

A column-switching two-dimensional liquid chromatography - high resolution/triple quadrupole dual mass spectrometry system for simultaneously untargeted metabolome and targeted exposome analyses

Guowang Xu

xugw@dicp.ac.cn

Dalian Institute of Chemical Physics, Chinese Academy of Sciences <https://orcid.org/0000-0003-4298-3554>

Yuting Wang

Dalian Institute of Chemical Physics, Chinese Academy of Sciences

Penglong Cao

The First Affiliated Hospital of Dalian Medical University

Pengwei Guan

Dalian Institute of Chemical Physics, Chinese Academy of Sciences

Tiantian Chen

Dalian Institute of Chemical Physics, Chinese Academy of Sciences

Xingyu Guo

Dalian Institute of Chemical Physics, Chinese Academy of Sciences

Xianzhe Shi

Dalian Institute of Chemical Physics, Chinese Academy of Sciences <https://orcid.org/0000-0001-9306-0130>

Xinyu Liu

Dalian Institute of Chemical Physics, Chinese Academy of Sciences

Article

Keywords: Two-dimensional liquid chromatography, Dual mass spectrometry, metabolome exposome-wide association study, Hyperlipidemia, Inflammatory bowel disease

Posted Date: February 3rd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-5820559/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: There is **NO** Competing Interest.

Version of Record: A version of this preprint was published at Nature Communications on August 14th, 2026. See the published version at <https://doi.org/10.1038/s41467-026-76696-w>.

Abstract

Metabolome-exposome association studies (mEWAS) demands high-coverage and high-sensitivity analytical methods for metabolites and exposures with diverse chemical properties and concentrations. Here, we present an integrated column-switching two-dimensional liquid chromatography (2DLC) - high resolution/triple quadrupole dual mass spectrometry (MS) method, achieving simultaneously untargeted metabolome and targeted exposome analyses including 598 chemical exposures in a single run. Polar and non-polar compounds with log P ranging from -8 to 15 were separated without unretained compounds in the dead time. Benchmarked against biological matrices, analyte coverage increased by 35-104% over one-dimensional LC method. Highly sensitive limits of detection with 0.001-1 $\mu\text{g/mL}$ for >90% metabolites and 0.001-1 ng/mL for >90% chemical exposures were obtained, matching the concentration difference between the metabolome and exposome in human blood. The method was demonstrated in patients with chronic diseases, showing the feasibility of establishing simultaneously untargeted metabolome and targeted exposome analytical methods for mEWAS by integrating 2DLC-dual MS techniques.

Introduction

Exposome study has revealed that environmental exposures are key contributors to most chronic noncommunicable diseases including cancer^{1, 2}, immune-mediated disease^{3, 4}, cardiometabolic diseases^{5, 6}, reproductive health issues^{7, 8}, and metabolic syndrome^{9, 10}. Especially due to the increasing incidence of metabolic diseases such as type 2 diabetes, hyperlipidemia, and fatty disease worldwide, the role of environmental exposures has garnered more attention⁹⁻¹³. Metabolite-exposome association studies (mEWAS) combine metabolomics and exposomes to further explore the relationship between environmental exposures and human health or disease states to identify the environmental drivers of disease. A certain chemical space overlap exists between metabolome and exposome, as both metabolic profiling and chemical exposures studies concentrate on the small molecule subsets (molecular weights ≤ 1500 Da) present in living systems, albeit with different research focuses^{14, 15}. With the development of untargeted approaches based on liquid chromatography (LC) - high-resolution mass spectrometry (HRMS), the analytical distinctions between metabolomics and chemical exposomics are further blurred. However, this does not mean the effective measurement of both the endogenous metabolome and the internal chemical exposome can be achieved simultaneously¹⁶.

Non-targeted methods are widely used in metabolomics research¹⁷, whereas most analytical methods employed to measure chemical exposures are piecemeal targeted methods tailored for different groups of specific exposures (e.g., insecticides¹⁸, flame retardants¹⁹, plasticizers²⁰, and per- and polyfluoroalkyl substances²¹), due to the requirement for high-sensitivity biomonitoring and obtaining reliable quantitative data²². A major proportion of mEWAS studies is to determine endogenous metabolites and exogenous chemical exposures by multiple independent analytical methods^{11, 23}. Although this multi-analysis strategy can partially satisfy the desire to explore the association between quantitative

exposure and adverse health outcomes, it also brings about notable problems including prolonged experiments, lowered analysis efficiency, and greater biological sample usage. Recently, an increasing number of studies are attempting to perform joint endogenous-exogenous analyses in a single run, such as simultaneous feature extraction of metabolites and exposures in a single untargeted analysis^{24, 25}, albeit with potential sacrifices in sensitivity and quantification performance of exposures, as the concentrations of exposures are 1–3 orders of magnitude lower than those of metabolites²⁶. Furthermore, Warth et al. reported a method that achieves both targeted (Zeno MRM-HR) and untargeted (SWATH) LC-HRMS analyses in one analytical run, enhancing the sensitivity of exposure detection by 2.1 to 2.3 times²⁷.

High-coverage analysis of the human metabolome and exposome is essential as metabolites, drugs and pollutant residues are numerous and exhibit a variety of physical and chemical properties²⁸. To expand analytical coverage, a notable trend is combining complementary chromatographic techniques, such as integrating gas chromatography (GC) with LC²⁹, or coupling reversed-phase (RP) with hydrophilic interaction liquid chromatography (HILIC) within LC³⁰. For example, the usefulness of a dual-column approach with RP and a HILIC column being rapidly operated in parallel to cover polar and nonpolar compounds was described by Flasch et al., providing new idea for integrating high-coverage and high-throughput of joint endogenous-exogenous analyses³¹. However, single-column one-dimensional (1D) analysis, whether based on HILIC or RPLC, cannot avoid the loss of compounds eluting close to the dead time. The inherent advantage of two-dimensional (2D) LC methods is the wider separation window producing higher coverage and separation selectivity achieved by combining different separation mechanisms³². Our previous work established a 2DLC metabolomics analysis method based on HILIC and RPLC column switching, which can reduce unretained analytes and effectively expand the log P coverage of analyzable metabolites³³. The method's design leads to a significantly higher utilization rate of the HILIC module than RPLC. To our knowledge, no column-switching 2DLC-based approach has been reported for mEWAS analysis.

It is still a challenge to achieve high coverage and high sensitivity analysis of endogenous metabolites and exogenous chemical exposures with widely different physicochemical properties and concentrations in a single run³⁴. A new analytical technology that can be used for mEWAS research is urgently needed. Here, we present an automatic HILIC/RP column-switching 2DLC coupled with high resolution (HR)/triple quadrupole (TQ) dual mass spectrometry (MS) system to perform simultaneously untargeted metabolome and targeted exposome analysis. The whole workflow for the study is shown in Fig. 1. Firstly, a column-switching 2DLC system was developed and optimized based on standards of metabolites and chemical exposures with different polarity for complementary analysis of polar and non-polar compounds in biological samples (blood, tissue, urine etc.). Secondly, simultaneously untargeted metabolome and targeted exposome analysis was performed by integrating column-switching 2DLC and dual MS system. The coverage and sensitivity of the method were evaluated across three common biological matrices and a highly diverse panel of more than 800 analytes including endogenous metabolites and xenobiotics, to demonstrate the power of the new approach. Finally, the applicability of

the method to real samples was demonstrated by analyzing exemplary serum samples from patients with hyperlipidemia and inflammatory bowel disease (IBD).

Results

Design and optimization of the 2DLC-HRMS/TQMS system for high-coverage analysis

In this study, a column switching 2DLC system combining HILIC and RP separation was developed for comprehensive analysis of polar and non-polar compounds in a fully automatic single-injection mode, avoiding the loss of non-retained compounds at the dead-time in 1DLC system. Firstly, weakly polar and non-polar compounds eluted nearly at dead time from HILIC column were pre-separated from polar ones and transferred onto a trapping column (**Supplementary Fig. 1a**). Subsequently, polar compounds were separated on HILIC column (**Supplementary Fig. 1b**). Finally, trapped compounds were backflushed to RP column and analyzed (**Supplementary Fig. S1c**). The key to achieve the aforementioned analysis is to ensure the weakly polar and non-polar fractions are effectively cut from the polar fractions and retained on the trapping column. Therefore, the fraction cutting time and trapping column selection of the column switching 2DLC system are to be optimized.

Optimization of fraction cutting time of pre-separation. In order to extend the retention coverage of compounds across a range of log P values in a 2DLC system, two complementary chromatographic systems were employed: an Amine-column-based HILIC system for polar compounds, and a C18-column-based RP system for weakly polar and non-polar compounds. The retention and separation performances of the HILIC and RPLC system were tested using 221 standards with log P ranging from – 6 to 8. Compounds with a log P below 2 were well separated on HILIC, while those with a log P above 2 were eluted near the dead time, indicating stronger retention of the polar compounds with log P less than 2 on HILIC. Conversely, weak polar compounds with a log P value above 2 were not optimally retained on the HILIC system (**Supplementary Fig. 2a**). A good separation of the compounds with a log P above 0 was obtained on RPLC, indicating the stronger retention of the weak polar compounds with log P more than 0 on RPLC (**Supplementary Fig. 2b**). Thus, the log P range of the boundary compound of the two fractions, which was the last compound eluted to trapping column, was narrowed to between 0 to 2.

To more intuitively compare the separation of standards on HILIC and RPLC, the extracted ion chromatogram of each compound was demanded to satisfy three conditions: (i) a better MS response with the peak intensity exceeding $1e5$, (ii) avoid multiple peaks or broad peak with full width at half maximum 0.3 min, (iii) a retention factor greater than 1. The results of all 221 standards are summarized in Fig. 2a, where green, yellow, red and black colors indicate satisfaction of three conditions, two conditions, one condition and no peak detection, respectively, meaning good, acceptable, unacceptable, and unacceptable. In detail, polar compounds with log P values from – 6 to 1 were better separated on HILIC and the non-polar compounds with log P values from 1 to 8 were well separated on RPLC. This distinction was particularly evident in carnitines (Cars) with different numbers of carbon atom, where

those with eight or more carbon atoms exhibited multiple peaks on HILIC and require transfer to RPLC for separation. Finally, Car C8:0 (log P = 1.1) was selected as the boundary of fraction cutting. When Car C8:0 was completely eluted from the HILIC, the pre-separation was stopped immediately, and the valve was switched to polar compound separation position. Consequently, polar compounds with log P value below and above 1.1 were separated on HILIC and RPLC respectively during one analysis process.

On the other hand, the effect of the water proportion in organic phase of HILIC on fraction cutting performance was evaluated within the range of 3–15% (Fig. 2b). Using Car C8:0 as the fraction cutting boundary, pre-separation effect was compared under isocratic elution conditions with organic phases containing varying water proportions. As shown in Fig. 2b, maintaining a low water proportion in the organic phase improved resolution of pre-separation, but increased pre-separation time, which may cause more compounds on the trapping column to waste. Conversely, a rapid pre-separation process was obtained under a high-water proportion in the organic phase, but resulted in insufficient separation of different polar compounds. To prevent the loss of the fraction on trapping column due to prolonged pre-separation and reduce the occurrence of split peaks from fraction cutting, a 10% water proportion in the organic phase was finally used for pre-separation on HILIC. The cutting time was set at 3.2 min, corresponding to the elution end time of Car C8:0 on HILIC. Therefore, Car C8:0 and other weak polar and non-polar compounds eluted before 3.2 min were transferred into RPLC, Car C6:0 and other polar compounds remained on HILIC with data acquisition starting at 3.2 min (Fig. 2c, d). Mixed standard solution of Car C6:0 and Car C8:0 was used to monitor the stability of fraction cutting throughout the entire experimental cycle.

Selection of trapping column. Ensuring that the unretained components can be captured on the trapping column with the lowest loss was crucial for the integration of the RPLC system and the HILIC system. Therefore, the retention of weak polar compounds was further evaluated by using 136 weak polar exposures (log P from 0 to 2) on four trapping columns with different packing materials and sizes, including PFP, C18, T3 and C8. At pre-separation position in **Supplementary Fig. 1a**, retention times of weakly polar compounds from injection to elution on four trapping columns were compared under isocratic elution conditions consisting of acetonitrile containing 10% water at 0.15 mL/min and pure water at 1 mL/min. More weak polar compounds were eluted from PFP and C18 after the fraction cutting time, than from T3 and C8, suggesting that PFP and C18 have enhanced capture properties for these compounds compared to T3 and C8 (Fig. 2e). Although the retention time of weak polar compounds from PFP were generally longer than from C18, more lost compounds were observed on PFP than C18, this may be due to the properties of different stationary phases (Fig. 2f). Consequently, taking both the retention ability and the capture coverage into account, a 3.5 cm C18 column was chosen as the trapping column to retain weak polar and non-polar compounds unretained in the pre-separation on HILIC.

Separation performance. The automatic HILIC/RP column-switching 2DLC system was established under optimized conditions of solvent composition, fraction cutting and trapping. It can be seen in **Supplementary Fig. 3a** that an excellent separation of compounds with different polarities was achieved

by integrating HILIC and C18 system in a single analysis, with no compounds eluted at the dead time. Compounds that eluted near dead time in IDLC analysis of HILIC were transferred to RPLC column in the 2DLC system, and detected after 11 min (**Supplementary Fig. 3b**). Similarly, unretained compounds in 1DLC-RP system were retained on the HILIC column of the 2DLC system and eluted at 3.2–10 minutes (**Supplementary Fig. 3c**). Thus, the analytical coverage of metabolites and exposures across a range of log P values based on this column-switching 2D-LC system can be significantly extended.

Development and evaluation of metabolome-exposome analysis method based on 2DLC-HRMS/TQMS system

Method development and validation results. The analytical method of simultaneously untargeted metabolome and targeted exposome was established based on an automatic HILIC/RP column-switching 2DLC coupled with HRMS/TQMS system which is connected with a 3-way valve. Polar and nonpolar metabolites, respectively, from HILIC and RPLC were detected via full-scan HRMS acquisition and identified using database searches, the exposures from HILIC and RPLC were detected by TQMS. The targeted exposome analytical method was optimized using individual standards containing 341 pesticides, 184 drugs, and 73 pollutants (Fig. 3a). In total 484 and 114 of the targeted exposomes were analyzed in positive ion mode and negative ion mode, respectively. Except for a few exposures with a single MRM transition, most of targets have one qualitative and one quantitative MRM transition (Fig. 3b). The optimized MRM transitions and parameters are detailed in **Supplementary Data 4**. TIC of serum QC obtained by 2DLC-HRMS is presented in Fig. 3c, d in the positive and negative ion mode. EIC of 598 mixed standard solution collected by 2DLC-TQMS is given in Fig. 3e, f.

Analytical performance of the method was evaluated as described in the supplementary information using different representative standards selected from different classes of metabolites and exposures. Overall, excellent linearity, sensitivity, accuracy, extraction recoveries and precision were obtained from both untargeted metabolome and targeted chemical exposure analysis, which are in details described in **Supplementary Results 1 and 2**.

Compounds annotation coverage comparison of 2DLC-HRMS/TQMS and 1DLC-HRMS analysis for three biological matrices. Untargeted metabolome analysis was traditionally performed on a 1DLC platform with HILIC³⁵ or RP^{36, 37} separation columns. The performance of untargeted metabolome analysis by our 2DLC-HRMS/TQMS system was evaluated and compared with 1DLC (HILIC and RPLC) system across three matrixes: serum, tissue and urine. Compounds from three matrices were respectively annotated utilizing both internal and public databases. To enhance the reliability of annotations reliant on public databases, only those with a signal-to-noise ratio exceeding 30 were considered. As shown in Fig. 4a, over 1,000 compounds were annotated in each matrix, with tissues displaying the richest profile, nearing 1,300 compounds. Compared with HILIC and RPLC system, the 2DLC system-based non-targeted analysis annotated the greatest number of compounds at both level 1 and level 2 across all matrices, covering a log P range from – 8 to 15, with no unretained compounds

eluted at the dead time. In the serum and tissue, a higher number of compounds were annotated based on the RPLC system compared to HILIC system due to the higher abundance of non-polar lipids. Conversely, the HILIC system-based method annotated more compounds in urine, which has a richer variety of polar compounds. Furthermore, more compounds eluted at the dead time on the 1DLC-RPLC than on 1DLC-HILIC system, suggesting a higher proportion of polar compounds in the extracts of three matrices, which was attributable to the properties of the extractant and solvent used in this study. Notably, the HILIC system's advantage in polar compound analysis was less pronounced than reported in other studies, speculated reason was higher noise levels in HILIC system led to the exclusion of more level 2 annotations with signal-to-noise ratios below 30.

A detailed comparison of the compound annotation results between 2DLC-based and 1DLC-based approaches was performed across different matrices. In serum, 986 compounds were annotated (level 1 and level 2) by 2DLC method, compared to 652 by RPLC and 483 by HILIC, showing a 51–104% increase in annotation coverage. There were 581 compounds annotated in both RPLC and 2DLC systems, and 436 compounds in both HILIC and 2DLC systems (Fig. 4b). Over 90% of analytes lost in 1DLC system were detected by 2DLC system. Among them, most compounds lost in the 1D-RPLC system, were polar compounds with a log P value less than 2 and detected before 9.4 minutes in the HILIC module of 2DLC system. Similarly, compounds lost on 1D-HILIC system were mainly weakly polar and non-polar compounds with log P greater than 0, and were detected after 9.4 minutes in the RPLC module of 2DLC system (Fig. 4c). In tissues, 1,204, 689, and 663 compounds were annotated by 2DLC, RPLC, and HILIC systems, respectively, with the 2DLC-based method increasing annotated coverage by 75–82% over RPLC and HILIC-based methods (**Supplementary Fig. 5a**). In urine, 1,006, 523, and 653 compounds were annotated by 2DLC, RPLC, with the 2DLC-based method increasing coverage by 35–82% compared to RPLC and HILIC-based methods (**Supplementary Fig. 5c**). Similar to serum, in tissue and urine, majority of polar compounds not retained on 1D RPLC were recovered in the HILIC module, whereas the lost weakly polar and non-polar compounds were captured on the RPLC module of the 2DLC system (**Supplementary Fig. 5b, d**). Consequently, by integrating two orthogonal chromatography systems, complementary analysis of compounds with different polarities was achieved on a HILIC module and a RPLC module based on a column switching 2DLC platform, capitalizing on the analytical advantages of HILIC and RPLC system while avoiding the compound loss caused by non-retention in single-dimensional separation mode, significantly improving the coverage of metabolome and exposome analysis and extending the separation window.

About 1–6% of compounds were lost in the 2DLC analysis compared with 1D RPLC + 1D HILIC, mainly because the inevitable peak broadening in 2DLC caused a slight decrease in detection sensitivity (Fig. 4d, **Supplementary Fig. 5e, g**), and some weak polar compounds were not retained in the HILIC or RP capture columns (Fig. 4e, **Supplementary Fig. 5f, h**). In addition, the elution time of the 2DLC method (100% B, 3 min) was shorter than that of the RPLC method, resulting in the loss of a few lipids in the tissue (**Supplementary Fig. 5f**). In the future, it is expected that the loss rate will be reduced by trying other capture columns with better performance to capture weakly polar compounds.

Comparison of LOD distribution of metabolites and exposures analyzed by 2DLC-HRMS/TQMS and 1DLC-HRMS. In this study, a dual MS system was constructed based on fractionation technology, directing fractions to HRMS for non-targeted metabolomics and TQMS for targeted exposure quantitation, respectively. The detection performance of the dual MS system was evaluated by comparing the LODs of 304 metabolites and 574 exposures between the optimized dual MS system and conventional HRMS system. For non-targeted metabolome analysis, the dual MS system employed the same MS platform and parameter settings as traditional HRMS. The LODs of the 304 metabolites were obtained ranging from 10^{-3} to $1 \mu\text{g/mL}$ (Fig. 4F). For exposures, the LODs based on conventional HRMS ranged from 10^{-4} to $10^{-1} \mu\text{g/mL}$, approximately an order of magnitude lower than metabolites, attributed to the higher ionization efficiency of exposures. In the dual MS system, exposures were targeted analyzed by TQMS, with LOD ranging from 10^{-5} to $10^{-3} \mu\text{g/mL}$, which was 1 to 2 orders of magnitude lower than traditional HRMS. Citing Rappaport et al ²⁶, concentration of pollutants in the human body was typically 1,000 times lower than metabolites. Based on our developed dual MS system for exposure detection with TQMS the LOD was 2 to 3 orders of magnitude lower than that of metabolite analysis with HRMS, achieving a sensitivity matching the concentration levels of metabolites and exposures in the human body. Overall, the 2DLC-HR/TQMS-based analysis method developed in this study made use of highly sensitive TQMS to enhance the responses of ultratrace exposures in complex matrices to a level comparable to those of metabolites detected by HRMS, providing a highly sensitive customized analytical method for human mEWAS research.

Demonstration application of the developed 2DLC-HRMS/TQMS method in the metabolite and exposure profiling of chronic disease cohorts

The developed HILIC/RPLC column switching 2DLC-HRMS/TQMS method for simultaneously untargeted metabolome and targeted exposome analysis was applied to explore the association between serum metabolic profiling in chronic disease patients and chemical residue exposures. Hyperlipidemia was widely reported to be associated with chemical residue exposures and was selected as one of the demonstration targets of this study. Additionally, inflammatory bowel disease (IBD) was also included and discussed in **Supplementary Results 4** considering its rising incidence and treatment difficulties in recent years.

Metabolite annotation and chemical residues detection results of chronic disease samples. In this study, a total of 79 serum samples from chronic disease patients were analyzed by the HILIC/RPLC column switching 2DLC-HRMS/TQMS method. In the non-targeted metabolic analysis, after removing the missing values based on 80% rule, 756 metabolites with an RSD of less than 30% were retained (RSDs of 46 xenobiotics were not calculated), covering 299 lipids and other various metabolites such as amino acids, nucleotides, carboxylic acids, dipeptides and so on (Fig. 5a). Detailed information and annotation levels of these metabolites are provided in **Supplementary Result 3 and Supplementary Data 8**.

In the targeted exposure analysis, 146 of the 598 targeted exposures monitored were detected (**Supplementary Data 9**). Among these exposures, 129 were accurately quantified because their signal intensities exceeded the limit of quantification, and 44 were high frequently detected with a total detection rate of more than 30% (Fig. 5b). Detailed information on the detection frequency and concentration levels of these chemical residues is summarized in Supplementary Table S8. 53 pesticides, 50 drugs, and 26 pollutants were included in the 129 quantified exposures. As shown in Fig. 5c, most pesticides and drugs were detected with low frequency rates, while pollutants were generally detected in most samples. Notably, the detection frequencies for 4-Nitro-3-cresol, Androstenedione, 9CI-PF3ONS, Cyclamic acid, Dazomet, Fipronil, Isopyrazam, Nicotine, PFHpA, PFHxS, PFOA, and PFUdA were 100%. Chemical residues detected in chronic disease samples were quantified ranging from 0.002 to 172.456 ng/mL. Among the 44 high-frequency quantitative chemical residues including 16 pesticides, 8 drugs and 20 pollutants, Isoruron, PFOS, PFOA, and Cyclamic acid were detected with the highest mean concentrations at 44.5 ng/mL, 16.3 ng/mL, 15.3 ng/mL, and 11.6 ng/mL, respectively (Fig. 5d).

Based on the positive list from targeted exposure quantitation obtained in serum samples, peak extraction of exposures was also performed in the untargeted analysis based on HRMS to verify the sensitivity of the dual MS system matching the analyte concentration. In untargeted acquisition data, 70.9% of all positive features obtained by targeted exposure quantitation could not be detected, and 16.9% were extracted with low responses less than 500,000, resulting in too few scanning points for stable quantification (Fig. 5e). Only 12.2% positive features were detected with high response. Even after the exclusion of exposure-positive features below the LLOQ, the detectability of the remaining targeted quantitative features in the untargeted analysis was not significantly improved, with 69% positive features remaining undetectable and 18.1% positive features still exhibiting responses below 500,000 (Fig. 5f). A further comparison of the detectability and concentration relationship of 7 high-frequency quantitative exposures was carried out between targeted and non-targeted analysis, exposures below 1 ng/mL in the sample were difficult to obtain in non-targeted analysis (Fig. 5g). While in the targeted analysis, exposures with concentrations two orders of magnitude lower than the LOD of the nontargeted analysis were still quantifiable. In summary, by applying the column switching 2DLC-HRMS/TQMS method to actual samples for untargeted metabolome analysis and targeted exposure analysis, a total of 756 annotated metabolites and 129 quantitative exposures were obtained, further demonstrating the high coverage and sensitivity of the dual MS system for ultratrace exposures and trace metabolites in complex samples.

Metabolome exposome-wide association study and risk analysis of hyperlipidemia. In the validation of the applicability of the column switching 2DLC-HRMS/TQMS method in mEWAS research, 18 serum samples from hyperlipidemia patients were analyzed to explore the correlation mechanism between chemical exposure and hyperlipidemia risk at the metabolic level. The research involved: identifying disease markers through metabolomics association studies, discovering exposure markers via exposomics association studies, and employing the meet-in-the-middle and mediation effect analysis to

identify key metabolite markers and related metabolic pathways that mediate the relationship between chemical exposure and hyperlipidemia risk.

PCA score plot of multivariate analysis revealed metabolic abnormalities in hyperlipidemia patients compared to control (**Supplementary Fig. 6b**). A total of 39 differential metabolites were further identified by nonparametric tests ($p < 0.05$), with 27 increased and 12 decreased in hyperlipidemia (**Supplementary Fig. 6c and Supplementary Data 10**). A binary logistic regression model was employed, revealing 22 metabolites associated with the risk of hyperlipidemia (**Supplementary Fig. 6d**). Among these, abnormal serum lipid metabolism is a direct manifestation of hyperlipidemia with general increase of PCs, phosphatidyl ethanolamine (PE), PIs and sphingolipids (SM), which were also reported by other serum lipid profiling of hyperlipidemia patients³⁸. Carnitine was elevated in the hyperlipidemia group, suggesting possible disturbance of energy metabolism. Unsaturated fatty acids were significantly reduced in the hyperlipidemia group, which has been repeatedly demonstrated in mice fed a high-fat diet, suggesting an increase in peroxidation and oxidative stress^{39–41}. Additionally, abnormalities were also observed in amino acid metabolism, including increasing derivatives of lysine and serine and decrease of serine in the hyperlipidemia group. The decrease in serine was related to the acetyl-CoA pathway disorder⁴², while the increase in lysine derivatives has not been reported.

In the association analysis between exposure and the risk of hyperlipidemia, 13 frequently detected exposures were significantly correlated with at least one clinical diagnostic parameter of hyperlipidemia by Spearman correlation analysis (Fig. 6a), including Dazomet, Fipronil-sulfide, Isopyrazam, Rabenzazole, Thiachlopid, Terbutcarb, Mono(2-ethyl-5-oxohexyl)phthalate (5-Oxo-MEHP), Bis[2-(perfluorohexyl)ethyl] Phosphate (6:2 diPAP), Avobenzene, 2,6-Di-tert-butyl-4-hydroxymethylphenol (BHT alcohol), 2,4-Diethylthioxanthone (DETX), Diethyltoluamide, and Perfluoroheptanesulfonic acid (PFHps). Binary logistic regression was further used to estimate the odds ratio of high-frequency exposures for the risk of hyperlipidemia, showing that Fipronil-sulfide, 5-Oxo-MEHP, and PFHps were associated with an increased risk of hyperlipidemia (Fig. 6b). The effects of PFHps and 5-Oxo-MEHP on the increased risk of hyperlipidemia have also been presented by other studies^{12, 29}. 6:2 diPAP was found to cause lipid metabolism disorders in zebrafish, suggesting a potential risk of hyperlipidemia⁴³. Fipronil-sulfide is a metabolite of Fipronil, and the risk effects of fipronil and its metabolite fipronil sulfoxide on hyperlipidemia have been reported¹¹.

To investigate exposure-related metabolic changes and their association with the risk of hyperlipidemia, a meet-in-the-middle approach was used to identify potential effect markers and evaluate the mediating effects of risk factor-mediated effect markers. After Spearman correlation analysis, 15 effect markers were obtained, most of which were positively correlated with exposure and associated with an increased risk of hyperlipidemia (Fig. 6c). 11 metabolites with mediating effects are summarized in **Supplementary Data 11**, among them (Fig. 6d), PFHps was positively correlated with most lipids, including fatty acids, PCs and PIs, suggesting that lipids are key metabolites between PFHps exposure and hyperlipidemia, especially PC 36:6 with a significant mediating effect of 58% in this association. This may be related to

the fact that binding to phospholipids is an important way to drive the high accumulation of perfluorinated compounds in cells ^{44, 45}. In addition, as a polycyclic aromatic hydrocarbon (PAH), 5-Oxo-MEHP has also been found to be positively correlated with most lipid metabolism abnormalities, with mediating ratio of NAE 17:0, LPI 20:4, PC 36:6, and FA 20:0 derivatives in the association between 5-Oxo-MEHP and the risk of hyperlipidemia ranges from 19–34%. Exposure to PAHs was reported significantly associated with abnormal electrocardiogram parameters, and was significantly related to dyslipidemia and inflammatory response, suggesting the possible pathogenesis mechanism of lipid-mediated PAHs and hyperlipidemia ^{46, 47}.

Discussion

In recent years, establishing a connection between environment exposure and disease metabolism has emerged as a novel approach to investigate the relationship between human health, disease states, and environmental factors. To address the complex data requirements in mEWAS, a variety of independent measurement techniques were employed to obtain comprehensively annotated metabolomes and accurately quantified exposomes, which is time-consuming and labor-intensive, and has hindered the rapid development of mEWAS research to a certain extent. In this work, we developed an integrated analytical method based on column switching 2DLC and dual MS for simultaneously non-targeted metabolome and targeted exposome analysis. This method facilitated the simultaneous analysis of polar and non-polar metabolites and exposures by tandem HILIC and RPLC system and showed a significant improvement in the coverage of annotated metabolites in all three matrices compared to the 1DLC-based method. Through the dual MS system, metabolite detection and exposure quantification with concentration difference by 2–3 orders of magnitude were simultaneously achieved for the first time in a single analysis. Finally, non-targeted metabolome analysis and 598 chemical exposure quantitation were simultaneously carried out in a single LC-MS run. It is currently the most integrated analytical method with the largest scale, highest coverage, and best sensitivity matching in LC-MS-based metabolomics and exposomics research. Demonstrative applications were performed in hyperlipidemia and IBD populations and revealed preliminary correlations between metabolites and chemical exposures. Ultimately, the potential impact between chemical exposures and disease metabolome was successfully derived directly from a single injection in an unprecedented way. In order to fully understand the environmental factors associated with complex diseases, it is necessary to monitor more highly diverse endogenous and exogenous compounds, accurately quantify more ultra-trace exposures, and analyze population cohort containing tens of thousands of samples. Therefore, the improved analytical coverage, sensitivity and efficiency of this method provide technical support for expanding the scale of mEWAS research for drawing reliable conclusions about the impact of environmental factors on disease development.

The 2DLC-TQ/HRMS method also plays an important role in independent metabolomics and exposomics studies. High coverage of non-targeted metabolome profiling and targeted quantification of key disease biomarkers can be integrated into a single analytical method, significantly improving analysis efficiency.

In the new definition of exposomes, the scope of health research was expanded, presenting the need for a comprehensive, discovery-oriented approach to identify environmental determinants on human health, rather than multiple piecemeal, hypothesis-driven approaches, and productiveness and integrity of exposomics research program were further emphasized⁴⁸. Although the exposure quantitation method used was still a hypothesis-driven targeted approach, it covers up to 600 common chemical exposures and is customizable for the analysis of other polar and non-polar exposures, maximizing the productivity of the hypothesis-driven approach and the completeness of relevant exposure studies. Additionally, with the improvement of sensitivity of HRMS, unknown exposures can be discovered in non-targeted analysis and supplemented into the study of environmental effects on health, thus achieving discovery-driven full-exposome research. However, the shortcomings of this method cannot be ignored, including the loss of some medium-polar compounds due to the inability to be retained on both the HILIC and trapping columns. Furthermore, it is unfortunate that the long distance between the two MS platforms resulted in significant chromatographic peak broadening in our study.

Methods

Chemicals and Reagents

HPLC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (HPLC-grade, > 98%), ammonium formate and ammonium bicarbonate were purchased from Sigma-Aldrich. Mill-Q water (Millipore, Billerica, MA, USA) was used to prepare mobile phases. Metabolite standards were purchased from Sigma-Aldrich (Saint Louis, Missouri, U.S.A.) and Avanti Polar Lipids (Alabaster, AL, USA), chemical standards were purchased from J&K Scientific (Beijing, China), ANPEL Laboratory Technologies (Shanghai, China) and ALTA Scientific (Tianjin, China). All standards ranged in purity from 90–99.9%. The metabolite and chemical internal standards used in the experiments were purchased from multiple companies and summarized in **Supplementary Data 1** and **Supplementary Data 2**, respectively.

Sample collection and preparation

Serum samples were collected from 79 patients with chronic diseases at Dalian Physical Examination Center (Dalian, China) for this study, including 18 with hyperlipidemia, 27 with ulcerative colitis (UC), 11 with Crohn's disease (CD) and 19 healthy controls. The remaining 4 samples were only utilized for chemical residue detection due to the lack of essential classification information. Approval was obtained from the ethics committee of The First Affiliated Hospital of Dalian Medical University (PJ-KS-KY-2024-404). Clinical information of chronic diseases was summarized in **Supplementary Table 1**. Sample pretreatment steps were as follows: 100 μ L serum was pipetted into 1.5 mL Eppendorf tube, 300 μ L methanol with internal standard and 100 μ L pure methanol were added to precipitate protein. After vortexed 60 s to complete liquid-liquid extraction, the mixture was centrifuged at 18,700 g, 4°C for 15 min. 400 μ L supernatant was directly transferred to low temperature freeze concentrator (JM CV600,

Beijing, China) for freeze-drying at vacuum condition. Finally, the dried mixture was stored at -80°C and reconstituted in 50 µL methanol/water (50:50, v/v) before analysis. The injection volume was 5 µL.

Analysis of metabolites and chemical exposures using 2DLC-HR/TQMS and 1DLC-HRMS

The column-switching 2DLC system was an upgraded version of our reported design for complementary analysis of polar and non-polar compounds in complex matrixes³³. As shown in **Supplementary Fig. 1**, in the initial position 1 (pre-separation position, **Supplementary Fig. 1a**), the sample was injected and polar compounds were retained on HILIC column. Unretained hydrophobic compounds were mixed with the aqueous diluent by pump P5 through the mixer and then captured on the trapping column. After the pre-separation of polar and non-polar compounds, the system was switched to position 2 (polar compound separation, **Supplementary Fig. 1b**). P5 was stopped and the polar compounds were eluted from HILIC to MS detector. During positions 1 and 2, the RPLC column was continuously equilibrated. Next, after the polar compound analysis was completed, the system was switched to position 3 (non-polar compound separation, **Supplementary Fig. 1c**), and the non-polar compounds captured on the trapping column were backflushed and transferred to RPLC column for further separation. Simultaneously, the HILIC column is constantly equilibrated. Finally, all valves were switched to their initial positions before the next analysis.

The column-switching 2DLC system was equipped with an SIL-30A autosampler (Shimadzu, Kyoto, Japan), five solvent delivery pumps, P1 to P4 were LC-30AD (Shimadzu) and P5 was LC-20AD (Shimadzu), three different columns (for details see below), two electronically controlled 6-port2-position valve (Valco, Houston, USA), and two system controllers (CBM-20Alite, Shimadzu). The dual mass spectrometer system consisted of a HRMS and a TQMS: Q Exactive HF System (Thermo Fisher, Waltham, MA, U.S.A.) for untargeted metabolome and QTRAP™ 6500 + system (AB SCIEX, Framingham, MA, USA) for targeted exposome data acquisitions. XBridge BEH C18 (5 µm, 2.1 mm × 30 mm), Acquity BEH Amide (1.7 µm, 2.1 mm × 100 mm) and Acquity BEH C18 (1.7 µm, 2.1 mm × 100 mm) column were used as the trapping column, HILIC and RPLC for unretained compounds capturing, polar compounds separation and non-polar compounds separation, respectively. Mobile phases A1 and B1 of HILIC column were water and 10% water in acetonitrile both containing 10 mM ammonium formate and 0.125% formic acid. Mobile phases A2 and B2 of RPLC column were water and methanol both containing 5 mM ammonium formate and 0.1% formic acid in positive ion mode, and water and 5% water in methanol both containing 6.5 mM ammonium bicarbonate in negative ion mode. Elution steps of separation by 2DLC system were summarized in **Supplementary Data 3**. The entire analysis process was carried out at an oven temperature of 50°C and lasted for 25 minutes.

Analysis of metabolites and chemical exposures using 1DLC-HRMS

Two 1DLC separation based on HILIC and RPLC system were performed on an ACQUITY UPLC (Waters, Milford, MA) and an Q Exactive HF System (Thermo Fisher, Waltham, MA, U.S.A.) respectively to compare with the developed 2DLC method.

Referring to the metabolome profiling method reported by Blaženović et al.⁴⁹, the 1DLC method based on HILIC system was conducted on an Acquity BEH Amide column (2.1 × 150 mm, 1.7 µm, Waters, Milford, U.S.A.) at 45°C oven temperature. The mobile phases A and B were water and 10% water in acetonitrile both containing 10 mM ammonium formate and 0.125% formic acid, respectively. The elution gradient started with 100% B and held for 2 min, then decreased linearly to 70% B from 2 min to 7.7 min, and continued decreased linearly to 40% B from 7.7 min to 9.5 min, and rapidly decreased linearly to 30% B from 9.5 min to 10.25 min for washing, and finally increased linearly back up to 100% B from 12.75 min to 17 min. One single analysis required 17 minutes to be completed. The flow rate was consistently maintained at 0.4 mL/min throughout the entire analysis cycle.

The 1DLC method based on RPLC system was modified with a total cycle time of 25 min from the method reported from our previous work³³. Acquity BEH C8 (1.7 µm, 2.1 mm × 100 mm) and Acquity HSS T3 (1.8 µm, 2.1 mm × 100 mm) column were respectively used for 1D-RPLC analysis in positive and negative ion mode at 50°C oven temperature. For positive ion mode, the mobile phases A1 and B1 were water and acetonitrile both containing 5 mM ammonium formate and 0.1% formic acid. The elution gradient started with 5%B and held for 1 min, then increased linearly to 100% B from 1 min to 18 min, and held for 4 min, and rapidly decreased back up to 5%B and held for 3 min. For negative ion mode, the mobile phases A2 and B2 were water and 5% water in methanol both containing 6.5 mM ammonium bicarbonate. The elution gradient started with 2%B1 and increased follow the same process with positive ion mode, finally decreased back up to 2%B2 and held for 3 min. The flow rate was set as 0.35 mL/min in both positive and negative ion mode.

MS Acquisition

High resolution mass spectrometry analysis of metabolites was achieved on Q Exactive HF System (Thermo Fisher, Waltham, MA, U.S.A.) with electrospray ionization (ESI) source. Parameters of data acquisition by three strategies for both positive and negative ion mode were set up as follows: (1) Full scan and data dependent acquisition (ddMS2) were used to generate MS2 fragment of metabolite standards for compounds identification in untargeted metabolome analysis. (2) Full scan and DDA with inclusion list containing ions were used to acquire MS2 information in QCs. All features in the full scan were divided into multiple lists and added to the DDA method, each list containing 300–500 precursor ions. (3) A single full scan mode was used for metabolome analysis of practical serum samples. Full scan was performed with m/z ranging from 85 to 1250 on resolution of 120,000. AGC target and maximum IT were set as 3×10⁶ and 100 ms. In MS2 acquisition, ddMS2 was performed with resolution 60,000, AGC target and maximum IT were set as 1×10⁵ and 50 ms. The top 10 abundant ions were selected for ddMS2, and the normalized collision energy was set at 15%, 30%, and 45%. Isolation window was set to 1 m/z. The top 10 abundant ions were selected for dd-MS2 acquisition. Inclusion lists were

added and set to on. The spray voltage in positive and negative ion modes was 3.5 kV and 3.0 kV, respectively, the temperature of the ion transfer tube aux gas was 320°C and 350°C. Sheath gas and aux gas flow rates were set at 45 and 10 (in arbitrary units). A QTRAPTM 6500 + mass spectrometer (AB SCIEX, Framingham, MA, USA) was used for targeted exposures detection. Ionization of targeted analytes was performed by electrospray ionization (ESI) in positive and negative switching mode. Declustering potential (DP) and collision energy (CE) were optimized for each chemical exposure. Detail information is presented in **Supplementary Data 4**.

Metabolite annotation

After obtaining the metabolic profile data, metabolite annotation was performed by utilizing accurate mass, retention time and MS² fragments through database matching combined with subsequent manual verification to ensure the reliability of identified metabolites. Multiple databases constructed based on experimental MS² information were used for metabolite annotation, including the laboratory's in-house database, MassBank of North America (MONA) (<https://mona.fiehnlab.ucdavis.edu/>), NIST mass spectral library (<https://chemdata.nist.gov/>), and LipidBlast (<https://fiehnlab.ucdavis.edu/projects/LipidBlast>). The specific parameter settings were as follows:

(1) Metabolite annotation based on in-house database: Metabolite annotation was performed using accurate mass, retention time and MS² fragments, which were obtained from in-house database by analyzing the standards on 2DLC-HRMS platform. A custom code program was developed for targeted database extraction, which was set with a tolerance of 10 ppm for MS¹ and MS², a MS² similarity score threshold of 0.6. Retention times matching was set with a tolerance of 18 seconds after being calibrated using internal standards.

(2) Metabolite annotation based on public database: Since retention time was not available in public databases, only accurate mass and MS² fragmentation were used for targeted database extraction by MSDIAL 4⁵⁰ for metabolite annotation. The tolerance of MS¹ and MS² was set at 0.01 Da and 0.02 Da, respectively, and the cutoff for identification score was set to 60%. The annotation result was defined as Level 2.

Annotation result based on in-house database and public database were respectively defined as Level 1 and Level 2. For each feature with multiple annotation outcomes, result with higher annotation level or score was selected. To guarantee the precision of our annotations, all identified compounds were manually checked based on structure-retention time relationship and characteristic fragments.

Method validation

Analytical performances of the 2DLC-HRMS/TQMS simultaneously untargeted metabolome and targeted exposome analysis method were evaluated using representative metabolite and exposure standards.

For untargeted metabolome analysis validation, 21 serum-spiked isotopically labeled metabolites were used for evaluating linearity, lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ),

accuracy, and extraction recovery. Serum quality control (QC) samples spiked with 21 concentration levels across 6 orders of magnitude were prepared for the calculation of linear range, LLOQ and ULOQ, and conducting internal calibration curves. Six replicates of spiked QC samples at low, medium and high concentration levels were used for the test of accuracy. The extraction recovery at low, medium and high concentration levels was investigated by calculating the ratio of the area of representative metabolites spiked before and after extraction ($n = 4$), respectively. In addition, all features of serum QCs without spiking were detected in 6 replicates samples within a single day to determine intra-day precision. Furthermore, a total of 18 replicates were analyzed over three consecutive days to calculate the inter-day precision.

For targeted exposome analysis validation of the 2DLC-HR/TQMS method, 574 of all 598 targeted exposures were spiked into fetal bovine serum to evaluate LOD, precision and extraction recovery. Fetal bovine serum samples spiked with 11 concentration levels of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL were prepared for the determination of the LOD. Six replicates of spiked fetal bovine serum samples at 0.5, 5 and 50 ng/mL were used for the test of intra-day precision in one day. The inter-day precision was measured by analyzing six QC replicates of each spiked level per day for three serial days. The extraction recovery at 0.5, 5 and 50 ng/mL levels were obtained by calculating the area ratio of exposures spiked before and after extraction ($n = 4$), respectively. In addition, additional evaluations of linearity, LLOQ, ULOQ, and accuracy for 145 exposures were performed for further accurate quantification. Fetal bovine serum samples spiked with more concentration levels of 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL were used to obtain the linear range, LLOQ and ULOQ, and conducting internal calibration curves. Six replicates of spiked fetal bovine serum samples at 0.5, 5 and 50 ng/mL were analyzed and calibrated with internal standard calibration curve to calculate accuracy.

Data processing and statistical analysis

In the untargeted metabolome analysis, Compound Discovery software (Thermo Fisher) was utilized to extract peak areas for metabolites and internal standards from the raw data. Only peaks with areas exceeding 500,000 were retained for further analysis. After removing zero values by 80% rule, the resulting ion features were semi-quantified using internal standard correction. Quantification of isotopically labeled metabolites for method validation was performed by Tracefinder 5.1 EFS (Thermo Fisher).

In the targeted exposome analysis, SCIEX OS (AB SCIEX) software was used for integration and quantification of the chemical residues and internal standards. After internal standard correction, concentration of the target analyte was determined through calibration curve quantification, which was constructed using weighted linear regression ($1/x$). The concentration below LOQ in the sample was set to $LOQ/\sqrt{2}$. Internal standards for metabolites and exposures in this study were selected according to the method reported in the literature⁵¹, using the internal standard with the smallest RSD of the corrected peak area in QCs.

Statistical analyses of metabolome and chemical residues were performed by using statistical software and R package. Multi Experiment Viewer (MeV, version 4.7.4, TIGR, Boston, MA, U.S.A.) was used for nonparametric Mann-Whitney U test. Multivariate analysis, spearman correlation analysis and binary logistic regression were performed based on R package. Metabolites and exposures with $p < 0.05$ were considered as significant differential metabolites and exposures. Metabolite enrichment analysis was performed on Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>), and mediation effect value of metabolites was calculated based on “mediation” R package⁵².

Abbreviations

2DLC: two-dimensional liquid chromatography; HRMS: high resolution mass spectrometry system; TQMS: triple quadrupole mass spectrometry; mEWAS: metabolome-exposome wide association study; 1DLC: one-dimensional liquid chromatography; MRM: multiple reaction monitoring; GC: gas chromatography RP: reversed-phase; HILIC: hydrophilic interaction liquid chromatography; IBD: inflammatory bowel disease; UC: ulcerative colitis; CD: Crohn's disease; MONA: MassBank of North America; LLOQ: lower limit of quantitation; ULOQ: upper limit of quantitation; LOD: limit of detection; QC: quality control; RSD: relative standard deviation; KEGG: Kyoto Encyclopedia of Genes and Genomes; log P: logarithm of the partition coefficient; FA: fatty acid; PC: phosphatidyl choline; PI: phosphatidylinositol; PE: phosphatidyl ethanolamine; SM: sphingolipid; AA: arachidonic acid; 5-Oxo-MEHP: Mono(2-ethyl-5-oxohexyl)phthalate; BHT alcohol: 2,6-Di-tert-butyl-4-hydroxymethylphenol; DETX: 2,4-Diethylthioxanthone; PFHps: Perfluoroheptanesulfonic acid; PFAS: per- and polyfluoroalkyl substances; 6:2 diPAP: Bis[2-(perfluorohexyl)ethyl] Phosphate.

Declarations

Data availability

The data that support the findings of this study are openly available in the supplementary data source data and MassIVE at <https://massive.ucsd.edu>, reference number MSV000096809.

Code availability

The Python and R codes for data analysis and generating figures are available at GitHub (<https://github.com/wyt2025/2DLC-Dual-MS-Method.git>).

Acknowledgments

This work was supported by the National Key R&D Program of China [2019YFC1605100], the National Natural Science Foundation of China [22434006, 22474138], Liaoning Province Excellent Youth Fund Program [1726299025904], Youth Innovation Promotion Association CAS [2021186], and the innovation program [DICP I202225] of science and research from the DICP, CAS.

Author Contributions:

G. X., X. L. and X. S. developed the concept and designed experiments. P. C. collected and provided human serum samples. Y. W. and X. G. performed the experiments of LC-MS part. P. G. and T. Chen contributed data analysis. Y. W. drafted the manuscript. X. L. and G. X. contributed to article revisions.

Competing interests:

The authors declare no competing interests.

Supplementary information

Supplementary information contains Supplementary Figures 1-9, Supplementary Table 1 and Supplementary Results. Supplementary data contain Supplementary Data 1-13. Supporting information to this article can be found online.

References

1. Polemi KM, Nguyen VK, Heidt J, Kahana A, Jolliet O, Colacino JA. Identifying the link between chemical exposures and breast cancer in African American women via integrated in vitro and exposure biomarker data. *Toxicology* **463**, (2021).
2. Goodrich JA, et al. Exposure to perfluoroalkyl substances and risk of hepatocellular carcinoma in a multiethnic cohort. *JHEP Reports* **4**, (2022).
3. Cheng H, et al. Ambient Air Pollutants and Traffic Factors Were Associated with Blood and Urine Biomarkers and Asthma Risk. *Environ Sci Technol* **56**, 7298-7307 (2022).
4. Holtjer JCS, et al. Identifying risk factors for COPD and adult-onset asthma: an umbrella review. *Eur Respir Rev* **32**, (2023).
5. Lange S, et al. Effects of Short-Term Gluten-Free Diet on Cardiovascular Biomarkers and Quality of Life in Healthy Individuals: A Prospective Interventional Study. *Nutrients* **16**, (2024).
6. Prattichizzo F, et al. Micro-nanoplastics and cardiovascular diseases: evidence and perspectives. *Eur Heart J* **45**, 4099-4110 (2024).
7. Gao F, Zhang X, Shen X, Zhao F, Shen H, Hu J. Exposure assessment of aryl-organophosphate esters based on specific urinary biomarkers and their associations with reproductive hormone homeostasis disruption in women of childbearing age. *Environ Int* **169**, (2022).
8. Thacharodi A, Hassan S, Acharya G, Vithlani A, Hoang Le Q, Pugazhendhi A. Endocrine disrupting chemicals and their effects on the reproductive health in men. *Environ Res* **236**, (2023).
9. Sun J, et al. A review of environmental metabolism disrupting chemicals and effect biomarkers associating disease risks: Where exposomics meets metabolomics. *Environ Int* **158**, (2022).

10. Wu M, Zhu Z, Wan R, Xu J. Exposure to per- and polyfluoroalkyl substance and metabolic syndrome: A nationally representative cross-sectional study from NHANES, 2003–2018. *Environ Pollut* **346**, (2024).
11. You L, et al. Metabolome-wide association study of serum exogenous chemical residues in a cohort with 5 major chronic diseases. *Environ Int* **158**, 106919 (2022).
12. Ding E, et al. Exposome-Wide Ranking to Uncover Environmental Chemicals Associated with Dyslipidemia: A Panel Study in Healthy Older Chinese Adults from the BAPE Study. *Environ Health Perspect* **132**, (2024).
13. Maitre L, et al. Multi-omics signatures of the human early life exposome. *Nat Commun* **13**, (2022).
14. Scholz S, et al. The Eco-Exposome Concept: Supporting an Integrated Assessment of Mixtures of Environmental Chemicals. *Environ Toxicol Chem* **41**, 30-45 (2022).
15. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem Soc Rev* **40**, 387-426 (2011).
16. Vermeulen R, Schymanski EL, Barabasi AL, Miller GW. The exposome and health: Where chemistry meets biology. *Science* **367**, 392-396 (2020).
17. Cajka T, Fiehn O. Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics. *Anal Chem* **88**, 524-545 (2016).
18. Mouskeftara T, Virgiliou C, Iakovakis A, Raikos N, Gika HG. Liquid chromatography tandem mass spectrometry for the determination of nine insecticides and fungicides in human postmortem blood and urine. *J Chromatogr B* **1179**, (2021).
19. Jayatilaka NK, Restrepo P, Williams L, Ospina M, Valentin-Blasini L, Calafat AM. Quantification of three chlorinated dialkyl phosphates, diphenyl phosphate, 2,3,4,5-tetrabromobenzoic acid, and four other organophosphates in human urine by solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* **409**, 1323-1332 (2016).
20. Silva MJ, Wong L-Y, Samandar E, Preau JL, Calafat AM, Ye X. Exposure to di-2-ethylhexyl terephthalate in a convenience sample of U.S. adults from 2000 to 2016. *Arch Toxicol* **91**, 3287-3291 (2017).
21. Narasimhan K, et al. Integrated analysis of per- and polyfluoroalkyl substances and plasma lipidomics profiles in multi-ethnic Asian subjects for exposome research. *Environ Health* **23**, (2024).
22. HBM4EU. The human biomonitoring for the European union network -. (2022).
23. Fang Y, et al. Adverse impact of phthalate and polycyclic aromatic hydrocarbon mixtures on birth outcomes: A metabolome Exposome-Wide association study. *Environ Pollut* **357**, (2024).
24. Ji X, Lakuleswaran M, Cowell W, Kahn LG, Sirota M, Abrahamsson D. Insights into the Chemical Exposome during Pregnancy: A Non-Targeted Analysis of Preterm and Term Births. *Environ Sci Technol* **58**, 20883-20893 (2024).

25. Sdougkou K, et al. Longitudinal Exposomics in a Multiomic Wellness Cohort Reveals Distinctive and Dynamic Environmental Chemical Mixtures in Blood. *Environ Sci Technol* **58**, 16302-16315 (2024).
26. Rappaport SM, Barupal DK, Wishart D, Vineis P, Scalbert A. The blood exposome and its role in discovering causes of disease. *Environ Health Perspect* **122**, 769-774 (2014).
27. Verri Hernandez V, Warth B. Bridging Targeted (Zeno MRM-HR) and Untargeted (SWATH) LC–HRMS in a Single Run for Sensitive Exposomics. *Anal Chem* **96**, 12710-12717 (2024).
28. Uppal K, Walker DI, Liu K, Li S, Go Y-M, Jones DP. Computational Metabolomics: A Framework for the Million Metabolome. *Chem Res Toxicol* **29**, 1956-1975 (2016).
29. You L, et al. An exposome atlas of serum reveals the risk of chronic diseases in the Chinese population. *Nat Commun* **15**, (2024).
30. Walker DI, et al. High-Resolution Exposomics and Metabolomics Reveals Specific Associations in Cholestatic Liver Diseases. *Hepatol Commun* **6**, 965-979 (2021).
31. Flasch M, Fitz V, Rampler E, Ezekiel CN, Koellensperger G, Warth B. Integrated Exposomics/Metabolomics for Rapid Exposure and Effect Analyses. *JACS Au* **2**, 2548-2560 (2022).
32. Lv W, Shi X, Wang S, Xu G. Multidimensional liquid chromatography-mass spectrometry for metabolomic and lipidomic analyses. *TRAC-TREND ANAL CHEM* **120**, (2019).
33. Wang Y, Lehmann R, Lu X, Zhao X, Xu G. Novel, fully automatic hydrophilic interaction/reversed-phase column-switching high-performance liquid chromatographic system for the complementary analysis of polar and apolar compounds in complex samples. *J Chromatogr A* **1204**, 28-34 (2008).
34. Wild CP. Invited Perspective: The Exposome—An Exciting Opportunity for Collaboration, Not Competition. *Environ Health Perspect* **132**, (2024).
35. Shen X, et al. Metabolic reaction network-based recursive metabolite annotation for untargeted metabolomics. *Nat Commun* **10**, (2019).
36. Zhao M, et al. Gut microbiota production of trimethyl-5-aminovaleric acid reduces fatty acid oxidation and accelerates cardiac hypertrophy. *Nat Commun* **13**, (2022).
37. Gentry EC, et al. Reverse metabolomics for the discovery of chemical structures from humans. *Nature* **626**, 419-426 (2023).
38. Zhao Q, et al. Chemometrics strategy coupled with high resolution mass spectrometry for analyzing and interpreting comprehensive metabolomic characterization of hyperlipemia. *RSC Adv* **6**, 112534-112543 (2016).
39. Chen Y-L, et al. Dynamic lipid profile of hyperlipidemia mice. *J Chromatogr B* **1055-1056**, 165-171 (2017).
40. González-Peña D, et al. Multiplatform metabolomic fingerprinting as a tool for understanding hypercholesterolemia in Wistar rats. *Eur J Nutr* **55**, 997-1010 (2015).
41. Wu Q, et al. UPLC-Q-TOF/MS based metabolomic profiling of serum and urine of hyperlipidemic rats induced by high fat diet. *J Pharm Anal* **4**, 360-367 (2014).

42. Wang X-f, Zhang Y-x, Ma H-y. Targeted profiling of amino acid metabolome in serum by a liquid chromatography-mass spectrometry method: application to identify potential markers for diet-induced hyperlipidemia. *Anal Methods* **12**, 2355-2362 (2020).
43. Chen P, et al. Hepatotoxicity and lipid metabolism disorders of 8:2 polyfluoroalkyl phosphate diester in zebrafish: In vivo and in silico evidence. *J Hazard Mater* **469**, (2024).
44. Sanchez Garcia D, et al. Cellular accumulation and lipid binding of perfluorinated alkylated substances (PFASs) – A comparison with lysosomotropic drugs. *Chem Biol Interact* **281**, 1-10 (2018).
45. Szilagyi JT, Avula V, Fry RC. Perfluoroalkyl Substances (PFAS) and Their Effects on the Placenta, Pregnancy, and Child Development: a Potential Mechanistic Role for Placental Peroxisome Proliferator–Activated Receptors (PPARs). *Curr Environ Health Rep* **7**, 222-230 (2020).
46. Adegoke SA, Okeniyi JAO, Akintunde AA. Electrocardiographic abnormalities and dyslipidaemic syndrome in children with sickle cell anaemia. *Cardiovasc J Afr* **27**, 16-20 (2016).
47. Lazzerini PE, Capecchi PL, Laghi-Pasini F. Systemic inflammation and arrhythmic risk: lessons from rheumatoid arthritis. *Eur Heart J*, (2016).
48. Safarlou CW, Jongsma KR, Vermeulen R. Reconceptualizing and Defining Exposomics within Environmental Health: Expanding the Scope of Health Research. *Environ Health Perspect* **132**, (2024).
49. Blaženović I, et al. Structure Annotation of All Mass Spectra in Untargeted Metabolomics. *Anal Chem* **91**, 2155-2162 (2019).
50. Tsugawa H, et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Anal Methods* **12**, 523-+ (2015).
51. Sysi-Aho M, Katajamaa M, Yetukuri L, Oresic M. Normalization method for metabolomics data using optimal selection of multiple internal standards. *Bmc Bioinformatics* **8**, (2007).
52. Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. mediation: R Package for Causal Mediation Analysis. *J. Stat. Softw.* **59**, (2014).

Figures

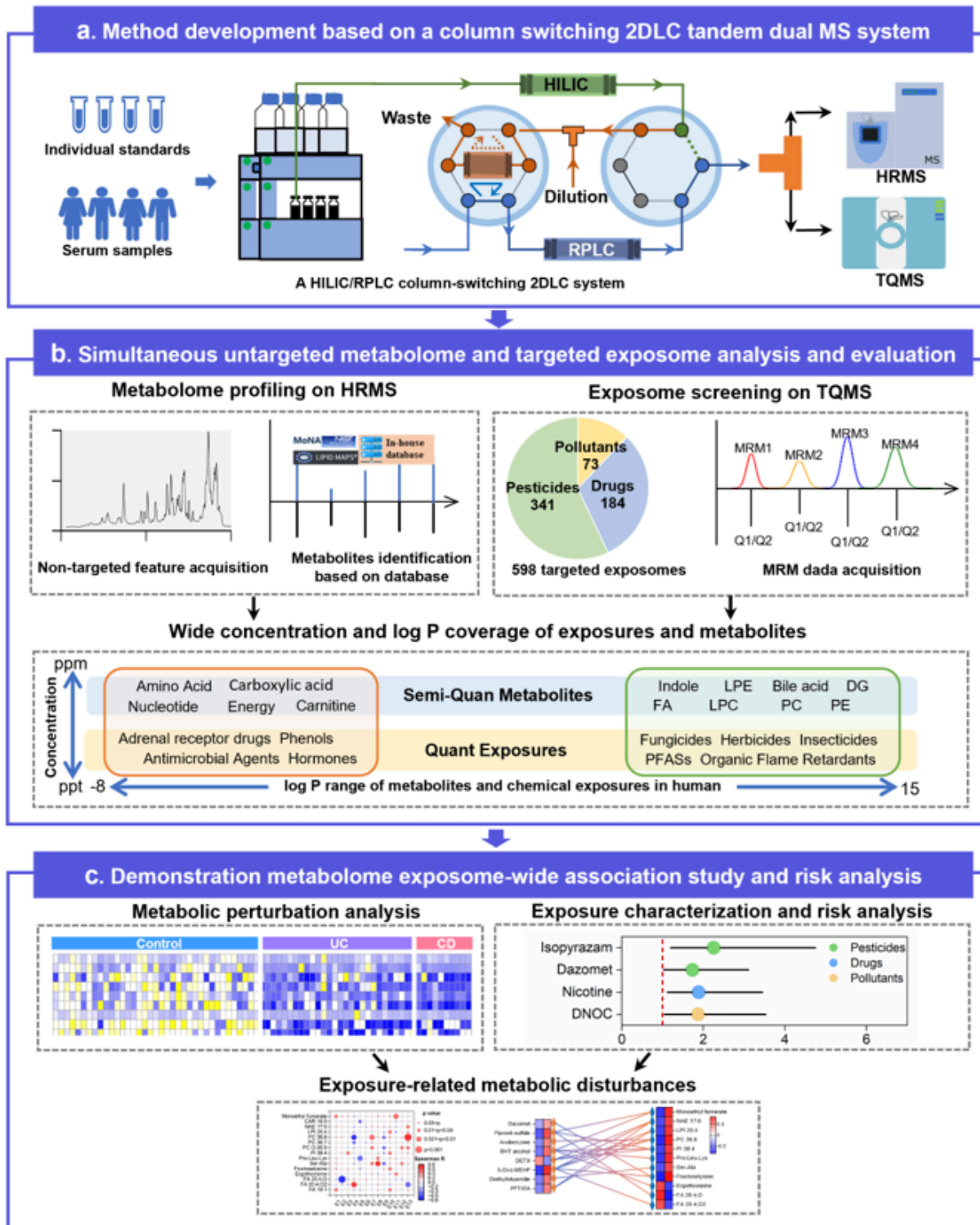


Figure 1

Workflow of this study. **a** Method development based on a column switching 2DLC-dual MS system. **b** Simultaneously untargeted metabolome and targeted exposome analysis and evaluation. Semi-quantification of metabolites and quantification of 598 chemical exposures across a range of concentration were obtained in a single run, with log P ranging from -8 to 15. The coverage and sensitivity of the method were further evaluated across three matrices and a highly diverse panel of

more than 800 analytes. **c** Demonstration metabolome exposome-wide association study and risk analysis. Metabolic profile analysis and chemical exposure risk analysis were performed on chronic disease serum samples. Exposure-related metabolic disturbances and intermediate metabolites that mediate the relationship between exposure factors and chronic disease risk were discovered through the meeting-in-the-middle method and mediation effect analysis.

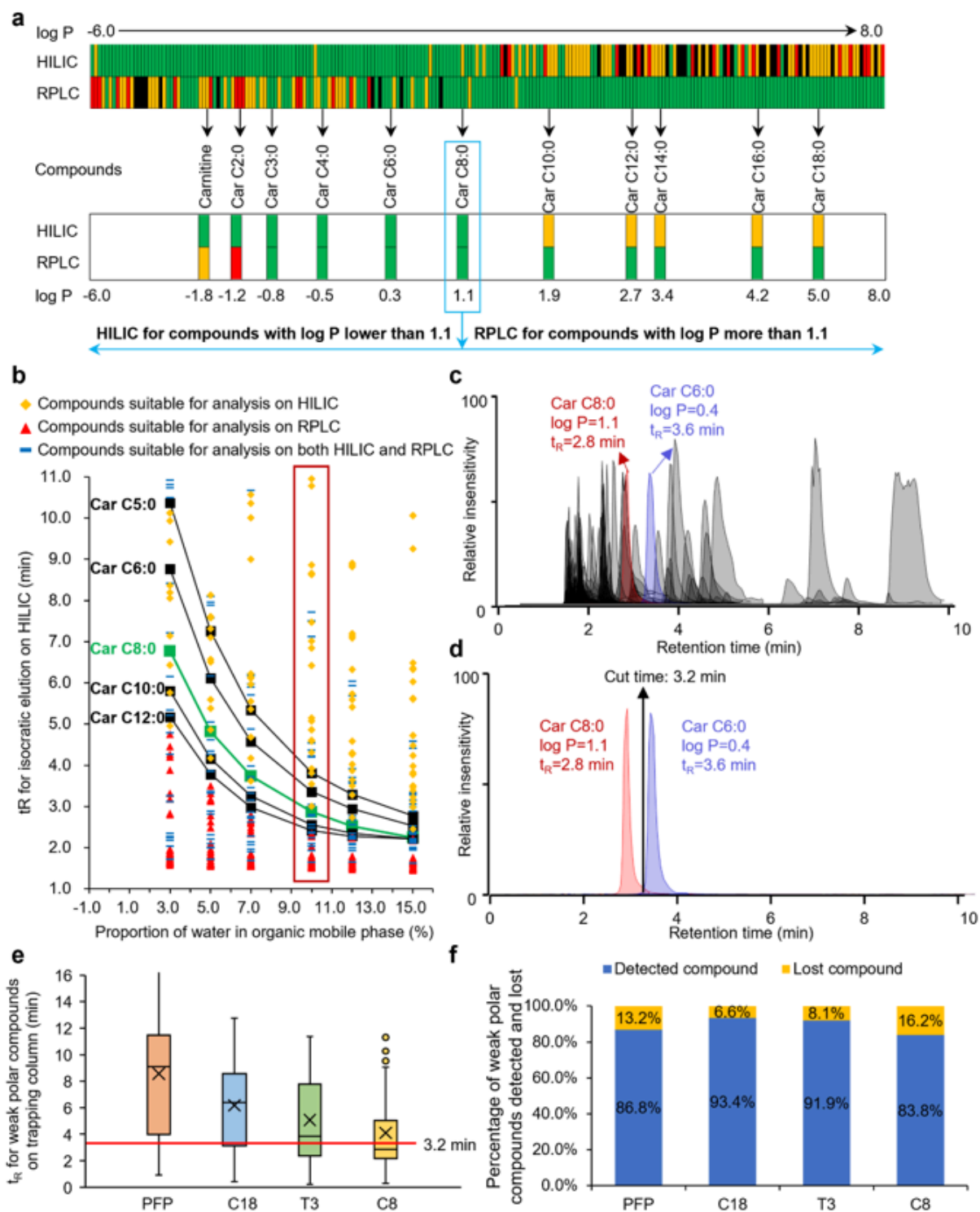


Figure 2

Optimization of the column-switching 2DLC system. **a** Separation of 221 standards on HILIC and RPLC columns. EIC of each compound was demanded to satisfy three conditions, green, yellow, red and black colors indicate the satisfaction of three conditions, two conditions, one condition and no peak detection, classified as good, acceptable, unacceptable, and unacceptable. **b** Retention time of 221 compounds pre-separated on HILIC under different proportions of water in organic mobile phase. Black squares represent the retention times of carnitines with different numbers of carbon atoms. Green square represents the retention time of carnitines 8:0. **c** EIC of carnitines with different chain lengths and other compounds pre-separated under 10% of water proportion in organic mobile phase. **d** EIC of Car C8:0 and Car C6:0 pre-separated under 10% of water proportion in organic mobile phase. **e** Retention time distribution of weak polar compounds eluted from four different trapping column including PFP, C18, T3 and C8, under a mix mobile phase composed of 100% organic mobile phase at 0.15 mL/min and water at 1 mL/min. **f** Percentage of weak polar compounds detected and lost from four different trapping column including PFP, C18, T3 and C8, under a mix mobile phase composed of 100% organic mobile phase at 0.15 mL/min and water at 1 mL/min.

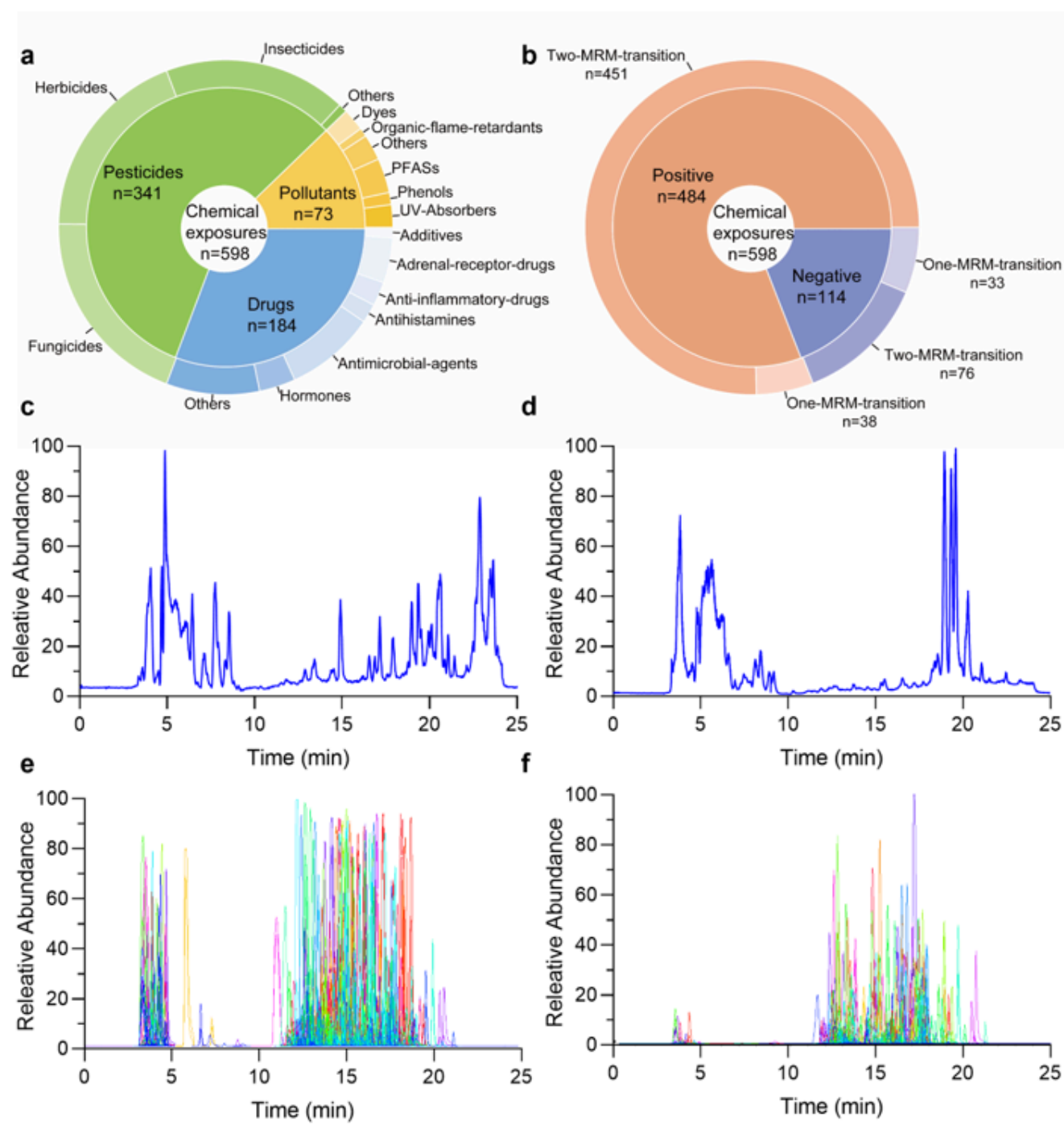


Figure 3

Chromatograms of the 2DLC-HRMS/TQMS method in positive and negative ion mode. **a** Chemical exposures are included in the method. **b** MRM transitions of the chemical exposures in positive and negative ion modes. **c** TIC obtained from HRMS in positive ion mode. **d** TIC obtained from HRMS in negative ion mode. **e** EIC obtained from TQMS in positive ion mode. **f** EIC obtained from TQMS in negative ion mode.

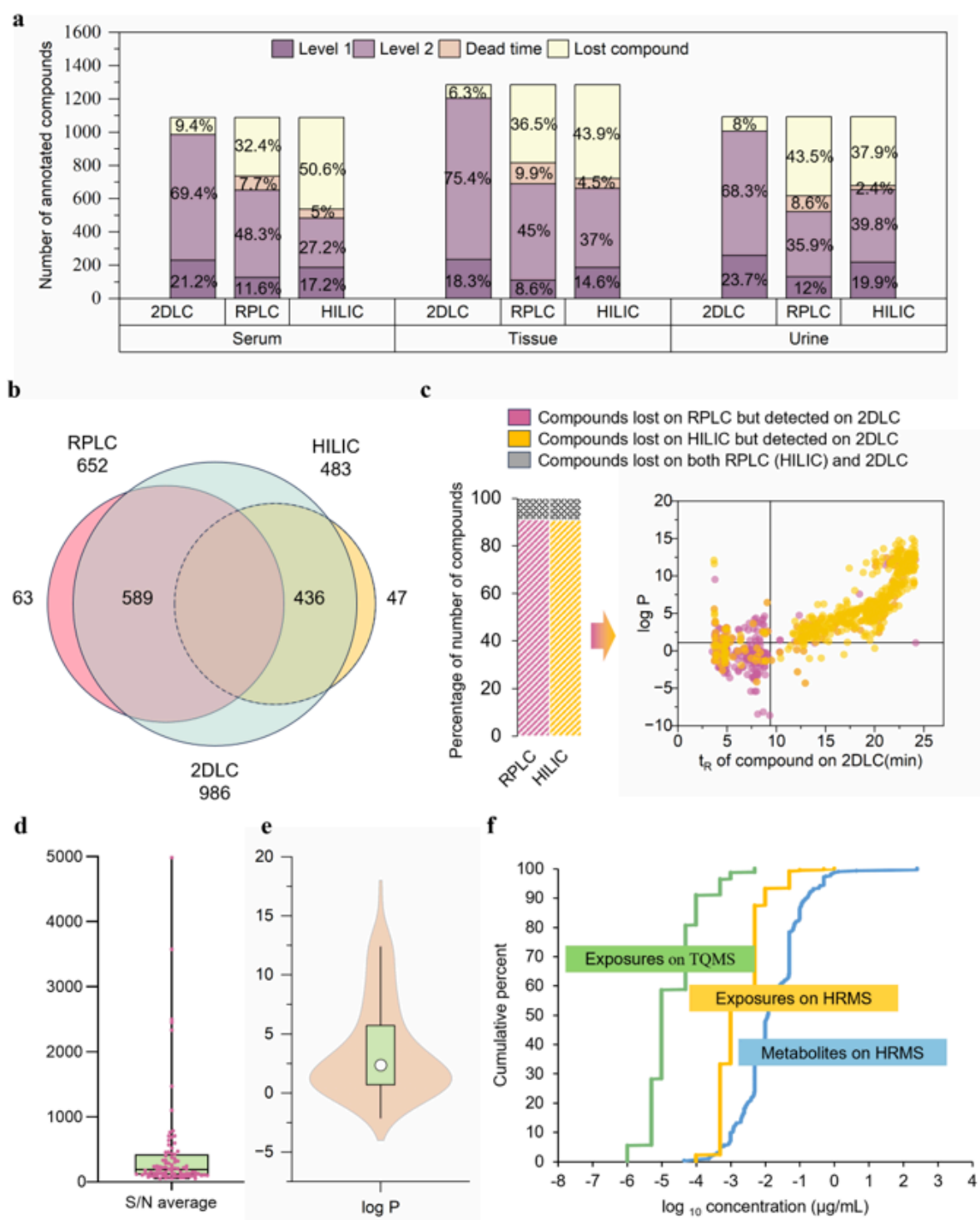


Figure 4

Evaluation of the annotation coverage and sensitivity of the 2DLC-HRMS/TQMS method. **a** Comparison of the compound numbers annotated from three different matrices (serum, tissue and urine) on three LC systems (2DLC, RPLC and HILIC). **b** Venn diagram of the compounds annotated in serum based on three LC systems. **c** Retention time and log P distribution of the compounds lost on 1DLC but detected on 2DLC in serum. **d** Box plot of S/N average of the compounds lost on 2DLC system in serum. **e** Violin plot

of log P distribution of the compounds lost on 2DLC system in serum. fLOD distribution of exposures on TQMS, exposures on HRMS, and metabolites on HRMS.

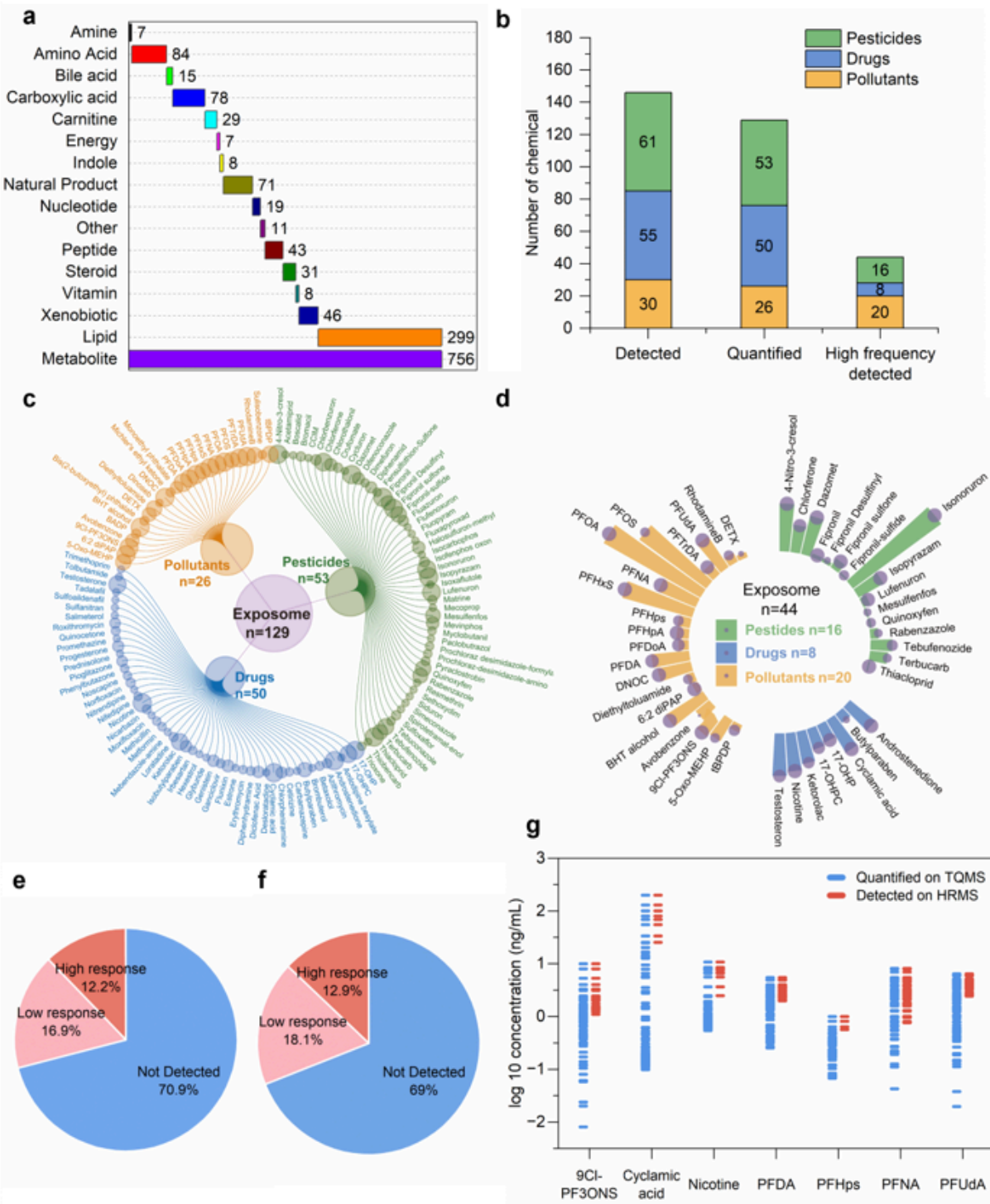


Figure 5

Untargeted metabolome and targeted exposome analysis results in serum samples based on 2DLC-HRMS/TQMS method. **a** Chemical classes and number of annotated metabolites in serum samples. **b**

Overview of chemical exposure quantitation results. **c** Classes and relative quantification rate of 129 chemical exposures (Circle area represents the relative quantification rate). **d** Relative quantification rate and average concentration of 44 high frequency quantified chemical exposures (Circle area and bar height represents relative quantification rate and average concentration). **e** Detection results of positive features detected from TQMS on HRMS. **f** Detection results of positive features quantified from TQMS on HRMS. **g** Relationship between exposure concentrations and the results of quantification on TQMS and detection on HRMS. DETX 2,4-Diethyl-9H-thioxanthen-9-one, PFOS Perfluorooctanesulfonate, 5-Oxo-MEHP Mono(2-ethyl-5-oxohexyl)phthalate, PFHps Perfluoroheptanesulfonic acid, Tbpdp tert-Butylphenyl diphenyl phosphate, 17-OHPC Hydroxyprogesterone, PFDoA Perfluorododecanoic acid, PFDA Perfluorodecanoic acid, PFTrDA Pentacosfluorotridecanoic acid, 17-OHP Hydroxyprogesterone caproate, BHT alcohol 3,5-Di-tert-butyl-4-hydroxybenzyl alcohol, 6:2 diPAP Bis[2-(perfluorohexyl)ethyl] phosphate, DNOC 4,6-Dinitro-O-cresol, PFNA Perfluorononanoic acid, PFHpA Perfluoroheptanoic acid, 9CI-PF3ONS Chloro-3-oxa-perfluorononanesulfonic acid, PFUdA Perfluoroundecanoic acid, PFHxS Perfluorohexanesulfonate, PFOA Perfluorooctanoic acid.

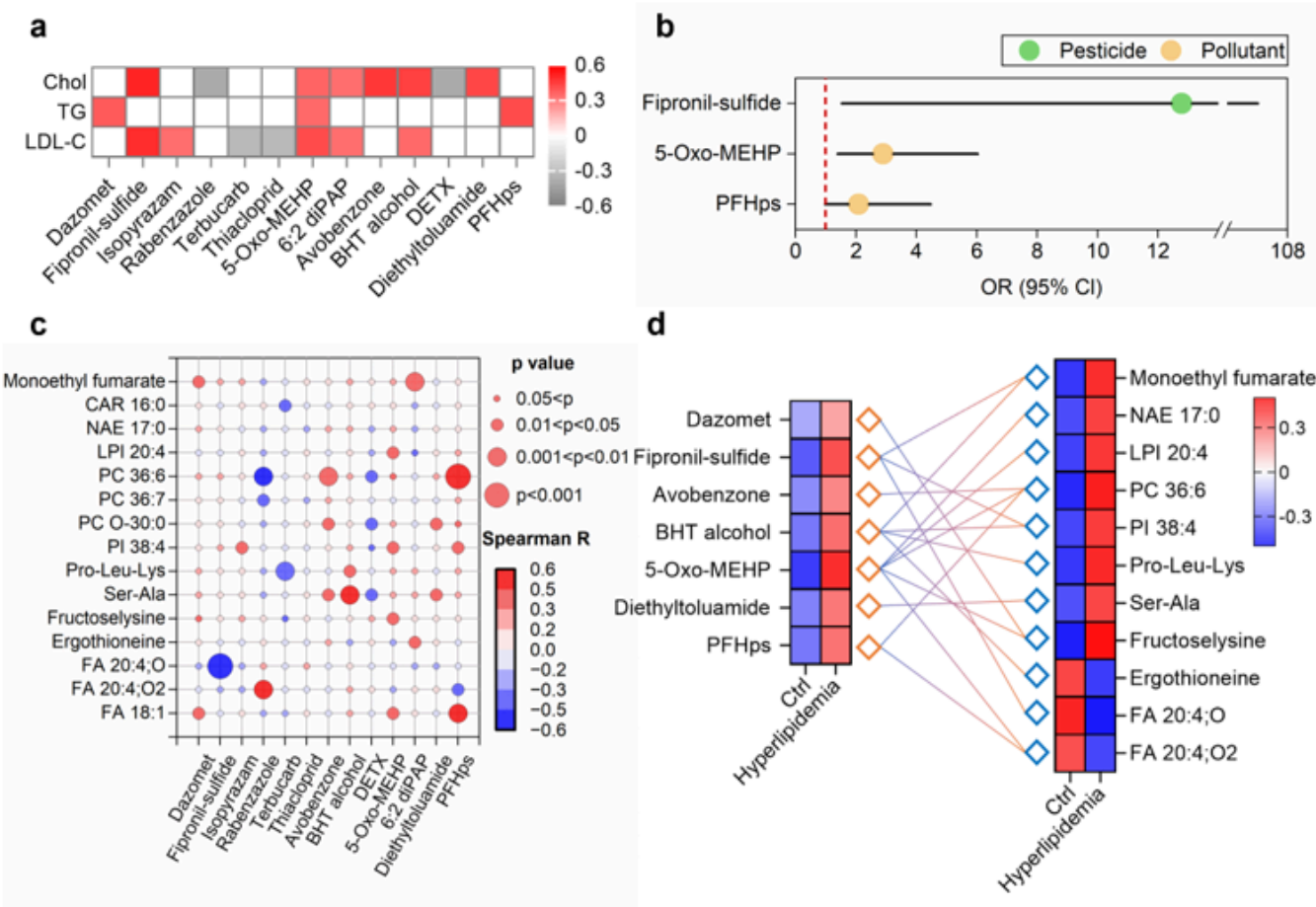


Figure 6

Metabolome-exposome wide association study and risk analysis of hyperlipidemia. a Chemical exposures significantly associated with clinical parameters of hyperlipidemia. **b**The odds ratio (OR) of

chemical exposures on hyperlipidemia. Error bars represent 95% confidence interval of ORs **c** Correlation bubble plot between 15 differential metabolites and 13 chemical exposures. **d** Paired heatmap of chemical exposures in hyperlipidemia and their mediating effector metabolites. Chol, Total cholesterol; TG, Triglyceride; LDL-C, low density lipoprotein cholesterol.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryData.xlsx](#)
- [Sourcedata.xlsx](#)
- [SupplementaryInformation.docx](#)