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Development of a plasma pseudotargeted metabolomics method based on ultra-high-performance liquid chromatography–mass spectrometry

Fujian Zheng[1,2], Xinjie Zhao[1,2], Zhongda Zeng[3], Lichao Wang[1,2], Wangjie Lv[1,2], Qingqing Wang[1,2] and Guowang Xu[1,2]✉

Untargeted methods are typically used in the detection and discovery of small organic compounds in metabolomics research, and ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) is one of the most commonly used platforms for untargeted metabolomics. Although they are non-biased and have high coverage, untargeted approaches suffer from unsatisfying repeatability and a requirement for complex data processing. Targeted metabolomics based on triple-quadrupole mass spectrometry (TQMS) could be a complementary tool because of its high sensitivity, high specificity and excellent quantification ability. However, it is usually applicable to known compounds: compounds whose identities are known and/or are expected to be present in the analyzed samples. Pseudotargeted metabolomics merges the advantages of untargeted and targeted metabolomics and can act as an alternative to the untargeted method. Here, we describe a detailed protocol of pseudotargeted metabolomics using UHPLC-TQMS. An in-depth, untargeted metabolomics experiment involving multiple UHPLC-HRMS runs with MS at different collision energies (both positive and negative) is performed using a mixture obtained using small amounts of the analyzed samples. XCMS, CAMERA and Multiple Reaction Monitoring (MRM)-Ion Pair Finder are used to find and annotate peaks and choose transitions that will be used to analyze the real samples. A set of internal standards is used to correct for variations in retention time. High coverage and high-performance quantitative analysis can be realized. The entire protocol takes ~5 d to complete and enables the simultaneously semiquantitative analysis of 800–1,300 metabolites.

Introduction

Metabolomics is a part of systems biology[1]. The concentration and variation of metabolites can offer fresh insight into disease biochemistry[2–4], toxicology[5–8], pharmacology[9,10], gene function[11,12], early disease diagnostics[13], the gut microbiome[14] and other areas[15–17]. The aim of metabolomics analysis is to comprehensively characterize the metabolites in biological samples both qualitatively and quantitatively. Because of the complexity of biological samples (serum, plasma, urine, tissue, etc.), fully characterizing all the metabolites in a sample is very difficult. The main analytical platforms used for metabolomics are ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). ^{1}H-NMR can do non-invasive analysis and enables better metabolite annotation but has lower sensitivity. Protocols on the NMR analysis of different biological samples have been published[18,19]. MS is often coupled with separation techniques such as liquid chromatography (LC), gas chromatography and capillary electrophoresis. Gas chromatography–mass spectrometry (GC-MS) is suitable for the analysis of volatile organic compounds. For non-volatile compounds, derivatization is required to increase thermal stability and volatility and reduce analyte polarity[20]. However, liquid chromatography–mass spectrometry (LC-MS) can be used to analyze thermally labile metabolites without derivatization and is thus the most frequently used platform for metabolomics analysis.

Untargeted approaches do not assume any knowledge of what compounds might be present and could theoretically detect all compounds analyzable by the method used. Targeted approaches have a starting hypothesis of what compounds are present and might change in response to a stimulus;

[1]CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China. [2]University of Chinese Academy of Sciences, Beijing, China. [3]Dalian ChemDataSolution Information Technology Co. Ltd., Dalian, China. ✉e-mail: xugw@dicp.ac.cn

the analysis is tailored to specifically look for these compounds. Untargeted methods are usually the first choice, because they provide non-biased, high coverage of the metabolome. This is important in the discovery phase where it is necessary to identify as many metabolites related to diseases or biological processes as possible. For untargeted LC-MS–based metabolomics analyses, the main MS platform is high-resolution MS (HRMS), which can be used to obtain the exact mass and tandem MS (MS^2) information of metabolites. Abundant mass spectral information enables better characterization of the global metabolome. In general, untargeted metabolomics experiments suffer from high complexity and limited repeatability and have limited linear ranges (for detection and quantitation). In addition, HRMS systems require frequent maintenance and are expensive.

Targeted metabolomics is usually used in the verification phase and can be used for absolute quantification of metabolites of interest. Multiple reaction monitoring (MRM) using triple-quadrupole mass spectrometry (TQMS), which monitors both the specific precursor ion and product ion of each metabolite, is the most frequently used technique in targeted methods because it enables high sensitivity, high specificity and excellent quantification ability[21,22]. The continuous development of TQMS in terms of the efficiency of ionization, scanning rate and other features enables the analysis of tens to hundreds of metabolites simultaneously[23,24]. Generally, targeted metabolomics requires standards (i.e., pure, well characterized versions of the compounds in which you are interested) to find out which MRM transitions to use. Targeted metabolomics is used to determine and verify the precise identity of the compounds of interest, and once this is known, the method will be used for absolute quantitation of these compounds.

The development of MS technology has enabled the detection of a large number of compounds within a targeted ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) run[25]. For the purpose of establishing an alternative to the untargeted method that has a higher sensitivity, higher specificity and excellent quantification ability, a new strategy named pseudotargeted metabolomics[26,27] has been proposed that can monitor hundreds to thousands of metabolites by dynamic MRM. Abundant metabolite information is gained from the untargeted metabolic profiling of HRMS, thereby ensuring high coverage of the metabolome, and ultimately, the detection is performed by dynamic MRM, which guarantees high data quality.

The pseudotargeted method is also suitable for the analysis of large-scale samples[28–30]. GC-MS–based pseudotargeted metabolomics by calibrating gross and systematic errors has been applied to a metabolic profiling study of 1,197 plant samples in nine batches analyzed with two instruments[29]. Similarly, the LC-MS–based pseudotargeted metabolomics method by integrating a blank-wash, a pooled quality control (QC) sample and post-calibration[28] has been used in a large-scale, multicenter serum metabolite biomarker identification study for the early detection of hepatocellular carcinoma; 1,448 participants were recruited from six clinic centers[30].

During the development of the pseudotargeted method, HRMS is used to acquire MS^2 information (the first step), and TQMS is used to perform high-coverage dynamic MRM analysis (the second step). The pseudotargeted methods are not limited to specific HRMS and TQMS instruments. For HRMS, Orbitrap and quadrupole time-of-flight (Q-TOF) have the ability to acquire abundant MS^2 spectra, and different TQMS in the dynamic MRM mode can be used in the second step. In the published articles, many combinations of HRMS and TQMS were reported, such as linear ion trap quadrupole (LTQ)-Orbitrap MS (Thermo Fisher) and QTRAP 5500 MS (AB Sciex)[31], Triple TOF 5600+ (AB Sciex) and QTRAP 5500 MS (AB Sciex)[32] and Agilent 6510 Q-TOF MS and Agilent 6460 triple-quadrupole (QQQ) MS[27]. If a sample is analyzed on multiple different instruments, the results are similar (more detail is included in Anticipated results).

Many researchers have done interesting work based on pseudotargeted metabolomics to study the biochemistry of disease[33–38], screening of biomarkers[31,39–44], plant metabolomics[32,45–49], traditional Chinese medicine[50,51], exposure to pollutants[52–56] and other topics. In addition, some researchers developed their own methods by using the concept of a pseudotargeted method[39–41,57–65]. Pseudotargeted methods have had a wide range of applications in metabolomics studies.

Development of the protocol

Our research group first introduced the concept of pseudotargeted metabolomics[26] in 2012. In that work, a GC-MS–based untargeted method was modified by incorporating an algorithm developed to select ions for selected ion monitoring from all the detected metabolites. With this method, data from both known and as yet unidentified metabolites present in the samples can be collected by using the retention time–locking GC-MS–selected ion monitoring. The pseudotargeted metabolomics method

was extended to LC-MS in 2013[27]. However, construction of the pseudotargeted metabolomics method was still time consuming and laborious, especially the process of choosing ion pairs for monitoring from thousands of candidates. To define ion pairs automatically and systematically, the in-house software[66] 'Multiple Reaction Monitoring–Ion Pair Finder (MRM-Ion Pair Finder)' was developed, which made the process of defining the MRM transitions for untargeted metabolic profiling easier and less time consuming.

Compared with information-dependent acquisition (IDA), sequential window acquisition of all theoretical fragment ion (SWATH) affords higher coverage of MS^2 information because it can, in theory, gain fragmental information on all precursor ions [67]. A method to define MRM transitions based on SWATH MS acquisition, named 'SWATHtoMRM', has also been published[41]. Our group also suggested a pseudotargeted ion-pair selection method based on UHPLC-SWATH MS[68].

Pseudotargeted metabolomics methods for urine[31], serum[27] and plant samples[69] have been developed and further extended to a high-coverage pseudotargeted lipidomics method[70]. In these applications with different research purposes, the pseudotargeted methods have shown very good performance.

Comparison with other methods

Pseudotargeted metabolomics has been thought of as the second-generation metabolomics method and merges untargeted and targeted data-acquisition strategies[25]. In an untargeted method based on HRMS, exact mass and MS^2 information are used for identification, usually generating thousands of features and hundreds of identified metabolites. Unfortunately, the quantitative performance of untargeted methods is not as good as that of targeted methods based on the MRM in TQMS. Pseudotargeted metabolomics is an alternative method to untargeted metabolomics because it has higher sensitivity and a wider dynamic range than untargeted metabolomics and does not need complex feature detection or peak alignment processes; in addition, data from pseudotargeted metabolomics from different analysis batches are easily calibrated and integrated. On the other hand, it is convenient to build a quantitative method by using authentic compounds because the pseudotargeted metabolomics is performed at MRM mode.

For typical targeted metabolomics, MRM transitions are obtained from standard compounds[23,71,72]; thus, targeted metabolomics is limited by the lack of standard compounds and is usually expensive. To improve coverage of the targeted method for the compounds without standards, the MS^2 spectral tag data library[73–75], which is typically constructed by total-scan electron spray ionization (ESI) MS^2[76], was reported. However, construction of an MS^2 spectral tag data library is complicated, and such a library contains a large amount of redundant data. Some public MRM transition repositories are available[77], but the compatibility between different systems or sample types is usually not good. The MRM transitions used in pseudotargeted metabolomics come from the biological samples to be analyzed. Pseudotargeted methods, in contrast with the above targeted methods, have higher coverage and wider applicability.

Advantages

In pseudotargeted metabolomics, quantitative analysis with both high coverage and high performance of quantitative analysis can be realized. There is no need for complex feature detection or peak-alignment processes, as in untargeted metabolomics. Moreover, data from pseudotargeted metabolomics are easily calibrated and integrated from different analysis batches in large-scale metabolomics analyses[28]. Most importantly, pseudotargeted metabolomics allows high coverage to be achieved with TQMS while retaining the original advantages of this detection technique so that laboratories with only TQMS and no HRMS can complete a conventional pipeline for high-coverage metabolomics research.

The MRM transitions in pseudotargeted metabolomics are based on the real biological samples to be analyzed; thus, more MRM transitions could be considered and included. IDA is performed at several different collision energy (CE) voltages, and abundant MS^2 information can be quickly acquired, greatly reducing the time required to optimize the TQMS parameters of each transition. XCMS and CAMERA are used to perform feature detection and annotation, and these software packages are suitable for the data formats of different manufacturers. The software MRM-Ion Pair Finder reported in 2016[3] is a systematic and automated software for acquiring characteristic MRM ion pairs by precursor ion alignment, MS^2 spectral extraction and reduction, characteristic product ion selection and ion fusion. In this protocol, MRM-Ion Pair Finder is updated to version 2.0 and reprogramed by R statistical scripting language (version 3.6.1). The codes were uploaded to GitHub

(https://github.com/zhengfj1994/MRM-Ion_Pair_Finder). The new version is more useful and is also suitable for negative ion mode.

Limitations

There are still several limitations of pseudotargeted metabolomics that need to be addressed. First, some detected metabolites are not identified because the MRM transitions come from biological samples rather than standard compounds. Identification of unknown metabolites must be performed by using UHPLC-HRMS/MS and other methods. Second, the number of metabolites that can be detected in pseudotargeted metabolomics is limited by the UHPLC resolution and HRMS and TQMS scan rate. For example, the TSQ Altis triple-quadrupole mass spectrometer (Thermo Scientific) can simultaneously detect ~100 compounds with a cycle time of 1 s and dwell time of 10 ms or ~200 compounds with a cycle time of 1 s and dwell time of 5 ms. Third, pseudotargeted metabolomics as a semiquantitative method is most useful in the discovery phase in which the absolute quantitation is not mandatory. The pseudotargeted metabolomics would form part of an overall research project that may start with untargeted metabolomics, and the findings from the pseudotargeted metabolomics can be conveniently taken to inform a targeted metabolomics study to further validate the results obtained.

Experimental design

The key factors to consider when designing pseudotargeted metabolomics experiments include (i) the sample for untargeted analysis, (ii) selection of internal standards (ISs), (iii) untargeted metabolic profiling data collection by using UHPLC-HRMS, (iv) MRM transition selection from the metabolic profiling data, (v) transformation of MRM transitions from HRMS to TQMS and (vi) validation of the pseudotargeted metabolomics method.

Pseudotargeted metabolomics methods have been developed for different body fluids[27,31] and plant samples[69], and a pseudotargeted lipidomics method has also been established[70]. In this protocol, we used a plasma sample as an example. For other samples, the procedures of sample preparation and selection of ISs should be modified based on research objectives.

Sample for untargeted analysis

To obtain comprehensive metabolite information for a given sample to be analyzed, untargeted metabolic profiling data collection by UHPLC-HRMS needs a sample that contains all the metabolites. A QC sample prepared by mixing equal quantities of the samples to be analyzed contains the most comprehensive set of metabolites and can be used to collect the metabolic profiling information via UHPLC-HRMS.

Some metabolites that exist in low concentrations in only one treatment group could be diluted to below detection limit if the QC mixture is prepared by mixing samples in all groups. To avoid this dilution effect, an equal aliquot from each treatment group can be mixed separately, and analysis of QCs from different groups can be performed separately[37]. In our routine experiment, in the sample preparation for untargeted metabolomics analysis step to extract the ion pairs, three times the volume of the QC sample as in the real sample analysis is used to obtain more information on metabolites in low concentrations. NIST Standard Reference Material for Human Plasma[78] (NIST SRM 1950) was used in this protocol so that other laboratories can repeat our experiment or directly use our MRM transitions to perform MRM analysis on UHPLC-TQMS.

Selection of ISs

In the pseudotargeted method, ISs are used for retention time calibration and peak area normalization. The selection of the ISs needs to satisfy the following conditions: (i) the retention times of the ISs should be evenly distributed in the chromatogram, (ii) ISs must not interfere with the detection of metabolites, (iii) the types of ISs should be diverse to meet the calibration needs of different metabolites and (iv) the IS must be stably detected in each sample.

It is also important that concentrations of each IS used result in a suitable response in the linear range of MS detection. Generally, the concentration of the standards should be similar to the concentration of the metabolites in the samples. The ISs must also be compounds that are not present in the sample itself, such as isotopically labelled versions of known metabolites (for example, L-phenylalanine-d_5) or compounds that do not occur naturally (for example, lyso-phosphatidylcholine (LPC) 19:0). Table 1 gives the ISs we used; different standards could be used depending on the research objectives.

Table 1 | ISs used in the pseudotargeted method

Name	Abbreviation	Volume (μl)	Concentration (μg/ml)	ESI+				ESI−			
				Retention time (min)	Precursor ion (Da)	Product ion (Da)	CE (V)	Retention time (min)	Precursor ion (Da)	Product ion (Da)	CE (V)
Cholic acid-2,2,4,4-d_4	CA-d_4	5	0.1	11.27	395.3099	359.2888	15	14.05	411.3054	347.2380	−30
Chenodeoxycholic acid-2,2,4,4-d_4	CDCA-d_4	15	0.3	13.05	379.3150	361.3044	30	15.00	395.3104	377.3784	−30
Decanoyl-L-carnitine-d_3 HCl (N-*methyl*-d_3)	Carnitine C10:0-d_3	7.5	0.15	10.14	319.2676	85.0306	30	-			
Hexadecanoyl-L-carnitine-d_3 HCl (N-*methyl*-d_3)	Carnitine C16:0-d_3	7.5	0.15	14.73	403.3615	85.0306	30	-			
Acetyl-L-carnitine-d_3 HCl (N-*methyl*-d_3)	Carnitine C2:0-d_3	7.5	0.15	0.81	207.1424	85.0306	15	-			
L-leucine-5,5,5-d_3	Leu-d_3	15	0.3	-				1.59	133.1062	133.1062	−15
Nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine	LPC19:0	30	0.6	16.76	538.3867	184.0744	30	-			
L-methionine-(*methyl*-d_3)	Met-d_3	160	3.2	1.03	153.0772	107.0420	15	1.11	151.0626	49.9290	−15
L-phenylalanine (Ring-d_5)	Phe-d_5	30	0.6	1.60	171.1176	124.9170	15	3.26	169.0320	152.0756	−15
N-lauroyl-D-erythro-sphingosylphosphorylcholine	SM (d18:1/12:0)	10	0.2	19.11	647.5122	184.0735	30	-			
L-tryptophan-d_5 (*indole*-d_5)	Trp-d_5	160	3.2	2.44	210.1285	192.0956	15	4.22	208.1139	120.0757	−15
L-valine-d_8	Val-d_8	160	3.2	1.02	126.1365	80.1250	15	0.88	124.1219	105.1550	−15
Palmitic acid-16,16,16-d_3	FFA 16:0-d_3	25	0.5	-				17.29	258.2518	258.2518	−15
Stearic acid-18,18,18-d_3	FFA 18:0-d_3	25	0.5	-				18.19	286.2831	286.2831	−15

Untargeted metabolic profiling data collection by using UHPLC-HRMS

To collect as much metabolite information as possible, untargeted metabolic profiling analysis by using UHPLC-HRMS is performed. Thousands of metabolites can be measured simultaneously. Parameter setting should follow the manufacturer's advice and be optimized for the actual demand. IDA/data-dependent acquisition (DDA) is used to obtain MS^2 information. The different CE voltages should be chosen for different metabolites because of their chemical property differences. Some metabolites will not fragment under low CE voltages, while under high CE voltages some metabolites may overly fragment to smaller product ions so that no suitable characteristic product ions can be selected. To obtain the suitable product ions for each metabolite, several different CE voltages are set in parallel LC-MS runs. The CE voltage range is chosen such that most of the metabolites have good responses. In this protocol, we set the CE voltages at 15, 30 and 45 V.

MRM transition selection from the metabolic profiling data

Next, metabolic profiling data containing MS^2 information is obtained. The objective of this step is to define the most appropriate product ions for every precursor ion. We developed software named MRM-Ion Pair Finder in 2016[66], which is a systematic and automated system for acquiring characteristic MRM ion pairs. In this protocol, MRM-Ion Pair Finder has been upgraded to version 2.0 and reprogramed by R statistical scripting language (version 3.6.1). The codes were uploaded to GitHub (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder).

Transformation of MRM transitions from HRMS to TQMS

To reduce the effect of different retention times between the different UHPLC systems of UHPLC-HRMS and UHPLC-TQMS, retention times are calibrated using ISs as described in Step 18 with the method described in ref. [79].

The TQMS parameters for each metabolite need to be optimized because the parameters of HRMS and TQMS are not the same for different instruments, especially when they are from different companies. The parameters of HRMS should be considered as references, and the instrument manufacturer's advice should also be followed. For convenience, to define optimum CE voltage of each metabolite, we recommend testing it with three injections near the recommended optimized CE value (CE of HRMS and ±5 eV); the optimal CE for each MRM transition is the voltage with the best MS response.

Validation of the pseudotargeted metabolomics method

This pseudotargeted method where the MRM transitions are derived from the real biological sample mix is robust and is not limited to a specific TQMS instrument; thus, researchers can choose their own available instrument for the experiments. However, before the established pseudotargeted method is used to carry out a metabolomics study, validation should be performed to optimize the analytical conditions. Pseudotargeted metabolomics merges untargeted and targeted methods; therefore, the analytical characteristics to optimize are similar to those of these two method types[68,80], with a focus on repeatability, stability and linearity (more detail is included in the Procedure (Step 21) and Anticipated results).

The intraday and interday repeatability is evaluated according to the calculated coefficient of variation (CV) of each metabolite analyzed in different LC-MS runs. In large-scale metabolomics, stability can be evaluated by analyzing QC samples in an analytical sequence.

The relationship between the concentration and response of each metabolite is evaluated by calculating the linearity over a 2^{10}-fold dilution series of the QC sample[27,41] because the detected metabolites in the pseudotargeted method are from biological samples, and it is impossible to get the standards of all metabolites.

Materials

Reagents

- Metabolites in Human Plasma (NIST, cat. no. NIST SRM 1950)
- Human plasma **! CAUTION** Follow all relevant ethical regulations and guidelines for the collection and use of human blood.
- HPLC-grade methanol (Merck, cat. no. 1.06007.4008) **! CAUTION** Methanol is toxic and highly flammable. Researchers should wear gloves and operate in a fume hood.
- HPLC-grade acetonitrile (Merck, cat. no. 1.00030.4008) **! CAUTION** Acetonitrile is toxic and highly flammable. Researchers should wear gloves and operate in a fume hood.

- Formic acid (J&K, cat. no. F0654) **! CAUTION** Formic acid is corrosive and volatile. Researchers should wear gloves and operate in a fume hood.
- Ammonium bicarbonate, an eluent additive for LC-MS (Fluka, cat. no. 40867-50G-F) **! CAUTION** Ammonium bicarbonate is toxic and hydroscopic. Researchers should wear gloves and operate in a fume hood.
- Acetyl-L-carnitine-d_3 HCl (N-*methyl*-d_3) (International Laboratory, cat. no. 377464)
- Decanoyl-L-carnitine-d_3 HCl (N-*methyl*-d_3) (International Laboratory, cat. no. 349681)
- Hexadecanoyl-L-carnitine-d_3 HCl (N-*methyl*-d_3) (International Laboratory, cat. no. 371939)
- L-leucine-5,5,5-d_3 (Sigma-Aldrich, cat. no. 486825)
- L-tryptophan-(*indole*-d_5) (Sigma-Aldrich, cat. no. 615862)
- L-valine-d_8 (Sigma-Aldrich, cat. no. 486027)
- L-methionine-(*methyl*-d_3) (Sigma-Aldrich, cat. no. 300616)
- Palmitic acid-16,16,16-d_3 (Sigma-Aldrich, cat. no. 615951)
- Stearic acid-18,18,18-d_3 (Sigma-Aldrich, cat. no. 490393)
- Cholic acid-2,2,4,4-d_4 (Sigma-Aldrich, cat. no. 614149)
- Chenodeoxycholic acid-2,2,4,4-d_4 (Sigma-Aldrich, cat. no. 614122)
- Choline-1,1,2,2-d_4 bromide (Sigma-Aldrich, cat. no. 615552)
- L-phenylalanine (Ring-d_5, 98% chemical purity) (Cambridge Isotope Laboratories, cat. no. DLM-1258-1)
- Nonadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-phosphatidylcholine 19:0) (Avanti, cat. no. 855776P)
- *N*-lauroyl-D-erythro-sphingosylphosphorylcholine (12:0 sphingomyelin (d18:1/12:0)) (Avanti, cat. no. 860583P)
- Ultrapure water

Equipment

- Vortex mixer (VWR, cat. no. 12620-850)
- Pipettes and tips
- Centrifuge tubes (1.5 ml; Eppendorf, cat. no. 0030120086)
- High-speed microcentrifuge (Hitachi, cat. no. CF16RN)
- Centrifugal vacuum evaporator (Labconco, cat. no. 7310031)
- UHPLC system (Waters) coupled with a Triple TOF 5600+ mass spectrometer (AB Sciex)
- LC (ExionLC AD) coupled with Triple Quad 6500 (AB Sciex)
- LC (Nexera x2)-MS (TQ8050) system (Shimadzu)
- LC system (Vanquish) coupled with a TSQ Altis triple-quadrupole mass spectrometer (Thermo Scientific)
- Acquity BEH C8 column (100 mm × 2.1 mm, 1.7 μm; Waters, cat. no. 186002878)
- Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, cat. no. 186003539)
- MSConvert (http://proteowizard.sourceforge.net/tools.shtml, provided by ProteoWizard)
- R statistical scripting language (version 3.6.1)
- Skyline[81] 19.1 (https://skyline.ms/project/home/software/Skyline/begin.view, used for processing of MRM data)

Reagent setup

IS solutions for retention-time calibration and normalization

- *IS storage solution*. Each IS is prepared as a 1-mg/ml solution in the appropriate solvent and stored in a 4 °C refrigerator.
- *IS extraction solution*. This solution is used for protein precipitation and metabolite extraction. The details are shown in Table 1, and 49.4 ml of acetonitrile is added to a mixed solution of ISs.
- *IS reconstitution solution*. This solution is used for gradient dilution to obtain a 2^{10}-fold dilution series with constant concentrations of ISs. The concentrations are the same as those for the IS extraction solution (Table 1), but 90% (vol/vol) H_2O/CH_3OH is used as the solvent ▲ **CRITICAL** All standard compound solutions are stored at 4 °C, and freshness is ensured.

90% (vol/vol) water/methanol

To prepare the 90% (vol/vol) water/methanol solution, 90 ml of ultrapure water is added to a clean glass bottle, followed by 10 ml of HPLC-grade methanol ▲ **CRITICAL** Because it contains a high proportion of water, this solvent should be prepared fresh before use.

Table 2 | Reversed-phase UHPLC-MS gradient for positive ion mode (30-min run)

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
Initial	0.35	95	5
1	0.35	95	5
24	0.35	0	100
28	0.35	0	100
28.1	0.35	95	5
30	0.35	95	5

Mobile phase solutions

These are prepared as described in ref. [82].

Mobile phase A used for positive ion mode is prepared by adding 1.0 ml of formic acid to 1,000 ml of HPLC-grade water and mixing thoroughly.

Mobile phase B used for positive ion mode is prepared by adding 1.0 ml of formic acid to 1,000 ml of HPLC-grade acetonitrile and mixing thoroughly.

Mobile phase C used for negative ion mode is prepared by dissolving 6.5 mmol of NH_4HCO_3 in 1,000 ml of HPLC-grade water.

Mobile phase D used for negative ion mode is prepared by dissolving 6.5 mmol of NH_4HCO_3 in 1,000 mL of 95% methanol and water.

Other solutions needed for UHPLC are prepared according to the manufacturer's instructions.

▲ **CRITICAL** All solutions should be prepared fresh before use, especially those containing water.

Equipment setup

UHPLC-HRMS instrument setup

Untargeted profiling is based on a Waters Acquity UHPLC system (Waters) coupled to an AB Sciex Triple Q-TOF 5600+ system (AB Sciex) in this protocol. Other HRMS instruments with IDA/DDA are also suitable.

Chromatographic separation is performed on a Waters Acquity BEH C8 column (100 mm × 2.1 mm, 1.7 μm) for positive ion mode and a Waters Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm) for negative ion mode. Mobile phases A and B are used for positive ion mode, and the column temperature is 50 °C. Mobile phases C and D are used for negative ion mode, and the column temperature is 55 °C. Gradient elution is performed for positive and negative ion mode detection, as detailed in Tables 2 and 3, respectively.

The following parameters are used for the triple TOF 5600+ mass spectrometer. The spray voltages are 5.5 kV and −4.5 kV for the positive and negative ion modes, respectively. The source temperature is 550 °C. Curtain gas, gas 1 and gas 2 are set at 35, 55, and 55 p.s.i., respectively. IDA-based auto-MS^2 was performed on the 20 most-intense metabolite ions in the cycle of a full scan. The mass-to-charge ratio (m/z) scan ranges of the precursor ions and product ions are set at 50−1250 Da. The CE voltage is set at 15, 30 and 45 V in positive ion mode. The CE voltage is set at −15, −30 and −45 V in negative ion mode. MS data are not acquired during chromatographic column equilibration.

Software for defining MRM transitions

MSConvert[83] (http://proteowizard.sourceforge.net/tools.shtml), provided by ProteoWizard, can convert the raw MS data format of some vendors into XCMS-supported data types and mgf file type.

XCMS[84] (http://www.bioconductor.org/packages/release/bioc/html/xcms.html) and CAMERA[85] (http://www.bioconductor.org/packages/release/bioc/html/CAMERA.html) are used for peak detection and peak annotation, respectively. To install the XCMS and CAMERA package, start R (version 3.6) and enter the following codes in the console:

```
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install("xcms")
BiocManager::install("CAMERA")
```

Table 3 | Reversed-phase UHPLC-MS gradient for negative ion mode (25-min run)

Time (min)	Flow rate (ml/min)	Mobile phase C (%)	Mobile phase D (%)
Initial	0.35	98	2
1	0.35	98	2
18	0.35	0	100
22	0.35	0	100
22.1	0.35	98	2
25	0.35	98	2

Box 1 | TQMS parameter setup

Triple Quad 6500 (AB Sciex)
The ionspray voltages are 5.5 and −4.5 kV for the positive and negative ion modes, respectively. The source temperature is 500 °C. Curtain gas, gas 1 and gas 2 are set at 35, 40, and 40 p.s.i., respectively.

TQ8050 (Shimadzu)
The interface temperature, desolvation line (DL) temperature and heat block temperature are set at 300, 250 and 400 °C, respectively. The nebulizing gas flow, heating gas flow and drying gas flow are 3, 10 and 10 l/min.

TSQ Altis (Thermo Scientific)
ESI spray voltage in positive ion mode is 3,500 V, and negative ion mode is 2,500 V. The ion transfer tube temperature and vaporizer temperature are 325 and 350 °C, respectively. Sheath gas, auxiliary (aux) gas and sweep gas are set at 50, 10, and 1 Arb, respectively.

A function named 'MRM_Ion_Pair_Finder' written by R (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder/tree/master/R) is used for defining MRM transitions.

UHPLC-TQMS instrument setup

To prove pseudotargeted metabolomics methods can be used in different TQMS systems, we used three TQMS instruments including Triple Quad 6500 (AB Sciex), TQ8050 (Shimadzu) and TSQ Altis (Thermo Scientific). The experimental conditions for LC separation are the same as those for UHPLC-HRMS described above. The detailed MS parameters are shown in Box 1.

Procedure

Plasma/serum QC sample preparation for defining MRM transitions ● Timing ~4 h

▲ **CRITICAL** To be simple and comparable in different laboratories, we have described the procedure for analysis of the commercially available plasma NIST SRM 1950. To prepare your own samples, follow Steps 1 and 2.

1 Store samples to be analyzed at −80 °C or in liquid nitrogen. When you are ready to analyze the plasma/serum samples, thaw on ice at 4 °C for 30–60 min.

2 Take 10–25 µl from each sample and separately mix them to produce QCs of different groups. The volume of each QC should be ≤200 µl.

■ **PAUSE POINT** For the large-scale cohort metabolomics study with more than hundreds of samples, if the sample number is too many, QC can be prepared by taking the representative samples from each group. In the following step, 200 µl of QC is used; the remaining volume can be stored at −80 °C for future use (e.g., in identification and method validation).

3 Add 800 µl of the IS extraction solution (4 °C) into 200 µl of plasma/serum sample for protein precipitation[66].

4 Thoroughly mix on a vortex mixer for 60 s.

5 Centrifuge for 10 min at 15,000*g* and 4 °C.

6 Transfer 900 µl of the supernatant to a centrifuge tube.

7 Lyophilize the supernatant in a centrifugal vacuum evaporator at 4 °C.

■ **PAUSE POINT** The lyophilized sample can be stored at −80 °C for up to several months.

8 Reconstitute the sample in 60 µl of 90% (vol/vol) H_2O/CH_3OH, vortex for 60 s and centrifuge for 10 min at 15,000*g* and 4 °C.
▲ **CRITICAL STEP** Take three times the volume of the QC sample as in the sample analysis to detect more metabolites.

9 Transfer the supernatant to a threaded screw–neck vial containing an insert for a large open vial. Place the vial in an autosampler operating at 6–8 °C.

UHPLC-HRMS analysis ● Timing 30 or 25 min per analysis

10 Inject 5 µl of the reconstituted sample onto a UHPLC-HRMS with IDA mode. Six independent analyses with different CE voltages (15, 30 and 45 V in positive ion mode and −15, −30 and −45 V in negative ion mode are recommended) are performed.
▲ **CRITICAL STEP** The range of CE voltages should roughly cover the optimal CE voltages of metabolites.
? TROUBLESHOOTING

11 Clean the UHPLC-HRMS following the manufacturer's instructions.

12 Archive the data.
■ **PAUSE POINT** The analyzed sample should be sterilized and handled at Biosafety Level (BSL) 2. The data can be analyzed at any time.

Definition of MRM transitions ● Timing ~5 h

13 Convert the raw UHPLC-HRMS data to XCMS-supported data type and mgf files using MSConvert.
▲ **CRITICAL STEP** The mgf files should be saved in a separate folder, and their file names should contain the value of the CE voltage without other numbers.

14 Open R statistical scripting language (version 3.6.1), invoke XCMS and then perform codes in the console to do peak detection.

15 Invoke CAMERA and annotate features that come from XCMS. First, create an xsAnnotate object, and then group features according to retention time. Finally, annotate isotopes and adducts. For information on how to do peak detection and annotation, refer to https://github.com/zhengfj1994/MRM-Ion_Pair_Finder for more help.

16 Remove redundant features. Output the result to a comma-separated values (csv) file. The recommended format of the csv file can be seen in Supplementary Table 1. The referential codes are provided in GitHub (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder).

17 Use 'MRM_Ion_Pair_Finder' to define MRM transitions. A list of defined MRM transitions is obtained (Supplementary Table 2). Based on the retention time and *m/z* information given in Table 1, the IS peaks in Supplementary Table 2 are defined.
▲ **CRITICAL STEP** The parameters of 'MRM_Ion_Pair_Finder' can significantly alter the number of MRM transitions. Each parameter setting should be carefully considered according to the quality of data collected. The information about parameters can be found at https://github.com/zhengfj1994/MRM-Ion_Pair_Finder?
? TROUBLESHOOTING

Retention-time calibration and CE voltage optimization for UHPLC-TQMS ● Timing ~10 h

18 *Retention-time calibration*. Inject ~5–10 µl of IS reconstitution solution onto the UHPLC-TQMS system to obtain the retention time of each IS under the chromatographic separation gradients given in Table 2 (positive ion mode) or Table 3 (negative ion mode). Perform retention-time calibration with the method described in Box 2 and Fig. 1. The retention times of ISs in UHPLC-HRMS are obtained from Step 16. We have provided a ready-made function in R statistical scripting language for retention-time calibration, and it has also been uploaded to GitHub (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder).
▲ **CRITICAL STEP** Each MRM transition is detected in a retention-time window. Retention-time drift should be calibrated to avoid false negatives.

19 *CE voltage optimization*. From UHPLC-HRMS analysis, the optimized CE has been suggested. For plasma/serum sample analysis, the CE value of HRMS can be directly used if TQMS and HRMS are from the same manufacturer because of the similar collision cell used. Otherwise, three injections are performed near the recommended optimized CE value (CE of HRMS and ±5 eV) to select the CE value with better response for UHPLC-TQMS analysis.

Box 2 | Retention-time calibration

Retention-time (tr) calibration was used to correct for differences between retention times of metabolites recorded on different instrument systems[79]. Here, the calibration from UHPLC-HRMS to UHPLC-TQMS is taken as an example. Figure 1 shows a schematic of the retention-time calibration. Suppose metabolite 'i' is eluted between ISs 1 and 2, the retention-time differences of these two ISs between the UHPLC-HRMS (^{HR}) and UHPLC-TQMS (^{TQ}) runs are calculated,

$$\Delta tr_1 = tr_1^{HR} - tr_1^{TQ} \& \Delta tr_2 = tr_2^{HR} - tr_2^{TQ} \quad (1)$$

Then, the UHPLC-TQMS retention time for metabolite 'i' could be calculated by Eq. 2 and Eq. 3.

$$\Delta tr_i = \Delta tr_1 + \frac{(\Delta tr_2 - \Delta tr_1)(tr_i^{HR} - tr_1^{HR})}{(tr_2^{HR} - tr_1^{HR})} \quad (2)$$

$$tr_i^{TQ} = tr_i^{HR} - \Delta tr_i \quad (3)$$

If retention time of metabolite 'j' is smaller than the first eluted IS or bigger than the last eluted IS, only one IS is used in the retention-time calibration. For example:

$$\Delta tr_{nearest\_standard} = tr_{nearest\_standard}^{HR} - tr_{nearest\_standard}^{TQ} \quad (4)$$

$$tr_j^{TQ} = tr_j^{HR} - \Delta tr_{nearest\_standard} \quad (5)$$

The method for batch retention time correction has been provided, and the codes have been uploaded to GitHub (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder/R).

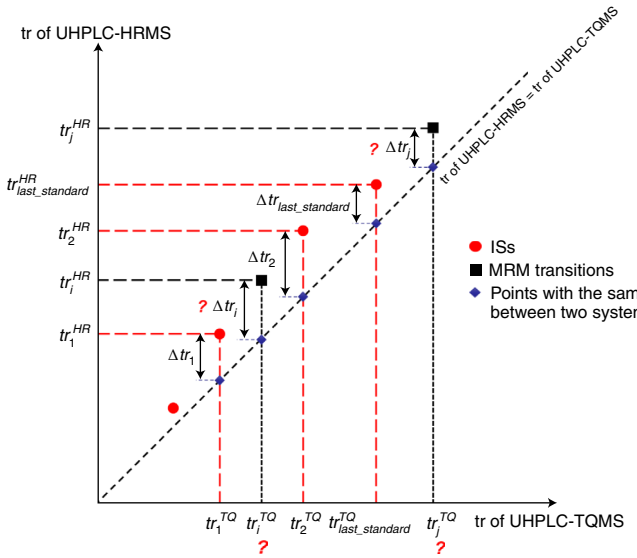

Fig. 1 | Schematic of the retention-time calibration method. The variables are defined in Box 2. ^{HR}, UHPLC-HRMS; ^{TQ}, UHPLC-TQMS; *tr*, retention time.

20 Delete undetected MRM transitions to ensure that the remaining MRM transitions have longer dwell times for optimal detection sensitivity.
? TROUBLESHOOTING

Evaluation of the quantitative performance of the pseudotargeted metabolomics method

● **Timing ~3 d**

21 Verify the analytical characteristics of the established pseudotargeted method, including the linearity, repeatability and stability. Detect the MRM transitions confirmed in Step 20 using the chromatographic separation gradient given in Table 2 (positive ion mode) or Table 3 (negative ion mode) and the detailed MS parameters of TQMS shown in Box 1. To evaluate linearity for

pseudotargeted metabolomics, choose option A. To evaluate repeatability, choose option B. Perform the steps in option C if you are evaluating stability.

(A) **Linearity**

(i) Add 880 µl of acetonitrile (4 °C) to 220 µl of QC sample prepared in Step 2.

(ii) Thoroughly mix on a vortex mixer for 60 s.

(iii) Centrifuge for 10 min at 15,000*g* and 4 °C.

(iv) Transfer 1,000 µl of the supernatant to a centrifuge tube.

(v) Lyophilize the supernatant in a centrifugal vacuum evaporator at 4 °C.

(vi) Reconstitute the sample in 200 µl of the IS reconstitution solution, vortex for 60 s and centrifuge for 10 min at 15,000*g* and 4 °C.

(vii) Transfer the supernatant to a centrifuge tube.

(viii) Perform gradient dilution with the IS reconstitution solution to obtain a 2^{10}-fold dilution series (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024).

▲ **CRITICAL STEP** Use the IS reconstitution solution as the diluting agent to ensure the same IS concentration in the 2^{10}-fold dilution series.

(ix) Transfer the 2^{10}-fold dilution series to threaded screw–neck vials containing an insert for large open vials and place the vials in the autosampler operating at 6–8 °C.

(x) Inject 5 µl of each sample onto the UHPLC-TQMS system. Analyze samples from low to high concentration and repeat three times.

(xi) Calculate the R^2 of each MRM transition. If >60% of the metabolites have an R^2 >0.95, and >80% of the metabolites have an R^2 >0.8, the results are considered to be adequate. If the proportion of transitions that behave linearly is lower than the 'cut-off' for non-linearity, step A should be done again. Also see Table 4.

? TROUBLESHOOTING

(B) **Repeatability**

(i) Add 200 µl of the IS extraction solution (4 °C) to 50 µl of the QC samples for protein precipitation.

(ii) Thoroughly mix on a vortex mixer for 60 s.

(iii) Centrifuge for 10 min at 15,000*g* and 4 °C.

(iv) Transfer 200 µl of the supernatant to a centrifuge tube.

(v) Lyophilize the supernatant in a centrifugal vacuum evaporator at 4 °C.

(vi) Reconstitute the sample in 50 µl of 90% (vol/vol) H_2O/CH_3OH, vortex for 60 s and centrifuge for 10 min at 15,000*g* and 4 °C. To reduce the chance that the metabolites will change while waiting in the autosampler, lyophilized samples should be stored at −80 °C and reconstituted only on the day they are to be analyzed.

(vii) Transfer the solution to threaded screw–neck vials containing an insert for large open vials and place the vials in the autosampler operating at 6–8 °C.

(viii) Inject 5 µl of the sample onto the UHPLC-TQMS system, repeat 10 injections within a day and repeat on three consecutive days.

(ix) Calculate the CV to evaluate the intraday and interday repeatability. More than 90% and 80% of metabolites with a CV value of <30% are considered to be adequate for intraday repeatability and interday repeatability, respectively.

? TROUBLESHOOTING

(C) **Stability**

Stability is evaluated by calculating the change in QC samples over the course of running a large-scale metabolomics analytical sequence.

(i) The plasma and QC sample pretreatment is the same as that in 21B(i)–(vi).

(ii) QC samples are injected at the start of the analytical batch[86], and one QC sample is injected at every 5th to 15th injection.

(iii) The QC samples in an analytical batch are used to validate the stability of the pseudotargeted metabolomics method.

? TROUBLESHOOTING

Application in large-scale metabolomics research ● Timing Depending on the number of analyzed samples

22 *Plasma/serum sample preparation*. Prepare the samples as described in Steps 1–8.

23 *Pseudotargeted metabolomics analysis based on UHPLC-TQMS*. Use the chromatographic separation gradients given in Table 2 (positive ion mode) or Table 3 (negative ion mode) and

the detailed MS parameters of TQMS shown in Box 1. At the start of each batch, 10 QC samples are run for instrument balance. Insert a QC in every 5–15 injections for signal drift calibration.

24 Archive the data.

PAUSE POINT The analyzed sample should be sterilized and handled at BSL2. The data can be analyzed later.

25 Extract peak areas using the software provided by instrument producers (MultiQuant from AB Sciex, TraceFinder from Thermo Fisher and LabSolutions from Shimadzu) or free software (Skyline).

26 Remove metabolites that are detectable in <80% of the samples in each sample group and metabolites with CV values >30% in QC samples after peak area standardization by internal standards.

27 The pre-processed pseudotargeted metabolomics peak table can be used to do statistical analysis including multivariate (principal component analysis (PCA), partial least squares–discriminant analysis (PLS-DA)) and univariate analysis (*P* value and false discovery rate (FDR)).

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Step	Problem	Possible reason	Solution
10	Low MS response	MS source contamination	Clean the MS source
17	Error when running 'MRM_Ion_Pair_Finder'	Incorrect MS^1 or MS^2 file	Check the files of MS^1 and MS2. MS^1 file should be a csv file. MS^2 folder should contain only mgf files
	Wrong retention time or *m/z*	Incorrect MS^1 format	The correct MS^1 format is shown in Supplementary Table 1
	Wrong CE voltages	Incorrect MS^2 file name	The MS^2 file should be named with CE voltage, and no other numbers should exist in the file name
20	Few detected MRM transitions	Retention-time drift	This is an indication of problems with the retention-time calibration in Step 16. Check the retention time of the IS, and recalibrate the retention time using the correct method
		Small dwell time	Use a longer cycle time and narrower detection window, reduce the number of MRM transitions to be detected or delete undetected MRM transitions and rerun
21A	Poor linearity of most metabolites	2^{10}-fold dilution series preparation errors	Reconstitute solutions with correct concentrations
		Inappropriate concentrations of 2^{10}-fold dilution series	Change the concentrations and repeat the experiment
		MS source contamination	Clean the MS source
21B	Poor interday repeatability	MS source contamination	Clean the MS source
		Batch interruption	Normalization using ISs
21C	Poor stability in the analytical sequence	MS source contamination	Clean the MS source
		The concentration of injected samples is too high	Dilute samples or reduce the injection volume. During the analytical sequence, clean the MS instrumentation at set intervals

Timing

Steps 1–9, plasma/serum QC sample preparation for defining MRM transitions: ~4 h
Steps 10–12, UHPLC-HRMS analysis: 30 or 25 min per analysis
Steps 13–17, definition of MRM transitions: ~5 h
Steps 18–20, retention-time calibration and CE voltage optimization for UHPLC-TQMS: ~10 h
Step 21, evaluation of the quantitative performance of the pseudotargeted metabolomics method: ~3 d
Steps 22–27, application in large-scale metabolomics research: depends on the number of analyzed samples

Anticipated results

Figure 2 shows an overview of the pseudotargeted metabolomics method development described in this protocol. 'MRM_Ion_Pair_Finder' can automatically define MRM transitions from MS/MS data,

and the codes were uploaded to GitHub (https://github.com/zhengfj1994/MRM-Ion_Pair_Finder). The codes for peak detection, peak annotation and retention-time calibration are also available on that website.

In this protocol, 1,490 and 984 MRM transitions in positive and negative ion mode, respectively, were obtained from NIST SRM 1950 via untargeted metabolic profiling with a triple TOF 5600+ (AB Sciex) system. Supplementary Tables 3 and 4 present the information on these metabolites. For plasma/serum pseudotargeted metabolomics MRM analysis, these MRM transitions can be used directly, skipping Steps 1–17.

To validate that MRM transitions from HRMS are suitable for different TQMS systems, Triple Quad 6500 (AB Sciex), TQ 8050 (Shimadzu) and TSQ Altis (Thermo Scientific) were used to perform validation of MRM transitions, respectively. MRM transitions verified by three mass spectrometers are highly coincident (Fig. 3). TSQ Altis (Thermo Scientific) was further used for evaluating the quantitative performance of the pseudotargeted metabolomics method. 880 and 552 metabolites were detected in NIST SRM 1950 in positive and negative ion modes, respectively. The linearity, repeatability and stability of pseudotargeted metabolomics with TSQ Altis were evaluated. To evaluate the linearity of the MRM transitions, R^2 values were calculated for the 874 metabolites in positive ion mode and the 552 metabolites in negative ion mode over the entire 2^{10}-fold dilution series of the NIST SRM 1950. The percentage of metabolites with R^2 values >0.95 was 85% and 62% in positive and negative ion modes, respectively (Fig. 4a and Supplementary Fig. 1a).

Repeatability was evaluated by calculating the CV of the repeated injections on the same day and on different days. In positive ion mode, after 10 injections on 1 d, 88.3% (737) of the metabolites, accounting for 98.8% of the total peak area, had an relative standard deviation (RSD) <15% (Fig. 4b). After repeated injections on three consecutive days, 76.6% (642) of the metabolites, accounting for 97.2% of the total peak area, had RSD values <15% (Fig. 4c). These results indicated that the established pseudotargeted method is stable and can be used in large-scale metabolomics analyses. Similarly, the repeatability in the negative ion mode can be seen in Supplementary Fig. 1b,c.

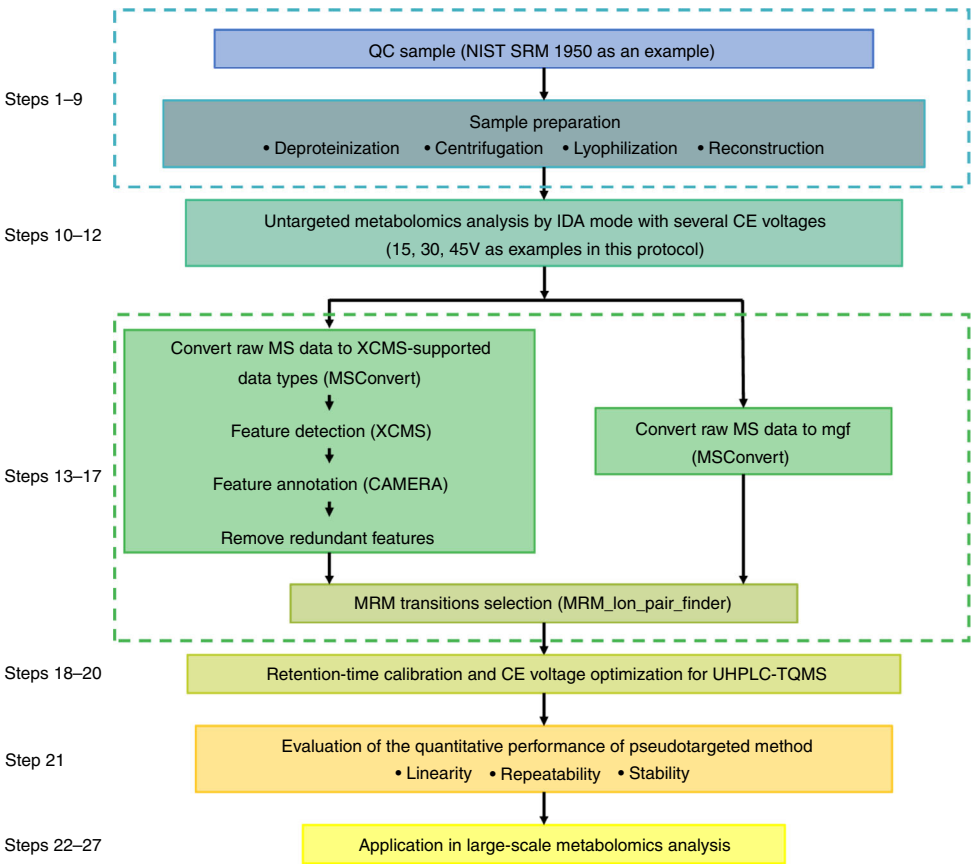

Fig. 2 | Overview of the pseudotargeted metabolomics method development.

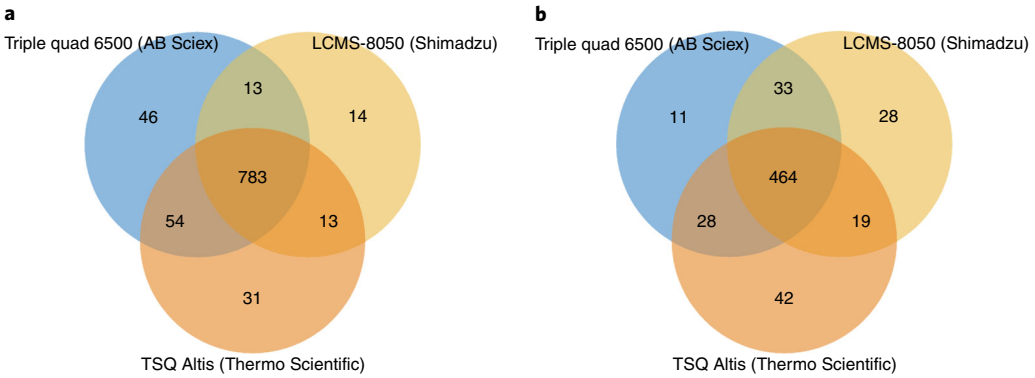

Fig. 3 | MRM transitions verified by different TQMS. a, Positive ion mode. **b**, Negative ion mode.

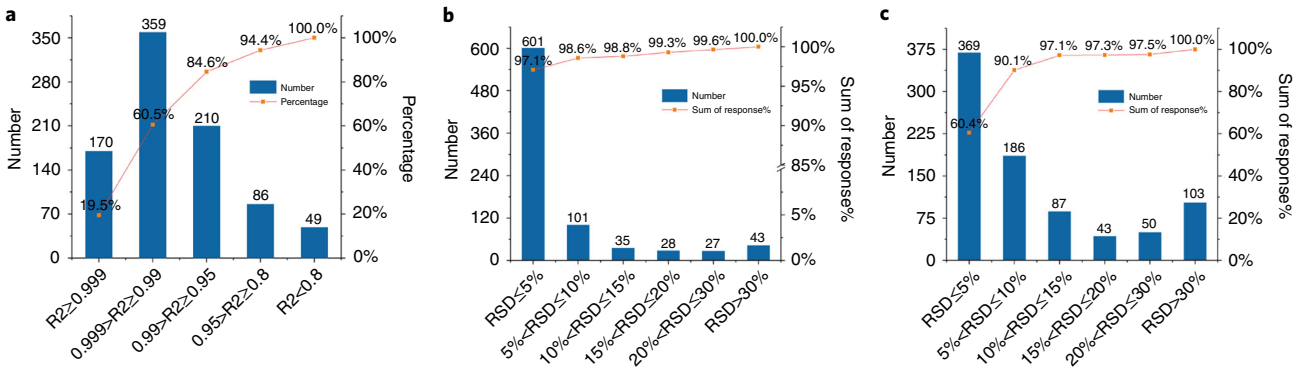

Fig. 4 | Quantitative performance of the pseudotargeted metabolomics method. The 'number' on the y-axis is the number of transitions that have the calculated validation measure; this corresponds approximately to the number of metabolites. **a**, Linearity of all MRM transitions in positive ion mode. Percentage, the percentage of the metabolite number with R^2 greater than the specific value to the total metabolite number. **b**, Intraday repeatability in positive ion mode evaluated from 10 injections in 1 d. Sum of response%, the percentage of the metabolite peak area with CV less than the specific value to the total peak area. **c**, Interday repeatability in positive ion mode evaluated from 10 replicates per day on three consecutive days.

As an application of the established pseudotargeted method, a childhood obesity metabolomics study, which was approved by the ethics committee of China Medical University and conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was performed. Children were classified as normal weight, overweight or obese according to their body mass index, and each group contained 30 children. The basic information is listed in Supplementary Table 5. QC samples were included in the analytical batch to evaluate the stability of the established pseudotargeted method. The distribution of values for the 'first component' in the PCA analysis of the QC samples in positive ion mode and negative ion mode is shown in Supplementary Fig. 2 panels a and b, respectively. These data show that the variation in the QC samples was within 2 s.d., indicating that the analytical sequence was stable[87].

On the basis of data-quality evaluation, multivariate and univariate analysis will be carried out. First, PCA is tried to know the whole difference in metabolic profiling. Then, PLS-DA is performed to establish the supervised classification model and define the differential metabolites (significantly changed compounds) between groups. The results of PLS-DA are shown in Supplementary Fig. 3a,b for positive ion mode. Univariate analysis usually includes *P* value and FDR, and SPSS and Matlab can be used to calculate these values of metabolites, respectively. Metabolites with a *P* value <0.05 and FDR <0.05 were chosen as significantly changed compounds between obese/overweight and normal-weight children. In total, 126 and 41 differential metabolites were defined between obese and normal-weight children and between overweight and normal-weight children, respectively. Similarly, the PLS-DA score plot and corresponding permutation test for negative ion mode can be found in Supplementary Fig. 3c.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Author contributions

G.X. and X.Z. developed the concept, designed experiments and optimized the manuscript. F.Z. performed the experiments, analyzed the data and wrote the manuscript. Z.Z. provided the software named 'MRM-Ion Pair Finder', and F.Z. modified this software. L.W., W.L. and Q.W. gave technical support and conceptual advice.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to G.X.

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Key data used in this protocol

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Corresponding author(s): Guowang Xu

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Data collection	UHPLC-HRMS vendor software which details was described in manuscript
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Sample size	For the application of pseudotargeted method, childhood obesity samples were used which were classified as normal-weight, overweight or obese according to their body mass index (BMI), and each group contained 30 samples
Data exclusions	No exclusions
Replication	No replication
Randomization	Samples are randomly analyzed in the UHPLC-TQMS
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☒	☐ MRI-based neuroimaging

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Policy information about studies involving human research participants

Population characteristics	For childhood obesity study, participants were healthy students aged 14 to 16 years with body mass indexes (BMIs) ranging between 14.6 and 41.9 kg/m2. Each group has 18 males and 12 females.
Recruitment	The participants were recruited from Bacui high school of Shenyang and they signed informed consent form.
Ethics oversight	The study was approved by the ethics committee of China Medical University

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