-
CUDA [M+H]+	PG 17:0/14:1-d5 [M-H]-
DG 17:0/22:4-d5 [M+NH4]+	PE 17:0/14:1-d5 [M-H]-
LPC 17:0-d5 [M+H]+	Cer 16:1 d18:1-d7/16:1 [M+Hac-H]-
LPE 19:0-d5 [M+H]+	PC 17:0/16:1-d5 [M+Hac-H]-
PC 17:0/14:1-d5 [M+H]+	SM 22:1-d9 d18:1/22:1 [M+Hac-H]-
PC 17:0/18:1-d5 [M+H]+	Cer 20:1 d18:1-d7/20:1 [M+Hac-H]-
PC 17:0/20:3-d5 [M+H]+	SM 24:1-d9 d18:1/24:1 [M+Hac-H]-
SM 16:1 d18:1/16:1-d9 [M+Na]+	Cer 24:1 d18:1-d7/24:1 [M+Hac-H]-
Sphingosine d17:1 [M+H]+	
TG 14:0/13:0/14:0-d5 [M+NH4]+	
TG 14:0/17:1/14:0-d5 [M+NH4]+	
TG 16:0/17:1/16:0-d5 [M+NH4]+	

Table S3. Internal standards used for GC-MS in the MSTFA.

C08 FAME
C09 FAME
C10 FAME
C12 FAME
C14 FAME
C16 FAME
C18 FAME
C20 FAME
C22 FAME
C24 FAME
C26 FAME
C28 FAME
C30 FAME

Table S4. Internal standards used for HILIC-MS in the 80:20 (v/v) ACN:H₂O buffer.

CUDA	D4-Cystine
D10-Isoleucine	D4-Glycocholic acid
D10-Leucine	D4-Niacin
D2-Biotin	D4-Taurochenodeoxycholic acid
D2-Dodecanoic-2,2 Acid	D4-Thymine
D2-Ornithine	D5-Histidine
D3-1-Methylnicotinamide	D5-L-Glutamine
D3-2-Hydroxyglutaric acid	D5-Phenylacetylglutamine
D3-ACar(2:0)	D5-Threonine
D3-Asparagine	D7-Acetaminophen
D3-Aspartic Acid	D7-Arginine
D3-Cotinine	D7-Proline
D3-Creatine	D8-Lysine
D3-Creatinine	D8-Methionine
D3-DL-Aspartic acid	D8-Phenylalanine
D3-DL-Glutamic acid	D8-Tryptophan
D3-Histamine, N-methyl-	D8-Valine
D3-L-Carnitine	D9-Betaine
D3-N-Acetyl-5-methoxytryptamine	D9-Choline
D3-Serine	D9-TMAO
D4-Alanine	Val-Tyr-Val

Table S5. Internal standards used for the retention times correction in HILIC-MS.

Positive

CUDA [M+H]⁺
D10-Isoleucine [M+H]⁺
D10-Leucine [M+H]⁺
D2-Biotin [M+H]⁺
D2-Ornithine [M+H]⁺
D3-1-Methylnicotinamide [M]⁺
D3-ACar(2:0) [M+H]⁺
D3-Asparagine [M+H]⁺
D3-Aspartic Acid [M+H]⁺
D3-Cotinine [M+H]⁺
D3-Creatine [M+H]⁺
D3-Creatinine [M+H]⁺
D3-DL-Glutamic acid [M+H]⁺
D3-Histamine, N-methyl- [M+H]⁺
D3-L-Carnitine [M+H]⁺
D3-N-Acetyl-5-methoxytryptamine [M+H]⁺
D3-Serine [M+H]⁺
D4-Alanine [M+H]⁺
D4-Cystine [M+H]⁺
D4-Glycocholic acid [M+H]⁺
D4-Niacin [M+H]⁺
D4-Taurochenodeoxycholic acid [M+H]⁺
D4-Thymine [M+H]⁺
D5-Histidine [M+H]⁺
D5-L-Glutamine [M+H]⁺
D5-Phenylacetylglutamine [M+H]⁺
D5-Threonine [M+H]⁺
D7-Acetaminophen [M+H]⁺
D7-Arginine [M+H]⁺
D7-Proline [M+H]⁺
D8-Lysine [M+H]⁺
D8-Methionine [M+H]⁺
D8-Phenylalanine [M+H]⁺
D8-Tryptophan [M+H]⁺
D8-Valine [M+H]⁺
D9-Betaine [M+H]⁺
D9-Choline [M]⁺
D9-TMAO [M+H]⁺
Val-Tyr-Val [M+H]⁺

Negative

CUDA [M-H]⁻
D10-Isoleucine [M-H]⁻
D10-Leucine [M-H]⁻
D2-Biotin [M-H]⁻
D2-Dodecanoic-2,2 Acid [M-H]⁻
D2-Ornithine [M-H]⁻
D3-2-Hydroxyglutaric acid [M-H]⁻
D3-Creatinine [M-H]⁻
D3-DL-Aspartic acid [M-H]⁻
D3-DL-Glutamic acid [M-H]⁻
D3-N-Acetyl-5-methoxytryptamine [M-H]⁻
D3-Serine [M-H]⁻
D4-Alanine [M-H]⁻
D4-Cystine [M-H]⁻
D4-Glycocholic acid [M-H]⁻
D4-Niacin [M-H]⁻
D4-Taurochenodeoxycholic acid [M-H]⁻
D5-Histidine [M-H]⁻
D5-L-Glutamine [M-H]⁻
D5-Phenylacetylglutamine [M-H]⁻
D5-Threonine [M-H]⁻
D7-Acetaminophen [M-H]⁻
D7-Arginine [M-H]⁻
D8-Lysine [M-H]⁻
D8-Methionine [M-H]⁻
D8-Phenylalanine [M-H]⁻
D8-Tryptophan [M-H]⁻
D8-Valine [M-H]⁻
Thymine-D4 [M-H]⁻
Val-Tyr-Val [M-H]⁻

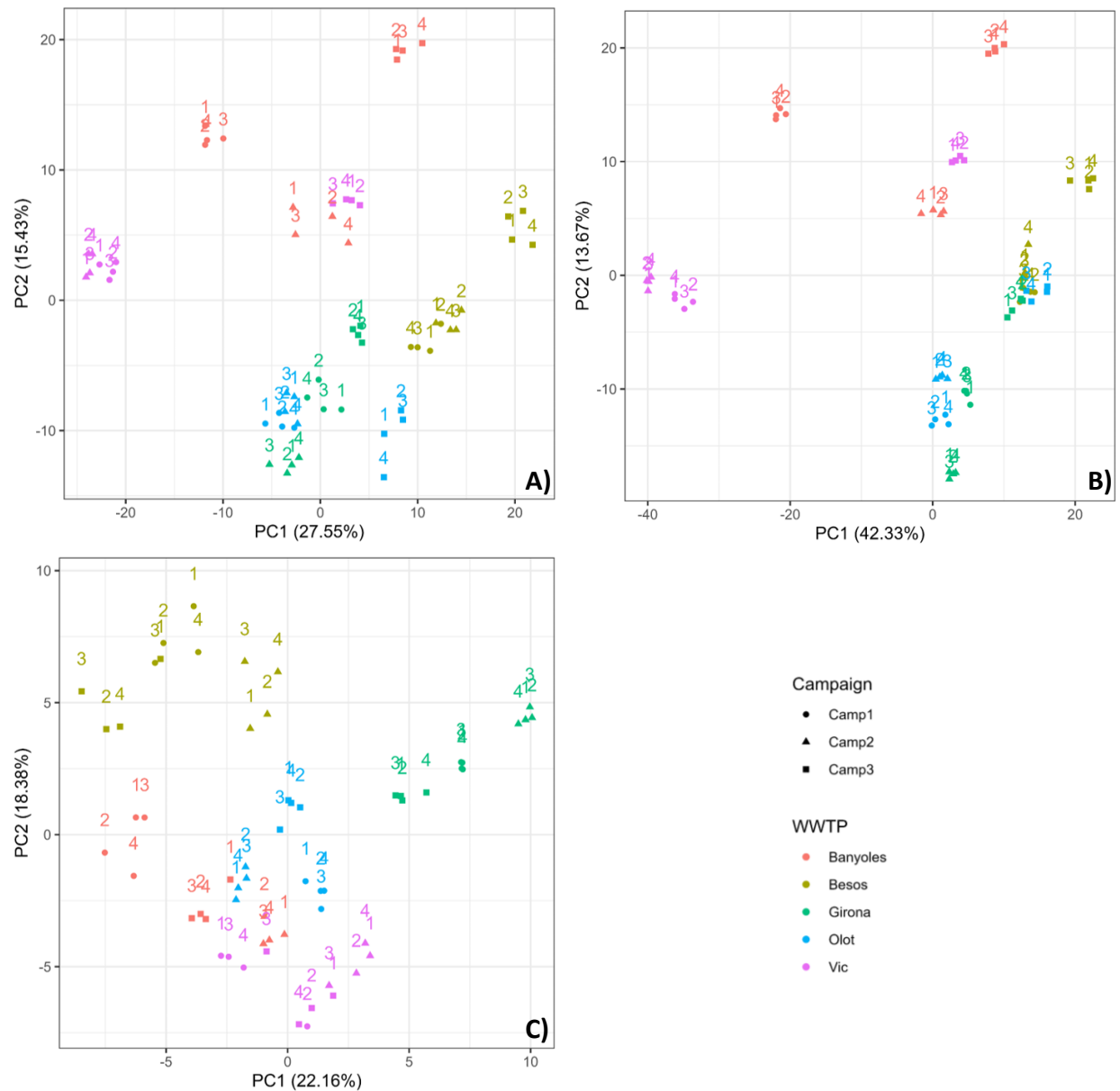


Figure S1. Principal Component Analysis (PCA) of the compounds from GC-MS (A), HILIC-MS (B) and RPLC-MS (C) without removing the campaign-associated variability. (Campaigns 1: winter, 2: spring, 3: summer)

MSI levels

RPLC-MS (positive and negative):

- IS: internal standards used for the correction of the retention times.
- 1a: unique MS/MS match with 2 fragments + m/z-RT match + accurate mass <1 mDa.
- 1b: non-unique MS/MS match + m/z-RT match + accurate mass <1 mDa.
- 1c: non-unique MS/MS match + m/z-RT match + accurate mass <2 mDa.
- 2a: unique MS/MS match with 3 fragments + accurate mass <2 mDa.
- 2b: non-unique MS/MS match + accurate mass <2 mDa.
- 2c: unique or non-unique MS/MS match with only the precursor + accurate mass <2 mDa.
- 3a: m/z-RT match + accurate mass <2 mDa + RT error <1 min.
- 3b: m/z-RT match + accurate mass 2-5 mDa + RT error <1 min.
- 4: RT error >1 min and/or mass error >5 mDa, but good peak; more than one peak.
- Unknown: peak in the noise level and no MS/MS / RT error > 5 / m/z error >10 / many possible MS/MS matches / RIKEN annotations.

GC-MS (positive):

- IS: FAMES internal standards used for the correction of the retention times.
- 1: unique, good fragmentation match, dot/rev >900, RI >950, good peak shape.
- 2: non-unique, good fragmentation match, dot/rev = 700-900, RI = 900-950, average peak shape, quant mass = 147.
- 3: bad fragmentation match, dot/rev = 500-700, RI = 800-900, bad peak shape or alignment.
- 4: very bad fragmentation match, dot/rev <500, RI <800, double peak, sugars.
- Unknown: noise level, wide peak, annotated as FAME but it's not, annotations starting with a number, duplicates, compounds mix.

HILIC-MS (positive and negative):

- IS: internal standards used for the correction of the retention times. Note: D3-Creatinine and D4-Niacin are not present in positive mode.
- 1a: unique MS/MS match with 2 fragments + m/z-RT match + accurate mass <1 mDa.
- 1b: non-unique MS/MS match + m/z-RT match + accurate mass <1 mDa
- 1c: unique or non-unique MS/MS match + m/z-RT match + accurate mass >1 mDa.
- 2a: unique MS/MS match with 2-3 fragments (including precursor) + accurate mass <2 mDa.
- 2b: non-unique MS/MS match with 2-3 fragments (including precursor) + accurate mass <2 mDa.
- 2c: unique with one fragment or non-unique MS/MS match with 2 fragments (including precursor) + accurate mass 2-5 mDa.
- 3a: m/z-RT match + accurate mass <2 mDa + RT error <1 min.
- 3b: m/z-RT match + accurate mass 2-5 mDa + RT error <1 min.
- 4: accurate mass 5-10 mDa and/or RT error 1-5 min, but good peak / no RT match + no MS2 match + <1 mDa m/z error / sugars.
- Unknown: peak in the noise level / peaks not well aligned / no RT match + >1 mDa m/z error + no MS2 match.

Significant metabolites (p_adj < 0.05 and |FC| > 1.5) across any contrast: 241



HILIC-MS

Significant metabolites (p_adj < 0.05 and |FC| > 1.5) across any contrast: 485



Significant metabolites ($p_{\text{adj}} < 0.05$ and $|\text{FC}| > 1.5$) across any contrast: 122

