Chapter 3

LC-MS-Based Metabolomics of Biofluids Using All-Ion Fragmentation (AIF) Acquisition

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Abstract

The field of liquid chromatography-mass spectrometry (LC-MS)-based nontargeted metabolomics has advanced significantly and can provide information on thousands of compounds in biological samples. However, compound identification remains a major challenge, which is crucial in interpreting the biological function of metabolites. Herein, we present a LC-MS method using the all-ion fragmentation (AIF) approach in combination with a data processing method using an in-house spectral library. For the purposes of increasing accuracy in metabolite annotation, up to four criteria are used: (1) accurate mass, (2) retention time, (3) MS/MS fragments, and (4) product/precursor ion ratios. The relative standard deviation between ion ratios of a metabolite in a biofluid vs. its analytical standard is used as an additional metric for confirming metabolite identity. Furthermore, we include a scheme to distinguish co-eluting isobaric compounds. Our method enables database-dependent targeted as well as nontargeted metabolomics analysis from the same data acquisition, while simultaneously improving the accuracy in metabolite identification to increase the quality of the resulting biological information.

Key words Metabolomics, Liquid chromatography-mass spectrometry (LC-MS), All-ion fragmentation (AIF), Metabolite annotation

Abbreviations

ACN	Acetonitrile
AIF	All-ion fragmentation
AM	Accurate mass
CID	Collision-induced dissociation
EIC	Extracted ion chromatogram
HILIC	Hydrophilic interaction liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
RP	Reverse phase
RT	Retention time

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1 Introduction

Mass spectrometry (MS)-based metabolomics has been used extensively to investigate biological processes and perform biomarker discovery work [1, 2]. In addition, it is considered an important part of precision medicine initiatives [3]. However, metabolite annotation is still a major challenge and significant bottleneck in translating metabolomics data into biochemical context [4, 5]. Following the identification criteria proposed by the Metabolomics Standard Initiative (MSI), the highest level of metabolite identification is based upon matching two or more orthogonal properties [e.g., accurate mass (AM), retention time (RT)/index, isotopic pattern, MS/MS spectrum] of an authentic reference standard analyzed under the same condition as the metabolite of interest [6].

Liquid chromatography-mass spectrometry (LC-MS) is one of the most commonly applied techniques in the field of metabolomics, offering high sensitivity and wide metabolite coverage [7]. In LC-MS-based nontargeted metabolomics approaches, metabolites are generally reported based on AM and RT matching; however, these criteria are often insufficient to conclusively identify a metabolite due to co-elution and/or the presence of metabolites of similar m/z and molecular formula. Accordingly, these data are frequently complemented with MS/MS spectra to increase the certainty in the identification of metabolites of interest. While useful, this approach relies heavily upon the use of spectral databases to confirm metabolite structure. The number and quality of spectral databases has steadily increased, improving the confidence in metabolite identification; however, additional resources and efforts are needed for comprehensive structural confirmation.

Herein, we describe a LC-MS metabolomics method using an all-ion fragmentation (AIF) approach. An AIF experiment uses the low-energy collision-induced dissociation (CID) to acquire precursor ion mass spectra, whereas the high-energy CID is used to obtain product ion information by tandem mass spectrometry [8]. In the subsequent data analysis, extracted ion chromatograms (EIC) from any precursor or associated product ions of interest are extracted from the low- or high-energy scan data. One EIC is chosen for relative quantification (the quantifier ion) of the metabolite, and further product ions from the same compound are used as qualifier ions. This approach has the advantage of simultaneously acquiring extensive fragmentation data on the sample, which can then be searched against spectral databases for MS/MS-based confirmation. For the purposes of the reported method, an in-house spectral database was constructed using commercially available analytical standards [9].

To further increase the metabolite identification specificity, the product/precursor ion ratio was calculated, and the % relative

error was calculated between each analytical standard and the corresponding compound in the biological matrix. The use of specific quantifier and qualifier ions is also used to distinguish co-eluting isobaric compounds. The described data acquisition strategy enables a simultaneous combination of database-dependent targeted and nontargeted metabolomics in combination with improved accuracy in metabolite identification, thus increasing the quality of the biological information acquired in a metabolomics experiment.

2 Materials

2.1 Samples

1. Plasma and/or serum samples kept at −80 °C until the day of analysis (*see* **Note 1**).
2. Urine samples kept at −80 °C until the day of analysis.

2.2 Chemicals and Standards

The analytical standards for the construction and expansion of the compound spectral database, as well as the internal standards, can be purchased from Sigma-Aldrich (St. Louis, USA), Cayman Chemical Company (Michigan, USA), Toronto Research Chemicals (Ontario, Canada), Zhejiang Ontores Biotechnologies Co., Ltd. (Zhejiang, China), and Avanti Polar Lipids, Inc. (Alabama, USA) depending upon availability. The standards are prepared at 1 mM concentrations in the appropriate solvent for dissolution, stored at −20 °C, and diluted appropriately on the day of analysis.

2.3 Solutions and Solvents

All solutions are prepared at room temperature (25 °C) using LC-MS grade solvents and analytical grade reagents.

1. ESI-low concentration tuning mix (Agilent Technologies, Santa Clara, USA) for calibrating the TOF-MS: For 100 mL of solution, mix 10 mL of ESI-low concentration tuning mix, 85.5 mL acetonitrile (ACN), 4.5 mL water, and 3 μL HP-321 (Agilent Technologies, Santa Clara, USA).
2. An internal lock mass mixture (Agilent Technologies, Santa Clara, USA) for continuous mass calibration during data acquisition: For 500 mL of solution, mix 475 mL ACN, 25 mL water, 0.2 mL purine [5 mM in (9:1, *v/v*) ACN/water], and 0.5 mL of HP-0921 [2.5 mM in (9:1, *v/v*) ACN/water].
3. Solvents for hydrophilic interaction liquid chromatography (HILIC).

   Solvent A (water with 0.1% formic acid): prepare by adding 1 mL formic acid to 1 L of water.

   Solvent B (ACN with 0.1% formic acid): prepare by adding 1 mL formic acid to 1 L of ACN.
4. Solvents for the reversed phase (RP) chromatography.

Solvent A (water with 0.1% formic acid): prepare by adding 1 mL formic acid to 1 L of water.

Solvent B [(90:10, *v/v*) 2-propanol:ACN]: prepare by adding 1 mL formic acid to 1 L of (90:10, *v/v*) 2-propanol:ACN.

5. A methanol (MeOH) crash solution for plasma/serum metabolite extraction or urine nonpolar metabolite extraction containing internal standards (Table 1) (*see* **Note 2**).
6. An ACN crash solution for urine polar metabolite extraction containing internal standards (Table 1) (*see* **Note 2**).

2.4 LC-MS System

1. Ultrahigh performance liquid chromatography (UHPLC) 1290 Infinity II system (including in-line filter 0.3 μm, Agilent Technologies, Santa Clara, USA) with 1260 Infinity II isocratic pump (including 1:100 splitter) coupled to a 6550 iFunnel quadrupole-time of flight (Q-TOF) mass spectrometer with a dual AJS electrospray ionization source (Agilent Technologies, Santa Clara, USA).
2. For separation of polar metabolites, HILIC SeQuant® ZIC®-HILIC column (100 mm × 2.1 mm, 100 Å, 3.5 μm particle size, Merck, Darmstadt, Germany) coupled to a guard column (2.1 mm × 2 mm, 3.5 μm particle size, Merck, Darmstadt, Germany).
3. For separation of nonpolar metabolites, reversed phase (RP) Zorbax Eclipse Plus C18, RRHD column (100 mm × 2.1 mm, 1.8 μm particle size, Agilent Technologies, Santa Clara, USA) coupled to a guard column (5 mm × 2 mm, 1.8 μm Agilent Technologies, Santa Clara, USA).

2.5 Software

1. Agilent MassHunter Acquisition for Q-TOF (version B.06.01, Agilent Technologies, Santa Clara, USA) for acquiring the data.
2. Agilent PCDL (version B.07.00, Agilent Technologies, Santa Clara, USA) for managing the compound database (AM, RT, and MS/MS spectra).
3. Agilent MassHunter TOF-Quant software (version B.07.00, Agilent Technologies, Santa Clara, USA) for construction of the data processing method and processing the acquired data for targeted metabolite screening.

2.6 Other Equipments

1. Pipettes and tips (2–20, 20–200, 100–1000, 500–5000 μL).
2. Specific gravity refractometer (Atago, Tokyo, Japan).
3. Eppendorf 1.5 mL tubes.
4. Vortex mixer.
5. Pyrex measuring cylinders (100 mL, 1000 mL).

Table 1
Composition of the crash solution (total volume of 500 mL in MeOH or ACN)

Compound	Stock concentration (μg/mL or ppm)	Solvent for the stock solution	Volume for crash solution (μL) in 500 mL	Concentration in crash solution (μg/mL or ppm)	Formula	Monoisotopic mass
L-phenylalanine-$^{13}C_9$,^{15}N	200	Water	1250	0.5	$C_6H_{14}[^{15}N]4O_2$	175.1062
L-arginine-$^{15}N_4$	500	Water	2600	2.6	$C_6H_9[^{15}N]3O_2$	178.0998
Uracil-$^{15}N_2$	500	Warm water/ ammonia water	500	0.5	$C_5H_{11}[^{15}N]O_2$	114.0213
L-valine-$^{15}N_3$	1200	Water	8333	20	$C_3[^{13}C]H_3D_2O_3$	118.0760
L-tyrosine-$^{13}C_9$,^{15}N	133	Water	3759	1	$[^{13}C]2H_7NO_3S$	191.1011
Taurine-$^{13}C_2$	1000	Water	1250	2.5	$C_{11}D_5H_7N_2O_2$	127.0214
L-asparagine-$^{15}N_2$	1000	Water	1250	2.5	$C_9H_{14}D_3NO_4$	134.0476
Acetyl-d_3-carnitine	1240	MeOH	202	0.5	$C_6H_5D_7O_6$	206.1346
Allantoin ^{13}C,^{15}N	1300	Water	385	1	$C_3[^{13}C]H_6N_3[^{15}N]O_3$	160.0444
Arachidonic acid-d_8	100	Ethanol	5000	1	$C_{20}H_{24}D_8O_2$	312.2904
Linoleic acid-d_{11}	100	Ethanol	5000	1	$C_{18}H_{21}D_{11}O_2$	291.3093
Docosahexaenoic acid-d_5	100	Ethanol	5000	1	$C_{22}H_{27}D_5O_2$	333.2716
Eicosapentaenoic acid-d_5	100	Ethanol	5000	1	$C_{20}H_{25}D_5O_2$	307.2560
S1P(d18:1/17:0)	456	MeOH	1000	0.186	$C_{17}H_{36}NO_5P$	365.2331
SM(d18:1/17:0)	1000	MeOH	5000	10	$C_{40}H_{81}N_2O_6P$	716.5832
Cer(d18:1/17:0)	500	MeOH	1000	1	$C_{35}H_{69}NO_3$	551.5277
Palmitoyl-L-carnitine (N-methyl-d_3)	100	MeOH	250	0.05	$C_{23}D_3H_{42}NO_4$	402.3537
Chenodeoxycholic acid-2,2,4,4,-d_4	1000	MeOH	200	0.4	$C_{24}D_4H_{36}O_4$	396.3178

6. Centrifuge.
7. Evaporator-concentrator.
8. HPLC vials (fixed insert 300 μL amber, Agilent Technologies, Santa Clara, USA) and screw caps (Agilent Technologies, Santa Clara, USA).

3 Methods

3.1 Processing of Plasma/Serum Samples

1. Thaw the samples on ice (~1 h, depending on volume), followed by vortexing for 30 s.
2. Place 150 μL of plasma or serum sample in a 1.5 mL Eppendorf tube (*see* **Note 3**).
3. Add 450 μL of ice-cold MeOH crash solution (sample/solvent 1:3, *v/v*) (*see* **Note 4**).
4. Vortex 10 s.
5. Centrifuge the sample to pellet the precipitate (13,000 × *g*, 10 min, 4 °C).
6. Transfer 70 μL of the supernatant containing extracted metabolites to a new 1.5 mL Eppendorf tube (*see* **Note 5**).
7. Evaporate the samples using an evaporator-concentrator. Set temperature at 30 °C, and dry for 45 min (drying time will vary depending on unit/volume) (*see* **Note 6**).
8. Reconstitute the evaporated samples on the day of the analysis in 50 μL (8:2, *v/v*) ACN/water for HILIC and 50 μL MeOH for RP metabolomics.
9. Centrifuge the reconstituted samples (13,000 × *g*, 2 min, 4 °C).
10. Transfer 35 μL to HPLC vial for analysis (*see* **Note 7**).

3.2 Processing of Urine Samples

1. Thaw the samples on ice (~1 h, depending on volume).
2. Vortex 30 s.
3. Centrifuge the sample (13,000 × *g*, 10 min, 4 °C), and use the supernatant in the following steps.
4. Measure the specific gravity by pipetting 100 μL of urine on the specific gravity refractometer (*see* **Note 8**).
5. Normalize all the urine samples to the sample with the lowest measured specific gravity using LC-MS grade water [10] (*see* **Note 9**).
6. Place 20 μL of normalized urine sample in a 1.5 mL Eppendorf tube.
7. Add 180 μL of crash solution containing internal standards (sample/solvent 1:9, *v/v*).

8. Vortex 5 s.
9. Centrifuge the sample (13,000 × *g*, 10 min, 4 °C).
10. Transfer 40 μL of supernatant to HPLC vial for analysis (*see* **Note 10**).

3.3 LC Parameters

To obtain consistently high-quality data, regularly inspect and maintain the system (*see* **Note 11**).

1. Maintain the samples in the autosampler module at 10 °C (*see* **Note 12**).
2. Injection volume 2 μL (injection loop 20 μL) (*see* **Note 13**).
3. The mobile phase gradient for both HILIC and RP chromatography is presented in Tables 2 and 3, respectively (*see* **Note 14**).

Table 2
Gradient settings for HILIC chromatography

Time (min)	Solvent A (%)	Solvent B (%)
0	5	95
1.5	5	95
12	60	40
14	60	40
14.2	75	25
17	75	25
18	5	95
25	5	95

Table 3
Gradient settings for RP chromatography

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
3	95	5
5	70	30
18.5	2	98
20	2	98
20.5	95	5
25	95	5

4. The mobile phase gradient flow rate is 0.3 mL/min for HILIC (*see* **Note 15**) and 0.4 mL/min for RP (*see* **Note 16**).
5. The column oven temperature is maintained at 25 °C for HILIC chromatography and 50 °C for RP chromatography.

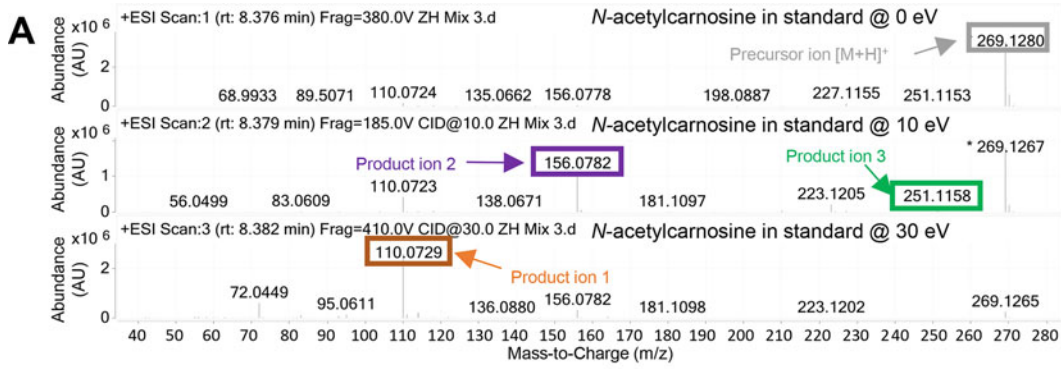
3.4 MS Parameters

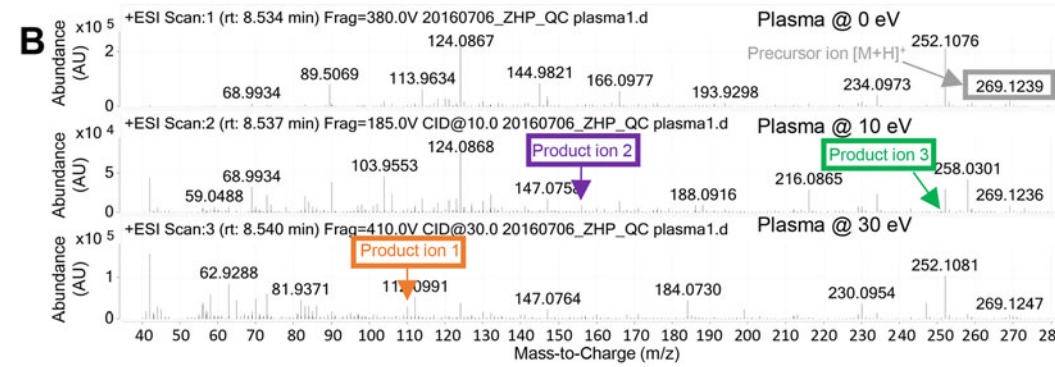
1. Tune the MS system before each project using the ESI-low concentration tuning mix solution.
2. The internal lock mass mixture is constantly infused at a flow rate of 1 mL/min using an isocratic pump (split ESI spray/return to the stock bottle 1:100; *see* **Note 17**) together with the LC eluent for constant mass correction. Positive ionization mode: purine ($[M+H]^+$ *m/z* 121.0509), HP-0921 ($[M+H]^+$ *m/z* 922.0098). Negative ionization mode: purine ($[M-H]^-$ *m/z* 119.0363), HP-0921 ($[M+COOH]^-$ *m/z* 966.0007). Use a detection window of 100 ppm and minimum height of 1000 counts.
3. Set the sheath and drying gas (nitrogen purity > 99.999%) flows to 8 L/min and 15 L/min, respectively. Set the temperature of the drying and sheath gas at 250 °C, with the nebulizer pressure at 35 psig. Set the voltages for positive and negative ionization modes at +3000 V and −3000 V, respectively.
4. Set the fragmentor voltages to 380 V at 0 eV, 185 V at 10 eV, and 410 V at 30 eV.
5. Acquire the data in centroid mode with a mass range of 40–1200 *m/z* for HILIC and 40–1200 *m/z* for RP.
6. Perform MS acquisition in AIF mode, where full-scan high-resolution data are acquired at three alternating collision energies (0 eV – full scan, 10 eV, and 30 eV) (*see* **Note 18**).
7. Set the data acquisition rate at six scans/s.

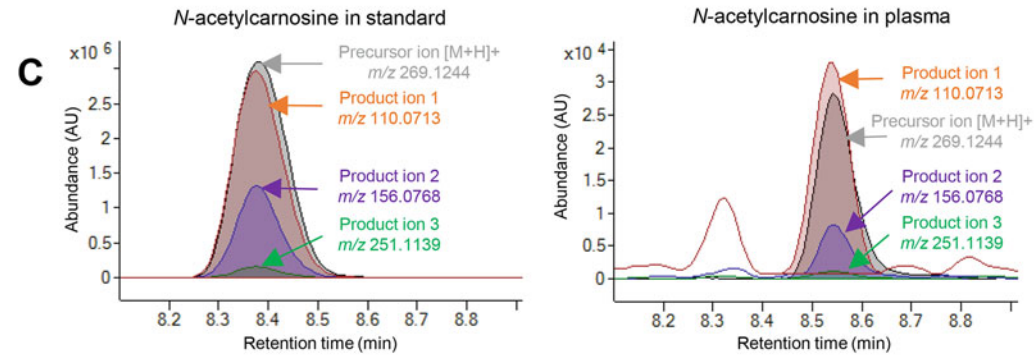
3.5 Data Processing and Metabolite Identification

A data processing method for database-dependent metabolite screening was constructed in the Agilent TOF-Quant software (version B.07.00, Agilent Technologies, *see* **Note 19**) using precursor and product ion information. The manually constructed data processing method is useful in improving metabolite identification as well as deconvoluting isobaric compounds. A list of 413 compounds with AM, RT (HILIC and RP chromatography), fragmentation, and ions ratios is provided in Naz et al. [9]. The level of confidence in the identification is reflected by the ranking: Rank 1, AM and RT; Rank 2, AM, RT, and MS/MS; and Rank 3, AM, RT, MS/MS, and ion ratio. Of the 413 compounds, 229 metabolites (Rank 1, 40; Rank 2, 86; Rank 3, 99) were detected in plasma. The steps for adding a compound to the data processing method are explained using the example of *N*-acetylcarnosine (Fig. 1).

1. Inject and measure appropriately diluted analytical standards using HILIC and/or RP chromatography in positive and/or negative ionization modes.







D

Ion ratio (peak area)	Standard	Plasma	Relative error (%)
Product ion 1 / Precursor ion	0.91	1.08	-18.6
Product ion 2 / Precursor ion	0.35	0.27	22.8
Product ion 3 / Precursor ion	0.043	0.045	5.0

Fig. 1 Metabolite identification with MS/MS fragments and ion ratio confirmation using all-ion fragmentation (AIF) data. (**a**) *N*-acetylcarnosine spectra obtained from an analytical standard (HILIC column, positive ionization mode) at 0 eV (upper panel), 10 eV (middle panel), 30 eV (lower panel). (**b**) *N*-acetylcarnosine spectra obtained in plasma (HILIC column, positive ionization mode) at the elution time of 8.37 min at 0 eV (upper panel), 10 eV (middle panel), 30 eV (lower panel). (**c**) Extracted ion chromatograms of *N*-acetylcarnosine precursor and MS/MS product ions overlaid in standard (left panel) and plasma (right panel). (**d**) Ion ratios based on the peak areas in standard and plasma samples and the respective relative errors

2. Characterize the analytical standard to obtain the precursor ion *m/z* and the retention time (RT) from the 0 eV scan. In the example of *N*-acetylcarnosine, the $[M+H]^+$ precursor ion is detected at *m/z* 269.1244 and RT 8.37 min (Fig. 1a upper panel).
3. When possible, select more than two product ions (from 10 to 30 eV scans; *see* **Note 20**) for each compound (as unique as possible), and calculate their ratios with the precursor ion. In the example of *N*-acetylcarnosine, three product ions can be observed: *m/z* 110.0713 at 30 eV, *m/z* 156.0768 at 10 eV, and *m/z* 251.1139 at 10 eV (Fig. 1a middle and lower panels). For isobaric compounds, a compound-specific highly abundant product ion is selected as the precursor ion. An example for deconvoluting isobaric compounds is shown in Fig. 2.
4. For each metabolite, merge the quantifier ion (precursor ion), qualifier ion(s) (product ions), their relative ion ratios, and RTs into the data processing method. Use a mass error of 20 ppm, Gaussian smoothing width of nine points, and a RT window of 10%.
5. The compound identification is then ranked based on the matching of the identification criteria as described above. In the example of *N*-acetylcarnosine (Fig. 1c, d), it is a Rank 3 compound in plasma sample (*see* **Note 21**).
6. After data processing, the quantifier ion peak area of each identified metabolite is exported in a .csv file format to be further used for statistical analysis.

3.6 Nontargeted Metabolomics Data Processing

With the presented AIF approach, data can be processed for targeted metabolite screening as described above, while at the same time also enabling nontargeted metabolomics analysis with the data collected at 0 eV (full scan) (Fig. 3). The workflow below can be used as a starting point from nontargeted to targeted metabolomics.

1. Process the data: Detect masses, build (perform smoothing if needed) and deconvolute chromatograms, remove isotopic peaks, align the peaks to obtain a feature list for the experiment (*see* **Note 22**).
2. Analyze the data to identify relevant peaks/features.
3. Perform database search to obtain candidate compounds, and if MS/MS spectra are available, search for fragments in the 10 eV and 30 eV scans (*see* **Note 23**).
4. Acquire an analytical standard, and add it to the database-dependent data processing method as described in Subheading 3.5.

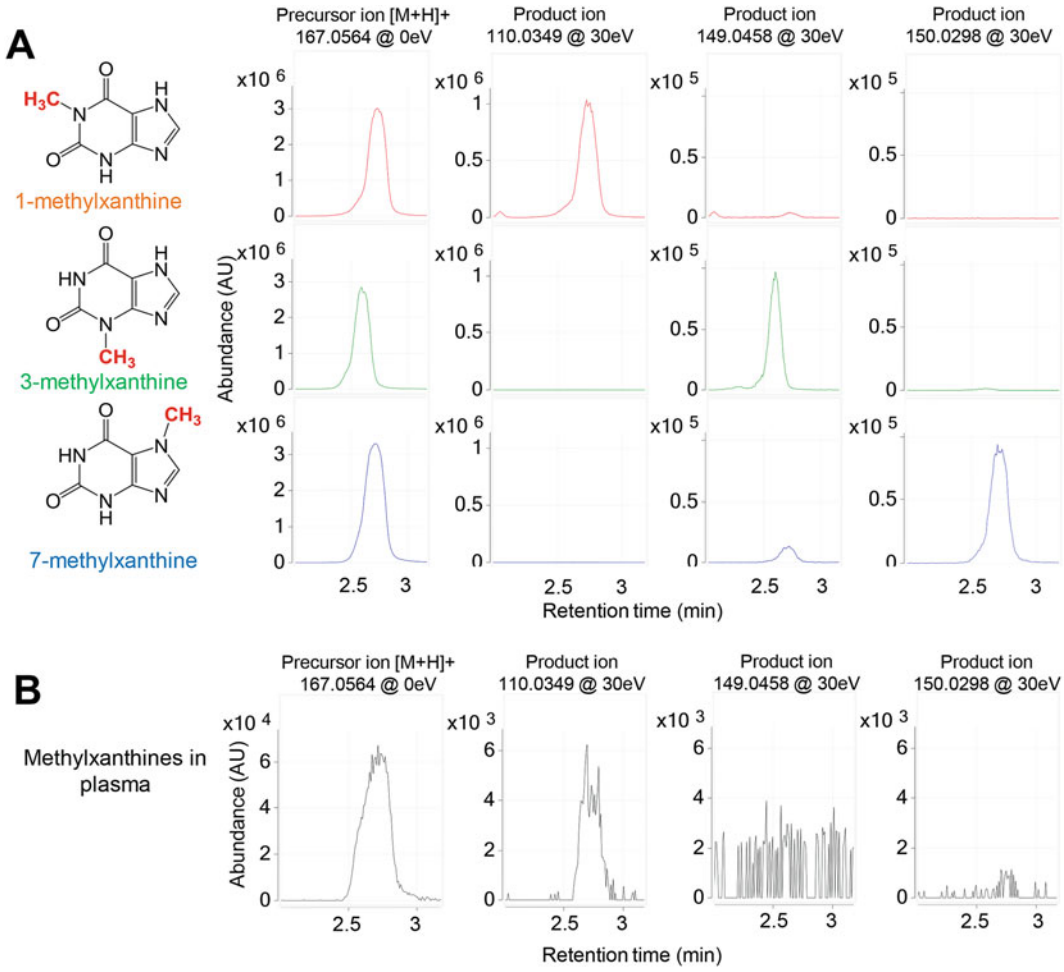


Fig. 2 Improving selectivity for co-eluting isobaric compounds using all-ion fragmentation (AIF) data. (**a**) Standards of 1-, 3-, and 7-methylxanthines (HILIC method, positive ionization mode) have very close retention times for the precursor $[M+H]^+$ ion at 0 eV (first column). However, each of the methylxanthines has a distinct product ion at 30 eV, *m/z* 110.0349, *m/z* 149.0458, and *m/z* 150.0298 for 1-, 3-, and 7-methylxanthines, respectively (second to fourth columns). (**b**) In plasma (HILIC column, positive ionization mode), the precursor ion for methylxanthines $[M+H]^+$ *m/z* 167.0564 is detected at 2.7 min, 0 eV (first column). In the 30 eV scans, product ion for 1-methylxanthine (*m/z* 110.0349, second column) and 7-methylxanthine (a low level of *m/z* 150.0298, fourth column), but no product ion for 3-methylxanthine, is observed, consistent with the literature [11]. Integration of the specific fragment ions enables the relative quantification of each co-eluting isobaric metabolite

4 Notes

1. While beyond the scope of the current protocol, sample collection and processing procedures are vital for data quality.
2. Compounds can be excluded/included as well as concentrations adjusted as required by the metabolomics experiment.

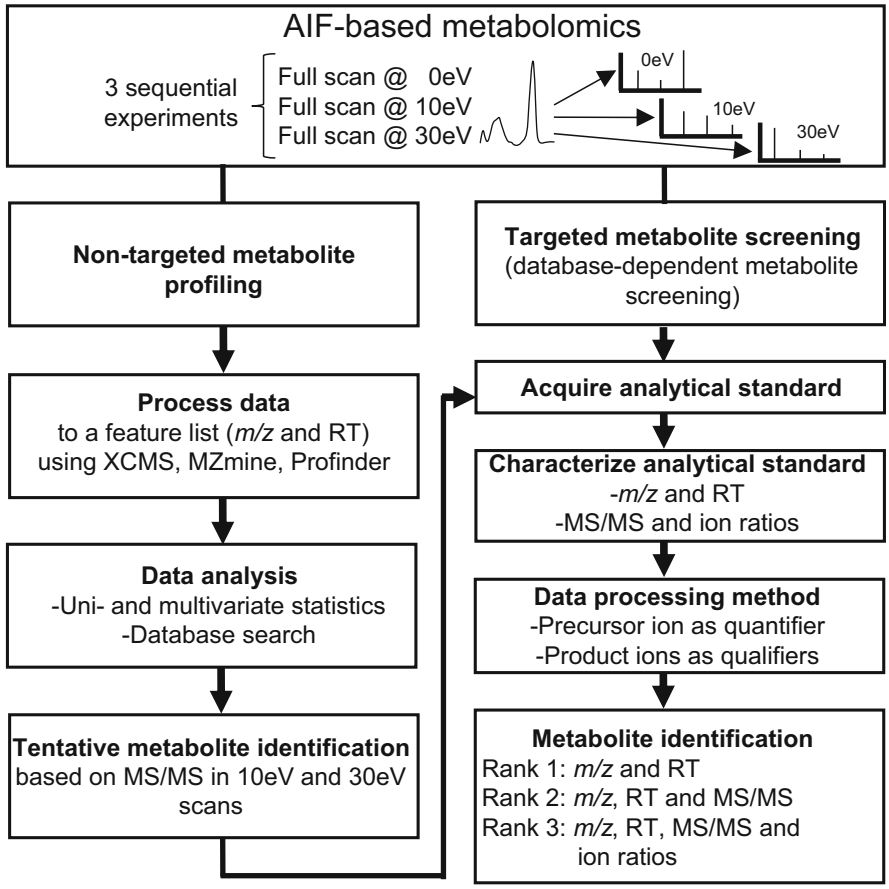


Fig. 3 Proposed workflow that can be used as a starting point from nontargeted to targeted metabolomics

3. This volume can be adjusted depending upon how many aliquots are needed (e.g., 100 μL are sufficient for four aliquots).
4. Crash solution containing standards should be stored at −80 °C until needed.
5. A maximum of six 70 μL aliquots can be obtained from 150 μL plasma or serum sample.
6. If required, the sample can be stored after this step at −80 °C.
7. Sometimes after reconstitution, a precipitate may form; avoid transferring it to the HPLC vial.
8. Wipe off the prism with lint-free tissue between measurements.
9. Create a dilution scheme by using the urine sample with highest specific gravity and subsequent 2-, 5-, 10-, and 50-fold dilutions.

10. Sometimes after adding crash solution, a precipitate (very little) may form; avoid transferring it to HPLC vial. A maximum of four 40 μL aliquots can be obtained from 20 μL urine sample.
11. Before running a new project, we recommend to clean the autosampler needle, change the in-line filter, and prepare fresh solvents.
12. For the strong needle wash, the current method uses ACN/-water ratios of 9:1(*v/v*) and 1:9 (*v/v*) for HILIC and RP chromatographic methods, respectively.
13. The draw speed for the syringe is 100 μL/min, the eject speed is 400 μL/min, and the wait time after the draw is 1–2 s.
14. For the seal wash of the pumps, use (9:1, *v/v*) water/MeOH.
15. Expected back pressure at initial conditions 65–70 bars.
16. Expected back pressure at initial conditions 365 bars.
17. Expected back pressure ~23 bars.
18. To increase sensitivity, use two instead of three alternating scans (e.g., 0 eV and 10 eV).
19. Alternatively, other metabolomics packages such as MS-DIAL [12] can be adopted to the workflow.
20. Alternative energies can be used if desired.
21. We consider compounds confirmed by ion ratio if the % relative error of ion ratio (peak area) for at least one product/product pair is <25%.
22. The parameters for data processing (mass detection, deconvolution, smoothing, etc.) are project and matrix dependent.
23. A list with a short description of online metabolomics databases is available under http://metabolomicssociety.org/resources/metabolomics-databases.

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