

Metabolomics of per- and polyfluoroalkyl substances (PFAS) exposure: Advances in LC-MS technology and applications

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic chemicals known for their high stability and bioaccumulation potential, leading to their widespread presence in the environment and human tissues. Exposure to PFAS has been associated with significant health concerns, including neurotoxicity, hepatotoxicity, and metabolic disturbances. Liquid chromatography–mass spectrometry (LC-MS) based metabolomics has emerged as a powerful and central tool for PFAS research, enabling the comprehensive analysis of metabolic changes induced by exposure. This review provides a critical summary of the applications of LC-MS metabolomics in both clinical studies and various experimental models, including *in vivo* models (mice) and *in vitro* systems (cell cultures and zebrafish embryos). We highlight key findings from these studies, specifically focusing on the identification of altered metabolic pathways (e.g., lipid, amino acid, and purine metabolism) and the discovery of specific metabolic biomarkers. Furthermore, we explore the intricate interconnections among these metabolic perturbations, which collectively provide a clearer picture of PFAS toxicological effects. By integrating systematic experimental design with advanced LC-MS techniques, PFAS metabolomics offers invaluable insights into the molecular-level responses to exposure, helping to elucidate toxicological mechanisms and identify specific metabolic biomarkers. The findings synthesized in this review are crucial for supporting a more standardized approach to risk assessment and for informing evidence-based public health policies related to PFAS monitoring and regulation.

Keywords: Exposure, LC-MS, Metabolomics, PFAS

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large class of synthetic fluorinated organic chemicals characterized by a highly stable carbon-fluorine backbone, one of the strongest chemical bonds in organic chemistry. This unique structure confers exceptional thermal and chemical stability, making them widely used in non-stick cookware, water-repellent textiles, and firefighting foams. The structural diversity of PFAS structures, including varying C–F backbone lengths, hydrocarbon

domains, and ether bonds, allows for subclass-level differentiation [1]. This chemical durability also makes PFAS environmentally persistent, bio-accumulative, and resistant to degradation, leading to their classification as “forever chemicals [2].” PFAS are now widely detected in environmental and human biological matrices, including water, soil, and wildlife [3].

While the chemical durability of PFAS makes them valuable for industrial applications, their potential toxicity to environmental biota and human health is a major concern. Numerous epidemiological and

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experimental studies have linked PFAS exposure to hepatotoxicity, endocrine disruption, immune modulation, reproductive effects, and metabolic disturbances, raising significant public health and regulatory concerns [4,5]. As a result, regulatory frameworks have expanded from addressing individual compounds to including broader classes of PFAS, reflecting a shift toward more comprehensive management strategies [6].

Metabolomics, defined as the comprehensive characterization of small-molecule metabolites within biological systems, has gradually emerged as a pivotal discipline in the field of environmental health research [7,8]. By providing a functional readout of biochemical activity, it offers a highly sensitive approach to reveal early signatures of toxicant-induced perturbations. The approach is broadly divided into targeted and untargeted strategies, with untargeted metabolomics being particularly valuable for comprehensive screening of metabolic perturbations and biomarker discovery [9]. Given these advantages, metabolomics demonstrates high sensitivity and efficiency in analyzing metabolic pathway responses in cells, tissues, and body fluids exposed to environmental stressors, playing a significant role in ecological risk assessment and elucidating the toxicological mechanisms.

In the context of PFAS exposure, metabolomics has emerged as a key approach in exposome research [10]. Untargeted metabolomics, in particular, has revealed significant perturbations in metabolic pathways, including lipid, amino acid, and energy metabolism. However, these investigations face distinctive challenges. The chemical complexity and structural diversity of PFAS complicate their detection and metabolite annotation, as many compounds are not represented in existing reference libraries [11]. Moreover, low-dose effects and subtle metabolic signatures pose analytical challenges. In addition, matrix effects and interference from endogenous metabolites in complex biological samples can compromise analytical accuracy.

To achieve comprehensive metabolite information, the integration of multiple analytical platforms is often required. Among these, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and Fourier-transform infrared spectroscopy (FT-IR) are the most widely used core technologies [12,13]. However, with the rapid development of liquid chromatography–mass spectrometry (LC-MS) technology, its advantages in sensitivity, resolution, and metabolite coverage

have gradually established it as the dominant platform in metabolomics research.

The widespread application and persistence of PFAS make human exposure a significant concern. This underscores the urgent need to establish metabolic connections between exposure and health outcomes, and to identify potential biomarkers. While previous studies using cellular and animal models have provided valuable insights into the molecular mechanisms of PFAS toxicity, a systematic synthesis on how analytical tools can be applied to unravel these biological pathways is currently lacking. This review focuses on LC-MS as a key platform in PFAS metabolomics, providing a comprehensive overview of its applications and highlighting how it advances understanding of PFAS exposure and associated biological responses.

2. Clinical studies of PFAS exposure

2.1. Clinical population exposure

Human exposure to natural and synthetic chemicals is influenced by diet, lifestyle, occupation, medication use, and psychosocial stress, with notable variations across populations [14]. Increasing evidence suggests that environmental factors, rather than genetic variation, play a more critical role in the etiology and pathogenesis of cardiometabolic diseases, including most cancers [14]. Currently, PFAS have been detected in the environment globally. These chemicals are prevalent in consumer products and their manufacturing processes, leading to widespread environmental contamination and human exposure. Consequently, PFAS are present in nearly all human populations. Metabolomics studies using *in vivo* and *in vitro* models have linked PFAS exposure to alterations in lipid, amino acid, and purine metabolism. However, the doses used in these experimental studies may not accurately reflect the exposure levels in the general population [15].

Increasingly, epidemiological research employs untargeted metabolomics to identify early biomarkers of health effects resulting from PFAS exposure [16,17]. Despite this progress, there is a lack of studies evaluating the robustness of methodologies and findings in environmental metabolomics research [15,18]. While epidemiological studies on environmental exposures often focus on specific clinical endpoints, such as mortality or birth weight, exposome approaches can utilize internal biomarkers of exposure and response to investigate more subtle outcomes, such as early disease

indicators [10]. Human studies on biological responses to PFAS exposure have primarily concentrated on selective and targeted lipid or hormone biomarkers or liver function biomarkers [19,20].

PFAS exposure pathways can be viewed as a series of events linking pollution sources, environmental media, external exposures, and concentrations in blood through toxicokinetics. Common exposure routes include dietary intake, water consumption (especially in contaminated communities), and inhalation of air and dust particles [21], while hand-to-mouth contact and dermal absorption may also contribute. For LC-MS metabolomics studies, it is important to consider that different exposure pathways may lead to distinct metabolic responses, which is especially relevant in epidemiological research as it can help explain heterogeneity in blood metabolite levels [22]. By linking changes in blood metabolites to specific exposure pathways, researchers can identify exposure-related metabolic signatures.

2.2. Sample types and study design for LC-MS analysis in PFAS clinical studies

Table 1 summarizes nine clinical studies on PFAS exposure in various groups, including occupationally exposed individuals and patients with specific diseases. For our classification, we treated a number of frequently reported and toxicologically significant compounds—perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDoA), perfluoroheptanoic acid (PFHpA), and perfluorohexanoic acid (PFHxA)—as individual entries. Other PFAS were grouped by their chemical class, such as perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSA), fluorotelomer sulfonates (FTS), chlorinated perfluoroether sulfonates (PFESA), perfluorodecanoic acid (PFDA), and per-/poly-fluoroether carboxylic acids (PFECA). This approach streamlines the presentation while preserving important distinctions in source, environmental behavior, and regulatory treatment across different PFAS classes. The major PFAS compounds identified across these studies included PFOA ($n = 7$), PFOS ($n = 7$), PFHxS ($n = 8$), and PFNA ($n = 3$). For individuals with occupational exposure or those in environments with long-term, high concentration PFAS contamination, exposure levels were found to be closely associated with clinical parameters such as age, BMI, and body weight. For example, firefighters who frequently handle aqueous film-forming foams (AFFF) are recognized

as a high-risk population [23]. The study reported that the PFOS concentrations of serum in primary firefighters ranged from 4.67 to 70.27 ng/mL, with a significant positive correlation between PFOS levels and age. In addition, several studies reported detection frequencies of PFAS above 50% among participants, underscoring the widespread nature of exposure [24–26].

Regarding the sample matrices, LC-MS analysis in the summarized studies was conducted on six serum samples and three plasma samples (Table 1). Previous research has highlighted that while many clinical parameters—such as metal ions, proteins, and enzymes—can differ between matrices, the small molecule composition of serum and plasma is considered highly similar [13]. Furthermore, long-chain PFAS compounds have been reported to exhibit longer half-lives in serum, making it a particularly suitable matrix for biomonitoring [27]. It is important to note that among the more than 9000 PFAS identified to date, only a small subset of legacy compounds has robust evidence regarding exposure, toxicity, and human health impacts. PFOS and PFOA are the most well studied representatives.

These considerations regarding sample type, PFAS prevalence, and compound selection provide the context for understanding LC-MS based metabolomics in clinical studies, which will be systematically compared with applications in experimental models in Section 4.

2.3. Biological pathway and toxicity in PFAS clinical studies

In order to better capture real-world exposure scenarios and cumulative effects, PFAS-related metabolomics studies frequently prioritize high risk cohorts as sentinel populations, including residents of industrially contaminated areas with chronic PFAS exposure, occupationally exposed groups such as workers and firefighters, and vulnerable populations such as pregnant women and children [23,28,29]. From a biological pathway perspective, the studies summarized in Table 1 include investigations of diseases such as non-alcoholic fatty liver disease (NAFLD) [30] and cancers, including thyroid and gastric cancer [31]. Among these, NAFLD serves as a representative condition and highlights the hepatotoxic effects associated with PFAS exposure. To date, the only published study has reported an inverse association between prenatal and childhood plasma PFAS concentrations and alanine aminotransferase (ALT) levels at age 8 [32], which is commonly used as a clinical

Table 1. Applications of LC-MS based metabolomics in human PFAS exposure studies.

PFAS types	Study designs	Separation methods	Scan modes	Extraction solvents	Biological pathways	Ref.
PFCA, PFESA, PFHxS, PFSA, PFOA, and PFOS	Plasma (PFAS-exposed workers)	RPLC (Waters ACQUITY UPLC HSS T3)	Full scan (Thermo Scientific Q Exactive Focus Orbitrap)	Methanol	Lipid, amino acids, purine, inositol, retinol, and alkaloids metabolisms	[59]
PFHxS, PFOA, and PFOS	Serum (PFAS-exposed workers)	(Not mentioned)	DDA (AB Sciex tripleTOF 5600)	Acetonitrile	Fatty acid, amino acid and sphingolipid metabolisms	[23]
PFHxS, PFNA, PFOA, and PFOS	Serum (PFAS-exposed pregnant)	HILIC (Waters XBridge BEH Amide XP HILIC) RPLC (Higgins Targa C18)	Full scan (Thermo Scientific Q-Exactive HF)	Acetonitrile	Taurine, hypotaurine, pantothenate, CoA biosynthesis, and beta-alanine metabolisms	[60]
PFESA, PFHxS, PFNA, PFOA, and PFOS	Serum (PFAS-exposed residents)	RPLC (Zorbax Eclipse Plus C18)	Full scan (Thermo Scientific LTQ Orbitrap XL) (Agilent 6540 QTOF)	QuEChERS 95% acetonitrile	Steroid hormone biosynthesis, primary bile acid biosynthesis, and fatty acid-related metabolisms	[61]
PFHxS, PFNA, PFOA, and PFOSs	Serum and liver (laparoscopic bariatric surgery patients)	RPLC (Waters Acquity UPLC BEH C18)	Full scan (Waters QTOF Premier)	Chloroform/methanol = 2/1, 0.9% NaCl	Bile acid synthesis, various hepatic lipid and glucose metabolisms	[30]
PFHxS, PFOA, and PFOSs	Serum (maternal perinatal)	RPLC (Higgins Analytical C18)	Full scan (Thermo Q-Exactive HF)	Acetonitrile	Urea cycle and key amino acid, and energy metabolisms	[28]
PFHxS, PFOA, and PFOS	Plasma (pediatric liver disease patients)	RPLC (Waters BEH C18) HILIC (Waters Atlantis HILIC silica)	Full scan (Thermo Q-Exactive HF)	Acetonitrile	Amino acids and lipids metabolisms	[29]
PFCA, PFDA, and PFSA	Serum (first-trimester maternal)	HILIC (Waters XBridge BEH Amide XP HILIC) RPLC (Higgins Targa C18)	Full scan (Thermo Q-Exactive HF)	Acetonitrile	Butanoate, D-amino acid, histidine, and glycerolipid metabolisms	[16]

diagnostic marker for liver function. This highlights the potential impact of PFAS exposure on early-life liver function. Because metabolomics provides a comprehensive approach to capturing liver toxicity, the study identified elevated levels of phosphoethanolamine (glycerophospholipid metabolism), tyramine and phenethylamine (tyrosine metabolism), aspartate (aspartate and asparagine metabolism), and creatine (urea cycle metabolism). In contrast, plasma betaine levels (glycine, serine, alanine, and threonine metabolism) were reduced [29]. Collectively, these findings demonstrated that higher plasma PFAS concentrations were associated with an increased risk of developing non-alcoholic steatohepatitis (NASH).

3. Experimental models studies of PFAS exposure

To precisely elucidate the molecular toxicological pathways of PFAS and enable the translation of findings from *in vitro* cellular models to *in vivo* animal systems, and ultimately to humans, the construction of multi-tiered experimental models is essential. Relevant studies are summarized in Table 2.

3.1. Selection of experimental models

In vivo models, such as mice, provide metabolic network information that is more comparable to human exposure, while also allowing flexible control overdose, administration route, and sampling time. These features circumvent the ethical limitations associated with human studies, making *in vivo* models ideal platforms for method development and toxicity validation. Certain parameters, such as age and sex of the mice, can influence final analytical outcomes. Most of the literature summarized in this review used male mice (Table 2), as female mice were traditionally considered more variable due to hormonal cycles, and using males helps reduce experimental variability [33]. In terms of study design, the choice of mouse strain is particularly important, as PPAR α is a key nuclear receptor mediating PFAS hepatotoxicity and metabolism, and the humanized PPAR α model therefore provides a metabolic background more comparable to that of humans [34]. In addition, the use of animal models offers practical advantages, such as enabling comprehensive tissue sampling and mechanistic interrogation, which are often not feasible in clinical studies due to ethical constraints. These findings are also critical for supporting cross-species extrapolation and informing physiologically

based pharmacokinetic (PBPK) and *in vitro*–*in vivo* extrapolation (IVIVE) modeling.

Common *in vitro* PFAS exposure models include cultured cells and zebrafish embryos. Zebrafish embryos represent a highly sensitive developmental system, considered an *in vitro* model, and are widely used to monitor early metabolic perturbations induced by PFAS. For the widely studied PFOS, Xu et al. applied zebrafish embryo models combined with metabolomics to confirm the toxicity in different developing stage [35]. At the same time, cell culture technology has emerged as a powerful new approach methodology (NAM) for advancing our understanding of how environmental contaminants, including PFAS, affect cellular systems. Cellular systems commonly include immortalized cancer or normal cell lines and primary cells [36]. Immortalized lines are easy to maintain and highly reproducible, making them suitable for large-scale or long-term experiments. Still, they may carry mutations or altered regulatory pathways that limit their physiological relevance. In contrast, primary cells more closely reflect the *in vivo* phenotype and metabolic activity of the tissue of origin, but the cell exhibit limited lifespan and greater variability across donors [37]. Overall, the advantage of cell-based models lies in the ability to investigate metabolic processes under controlled conditions, which are more tractable than animal or human systems, though they cannot fully reproduce the metabolic and biological characteristics of human tissues.

Methodologically, two-dimensional (2D) cultures remain the most widely used due to their simplicity, but they diverge considerably from the architecture and physiology of actual tissues. To address this limitation, three-dimensional (3D) cell culture models have been developed to study the combined effects of PFAS exposure [38]. Compared with 2D culture models, 3D spheroids offer improved accuracy in monitoring cell proliferation, responses to stimuli, and drug metabolism. These advanced *in vitro* systems provide more physiologically relevant cell-cell and cell-matrix interactions, thereby yielding complementary insights into the mechanistic consequences of PFAS toxicity.

3.2. Sample types and study design for LC-MS analysis

Table 2 summarizes nine *in vivo* and *in vitro* studies on PFAS exposure. From an analytical perspective, the design of PFAS exposure experiments is the foundation for meaningful LC-MS metabolomics analysis, and PFOS and PFOA are the major PFAS compounds for *in vivo* and *in vitro*

Table 2. Applications of LC-MS based in-vivo and in-vitro metabolomics in PFAS exposure studies.

PFAS types	Study designs	Separation methods	Scan modes	Extraction solvents	Biological pathways	Ref.
FTS, PFCA, PFHpA, and PFOS	Brain and plasma (male C57BL/6 J mice)	RPLC (Waters ACQUITY UPLC HSS T3)	Full scan and DDA (Thermo Orbitrap Exploris 120)	Methanol/ acetonitrile = 1/1	Osmoprotectant metabolism, glutamatergic synapse function, tryptophan, purine and arachidonic acid metabolisms	[34]
PFOA and PFOS	Liver (CD-1 male mice)	RPLC (Phenomenex Kinetex C18)	Full scan (Thermo Q-Exactive Orbitrap)	Chloroform/ methanol = 2/1	Synthesis of lipids, steroidogenesis, amino acid, nitrogen and bile acid metabolisms	[45]
PFOA	Brain and liver (male Balb/c mice)	(Not mentioned)	MRM (AB Sciex 4000 QTrap)	Commercial kit	Amino acids, lipids, carbohydrates and energetics metabolisms	[62]
PFDA	Serum and liver (C57BL/6 J mice)	RPLC-(ACE C18-PFP) (Waters Acquity HSS T3 C18) (Thermo Accucore C30 core-shell) HILIC (Waters Acquity BEH Amide)	Full scan and DDA (Thermo Q Exactive Orbitrap)	MTBE	Dysregulated amino acid and lipid metabolisms	[39]
PFOS and PFOA	Cell (colorectal cancer cell)	RPLC (Waters Acquity UPLC BEH C18)	Full scan (Waters Xevo G2-XS hybrid QTOF)	Methanol	Fatty acid β -oxidation, protein, nucleotide and lipid synthesis	[38]
PFOA	Cell (SH-SY5Y cells)	RPLC (Thermo Hypersil Gold C18)	Full scan (Thermo Q-Exactive Orbitrap)	MeOH	ATP-related metabolites, neurotransmitter precursors, mitochondria-protective metabolites	[41]
PFDA, PFHxS, PFNA, PFOA, and PFOA	Cell (HepG2 and HepaRG)	RPLC (Waters ACQUITY UPLC BEH C18)	Full scan (Agilent QTOF-MS)	Folch	Bile acid and glucose metabolisms	[40]
PFOS	Embryo (zebrafish)	RPLC (Phenomenex Kinetex F5) (Phenomenex Kinetex C18) HILIC (Phenomenex Kinetex HILIC)	Full scan (Thermo Q-Exactive Focus quadrupole-orbitrap)	MTBE	Amino acids, purine, glycerolipids, FFA, and cholesterol-linked to energy metabolisms	[42]
PFHxS	Embryo (zebrafish)	RPLC (Waters XBridge C18) (Phenomenex Kinetex F5)	DDA (Bruker Compact QTOF)	MeOH	Energy, protein, and glycerophospholipid metabolisms	[35]

studies in Table 2. Notably, for *in vivo* sample, PFAS elimination exhibits pronounced sex- and species-specific differences, largely due to variations in renal transporter activity [27]. For example, female rats eliminate over 60% of an administered PFOA dose via urine and feces within 24 h, whereas male rats excrete only a small fraction over a much longer period, leading to a substantially longer half-life in males. Also, in the case of the PFOA exposure study, C57BL/6J mice received 8 mM PFOA in drinking water for 6–7 weeks. This design allows a direct comparison between vehicle and PFOA-treated groups [39]. The contrast enables identification of metabolic and lipidomic alterations driven by PFAS exposure and supports robust downstream LC-MS analysis.

For *in vivo* experiments, the sample type mainly include tissues, blood, and urine, depending on the specific toxicological endpoints to be analyzed. In cell-based studies, HepG2 and HepaRG cells are frequently employed for hepatotoxicity assessment due to their ease of culture, metabolic competence, and suitability for LC-MS metabolomics applications [40]. For neurotoxicity investigations, SH-SY5Y neuroblastoma cells are widely used, as they can be induced to differentiate into neuron-like phenotypes and provide a reproducible *in vitro* model for studying neuronal function and toxicant effects [41]. In addition to mammalian cell lines, alternative models such as zebrafish embryo models, offer flexibility in selecting developmental stages according to experimental objectives, making them particularly advantageous for toxicity studies and high-throughput screening [35]. A comprehensive summary of LC-MS methodologies across clinical, animal, and cell-based studies will be presented in Section 4.

3.3. Biological pathway analysis and toxicity

Based on the studies summarized in Table 2, metabolomics has been widely applied to investigate PFAS-induced toxicological effects, including neurotoxicity, hepatotoxicity, oxidative stress responses, mitochondrial dysfunction, and disturbances in energy metabolism.

For neurotoxicity, metabolomics profiling revealed metabolic alterations following PFAS mixture exposure. Specifically, 55 metabolites (30 upregulated, 25 downregulated) were identified in the low dose group, 47 metabolites (21 upregulated, 26 downregulated) in the medium dose group, and 62 metabolites (39 upregulated, 23 downregulated) in the high dose group [34]. Complementary RNA sequencing further indicated that key pathways,

including bile acid secretion, glutamatergic synapse transmission, and tryptophan metabolism, were significantly perturbed, suggesting that these metabolic and signaling processes underlie systemic responses to PFAS exposure. Glutamate-related amino acid metabolism was of particular importance, since glutamate release is essential for maintaining calcium homeostasis, which has been reported to be disrupted by PFAS (e.g., PFOA and PFOS)-induced neurotoxicity. Moreover, the observed decrease in tryptophan levels alongside elevated serotonin may disrupt the gut–brain axis, potentially affecting mood and cognition. Overall, impairment of neurotransmitter function appears to represent the most widely studied mechanism of PFAS-induced neurotoxicity. Supporting evidence also comes from zebrafish embryo to conduct MTBE-based extraction strategies, metabolic and lipidomic analyses revealed that PFOS exposure in zebrafish larvae led to disturbances in amino acid and lipid homeostasis (e.g., purines, glycerolipids, FFAs, and cholesterol) [42]. Sphingolipids, in particular, emerged as key targets implicated in PFOS-induced neurotoxicity.

For hepatotoxicity, PFAS are known to strongly bind to serum proteins (e.g., albumin) and hepatic transport proteins due to their high protein affinity and persistence [43]. Since the liver is central to metabolic regulation, these interactions make it a primary target for PFAS toxicity. Oxidative stress is a common feature, often accompanied by significant alterations in lipid metabolism and *de novo* lipogenesis. Reactive oxygen species (ROS)-induced oxidative stress promotes lipid peroxidation, a process closely linked to the pathogenesis of hepatic diseases such as alcoholic fatty liver [44]. *In vivo* evidence also supports these findings: long-term low-dose exposure to PFOA and GenX in mice disrupted hepatic lipid metabolism, while exposure to PFAS such as PFOA, PFOS, PFNA, and PFHxS increased circulating bile acids, leading to hepatic inflammation [45].

For the result of clinical and experimental model, both human cohort studies and PPAR α -humanized mouse models were employed to investigate the impact of environmental pollutants, particularly PFAS, on hepatic lipid metabolism in NAFLD [30]. PFAS exposure was associated with perturbations in key hepatic metabolic pathways previously implicated in NAFLD, especially those linked to bile acid and lipid metabolism [40]. By leveraging the flexibility of LC-MS based metabolomics, these studies were able to bridge human observational data with mechanistic insights from controlled animal experiments, thereby strengthening causal

inference and providing translational value for understanding PFAS-associated metabolic disruption in NAFLD.

From the perspective of pathway analysis, these model studies revealed partially overlapping metabolic alterations—particularly in lipid and energy metabolism—that provide a mechanistic basis for consensus in toxicology research (Fig. 1). However, significant differences in metabolic background, exposure routes, and toxicokinetics between zebrafish and mice pose challenges for extrapolation to humans. This underscores the necessity of leveraging LC-MS platforms for high resolution metabolic profiling and multi-omics integration, thereby enabling a more precise understanding of human metabolic responses to PFAS exposure.

4. LC-MS technologies in metabolomics

Recent advances in LC-MS have significantly expanded the capabilities of metabolomics for environmental research. By integrating improvements in sample preparation, chromatographic separation, mass spectrometric detection, and data process, researchers can now achieve higher sensitivity, broader coverage, and more reliable quantification of metabolites. A typical LC-MS workflow for metabolomics analysis includes sample pretreatment, data acquisition, statistical analysis, and biological interpretation, which together enable high coverage untargeted metabolomics to reveal exposure-associated metabolic perturbations (Fig. 2). This section outlines the key enabling

technologies in LC-MS based metabolomics, highlighting how each contributes to a comprehensive understanding of pollutant-induced metabolic changes.

4.1. Sample pretreatment

The choice of sample matrix is a critical first step in metabolomics, influencing the relevance and scope of the findings. Blood and urine are the most frequently used matrices for human biomonitoring due to their non-invasive collection and suitability for large scale exposure assessments. In contrast, tissues are valuable for revealing organ-specific accumulation and metabolic perturbations. For these matrices, as shown in Tables 1 and 2, protein precipitation with organic solvents (e.g., methanol or acetonitrile) is the most widely applied method for metabolomics analysis. This approach is straightforward and effective at reducing matrix interference while ensuring broad metabolite coverage. However, it's important to note the metabolic differences between serum and plasma, as the clotting process in serum can alter the levels of endogenous metabolites such as amino acids and lipids [46]. For cell culture, metabolic quenching with cold solvents like methanol/water mixtures is crucial to rapidly halt enzymatic activity and preserve intracellular metabolite profiles. Similarly, tissue samples require mechanical homogenization prior to solvent extraction to ensure the uniform release of intracellular metabolites.

While solvent precipitation offers broad applicability, more selective strategies are often required.

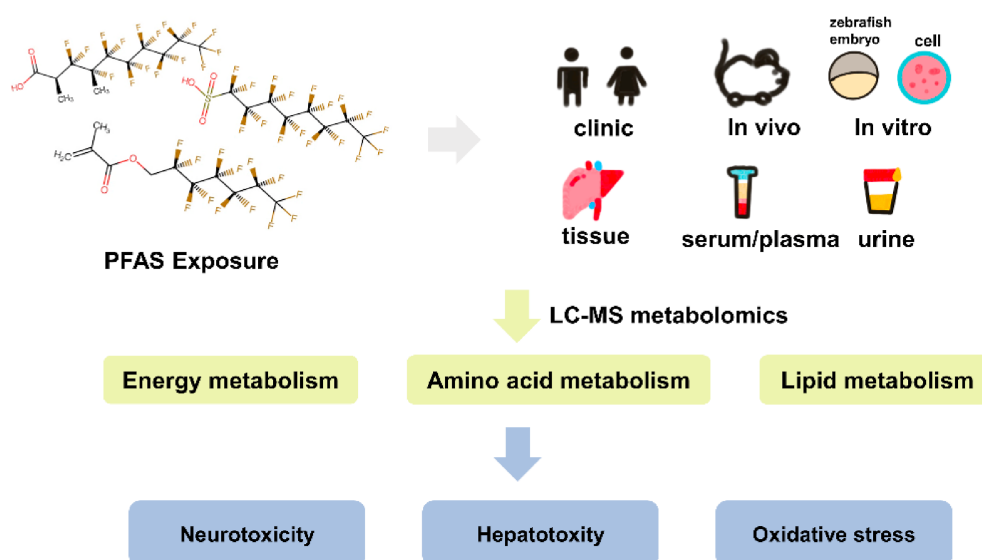


Fig. 1. PFAS-induced metabolic pathways underlying toxicity and oxidative stress.

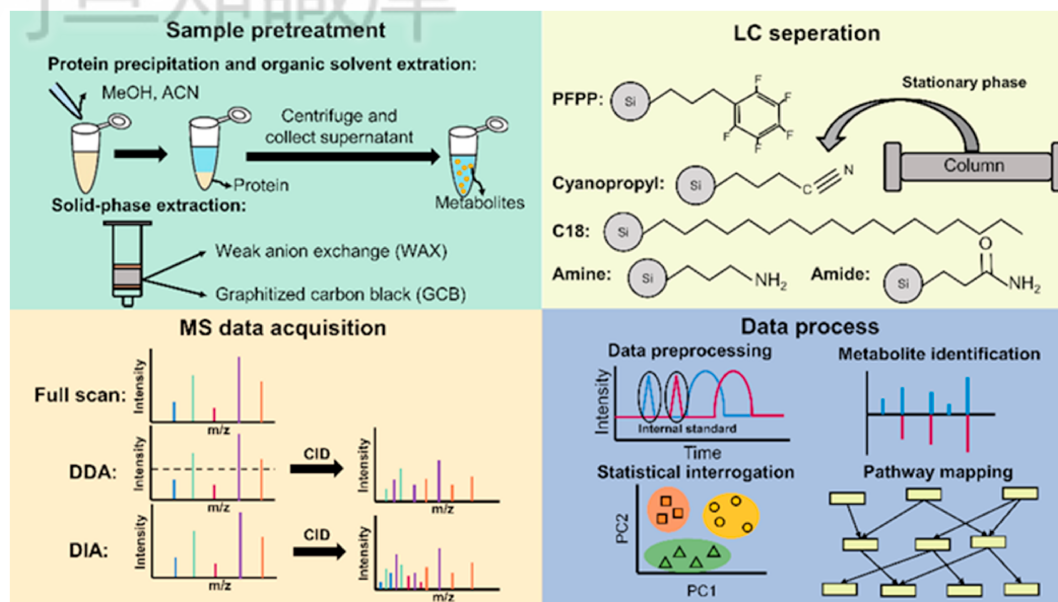


Fig. 2. Workflow for LC-MS based metabolomics analysis.

Solid phase extraction (SPE), offers higher selectivity and can complement precipitation-based methods. Protocols such as EPA (Environmental Protection Agency) method 1633A highlight the importance of homogenization, typically followed by cleanup steps including weak anion exchange (WAX) and graphitized carbon black solid phase extraction (GCB) to obtain clean extracts for LC-MS analysis [47]. When applied in combination, SPE improves reproducibility, minimizes matrix effects, and expands metabolome coverage, although it introduces additional complexity and potential challenges in reproducibility.

In addition to extraction strategies, the incorporation of internal standards during sample preparation is essential for achieving quantitative accuracy and data stability [48]. Internal standards, which are compounds with known concentrations and similar physicochemical properties to the analytes of interest, correct for variability introduced by factors such as extraction efficiency and ionization drift.

Derivatization has also emerged as a powerful complementary strategy in PFAS-related metabolomics to improve detection sensitivity, selectivity, and metabolite coverage. By chemically modifying functional groups of low abundance or poorly ionizable metabolites, derivatization enhances volatility, ionization efficiency, and chromatographic separation, thereby facilitating the detection of metabolite classes such as amino acids, fatty acids, and carbonyl-containing compounds. As a research summarized in Table 2, a combined

metabolomics and lipidomics study on mice exposed to PFDA demonstrated that derivatization enabled the identification of hepatic injury signatures and metabolic perturbations that would otherwise remain undetectable, underscoring its mechanistic value in toxicological investigations [39].

4.2. Liquid chromatography separation

In both non-targeted and targeted PFAS metabolomics, liquid chromatography (LC) is essential for achieving accurate and comprehensive analysis. For non-targeted approaches, its high resolving power is crucial for separating the wide structural and polarity range of metabolites. In targeted metabolomics, LC ensures the reproducible separation of specific compounds, which in turn maximizes quantification accuracy and minimizes matrix effects. Ultra performance liquid chromatography (UPLC) addresses this challenge by offering significantly enhanced resolution, reduced analysis times, and lower sample consumption than traditional LC systems. In complex biological matrices, such as serum or plasma, separation is indispensable, as it resolves co-eluting compounds that could cause ion suppression in mass spectrometry and provides retention time as an invaluable orthogonal identifier for metabolite annotation [49]. When high resolution LC is seamlessly combined with sensitive and selective MS detection, it enables the accurate trace-level quantification of PFAS and their metabolites [50].

While reversed phase liquid chromatography (RPLC) remains the backbone for separating non-polar to mid-polar analytes, standard C18 columns often fail to retain highly polar PFAS metabolites. To address this limitation, researchers have adopted several strategies. Advanced C18 phases, such as polar-embedded T3 columns, have been developed to improve the retention of polar compounds while tolerating 100% aqueous mobile phases [51]. Alternatively, hydrophilic interaction liquid chromatography (HILIC) excels in resolving strongly polar analytes and can enhance electrospray ionization efficiency. A variety of HILIC chemistries such as bare silica, amine-functionalized, and zwitterionic stationary phases can be selected based on analyte charge and polarity, and pentafluorophenyl (PFPP) column have also been used successfully to retain both polar and nonpolar analytes. Ultimately, the choice of column chemistry must be carefully matched to the specific biological matrix and the metabolite classes of interest to ensure robust and accurate data.

Mobile phase composition is central to both RPLC and HILIC separations. In RPLC, a mix of water (often buffered or acidified) and organic solvents such as acetonitrile or methanol is used, with acetonitrile typically preferred for its ability to produce sharper peak shapes and stronger signals. Conversely, HILIC commonly employs a high percentage of acetonitrile with a minor amount of water and buffer additives such as ammonium acetate or formate (with optional formic acid) to optimize pH and ionic strength [52]. For instance, optimized HILIC mobile phases with ammonium formate and formic acid have shown superior separation of polar metabolites like amino acids, nucleotides, and sugar phosphates. In contrast, RPLC is better suited for organic acids with formic acid [53]. For lipidomic analysis, mobile phases containing ammonium formate (in positive mode) or ammonium acetate with acetic acid (in negative mode) effectively balance signal intensity and retention time stability.

Combining RPLC and HILIC in a single workflow is a powerful strategy to achieve comprehensive coverage of PFAS-related metabolites. Innovations like dual-column configurations and gradient switching allow for the simultaneous analysis of a wide range of compounds, from hydrophobic species to early eluting polar metabolites [9]. This integrated approach is particularly valuable when analyzing diverse biological matrices—including serum, plasma, and cellular extracts—where metabolite composition and required sensitivity can vary significantly.

Ultimately, optimizing key LC parameters—such as using UPLC for high resolution, selecting appropriate stationary phases (RPLC or HILIC), and formulating precise mobile phases—is crucial for achieving sensitive and comprehensive PFAS metabolomics across all sample types.

4.3. Mass spectrometric detection and data acquisition strategies

The choice of MS acquisition mode is crucial in metabolomics workflows, largely determining metabolite coverage, quantitative precision, and downstream data processing complexity. Full scan acquisition on high resolution mass spectrometers (HRMS) instruments, such as orbitrap and QTOF instruments, provides broad coverage and exploratory sensitivity suitable for detecting unknown metabolic changes. This makes full scan the backbone of untargeted discovery experiments. For structural detail, many studies use MS/MS (tandem MS) and employ either data dependent acquisition (DDA) or data independent acquisition (DIA) [54]. DDA selects precursor ions for fragmentation based on their intensity in the preceding full scan, producing relatively clean MS/MS spectra that facilitate library matching and structural elucidation. However, DDA is intrinsically stochastic (biased toward high abundance ions), sensitive to parameter tuning (e.g., collision energy, isolation width, dynamic exclusion), and can miss low abundance species unless settings are carefully optimized. DIA approaches fragments all ions within sequential wide m/z windows, ensuring fragmentation data are acquired for virtually every detectable ion and increasing MS/MS coverage, but the resulting composite spectra require sophisticated deconvolution and spectral-matching algorithms, and may complicate confident annotation without high quality spectral libraries or advanced computational pipelines.

For quantitative analysis, such as validation of candidate biomarkers or high throughput screening, triple quadrupole instruments remain the gold standard. They operate in multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) mode, providing exceptional sensitivity and specificity. MRM can offer unparalleled sensitivity, linear dynamic range, and reproducibility for targeted metabolomics. While MRM offers unparalleled sensitivity and a wide linear dynamic range for targeted metabolomics, parallel reaction monitoring (PRM) on HRMS offers an attractive alternative. PRM provides full scan, high resolution MS/MS of selected precursors, which significantly improves specificity [55].

In practice, designing MS methods for PFAS-related metabolomics requires a balance between discovery and quantitation. Instrument performance is materially affected by several technical considerations, such as resolving power and mass accuracy, which are crucial for reducing isobaric interference. Other factors include scan speed, which impacts quantitation precision, and polarity switching, which is used to capture both positive and negative ions.

Beyond instrument-specific factors, ensuring the reliability of metabolomics data requires careful attention to sample preparation and data validation. This is achieved through the routine use of internal standards to correct for experimental variability, such as extraction efficiency and ionization drift. Furthermore, robust quality control (QC) procedures, including pooled QCs, blanks, and system suitability runs, are essential to monitor for matrix effects and signal drift throughout the entire analytical process.

4.4. Data process, annotation and biological pathway

Non-targeted metabolomics generates vast and complex LC-MS datasets that require rigorous processing and interpretation. The analytical workflow begins with raw data conversion, followed by peak detection and retention time alignment to correct for chromatographic drift. Data are then filtered to remove low-quality signals and normalized using methods like internal standards, TIC scaling, or QC-based approaches to reduce technical variation. Missing values are subsequently addressed through statistical imputation, preparing the data matrix for statistical analysis. For biological interpretation, univariate tests identify significantly altered metabolites, while multivariate models (e.g., PCA, PLS-DA) and machine learning algorithms are used for pattern recognition, classification, and biomarker discovery. A critical bottleneck remains in the accurate annotation of metabolites, which is addressed using spectral libraries (HMDB, METLIN) and in-silico fragmentation tools (MS-FINDER). These annotations are then categorized based on the Metabolomics Standards Initiative (MSI) confidence levels [56]. Mapping significant features onto biochemical pathways via resources like KEGG further helps reveal perturbed pathways and cross-talk. Increasingly, artificial intelligence and deep learning are being integrated into this workflow to automate peak picking, enhance annotation accuracy, and predict metabolite structures from MS/MS spectra. These AI-enabled tools promise to improve reproducibility, reduce manual bias, and accelerate the

discovery of exposure-related metabolic signatures, which is particularly valuable for PFAS research where subtle perturbations require highly sensitive strategies [31].

5. Methodological challenges and future perspectives

Although LC-MS has greatly advanced the characterization of metabolic responses to PFAS exposure, challenges remain that hinder data comparability and translation to risk assessment. These arise from heterogeneous experimental designs, diverse sample preparation and analytical protocols, and the complexity of metabolomics data. Biological matrices such as plasma and serum contain abundant proteins and lipids that interfere with PFAS detection, while endogenous metabolites often overlap with PFAS and their transformation products. This places high demands on sample preparation selectivity and LC-MS sensitivity, particularly for low-abundance PFAS substitutes and metabolites with potential biological activity. Reproducibility is another major issue. Variability in workflows, LC-MS configurations, acquisition modes (DDA vs. DIA), and data-processing pipelines complicates cross-study comparisons. This variability also hinders the establishment of unified biomarkers or pathway models. For example, DDA captures structural information but may miss low-abundance features. Moreover, stochastic precursor selection can reduce reproducibility. Limited clinical samples further constrain statistical power and amplify uncertainties. Because of these trade-offs, contemporary workflows often combine multiple acquisition modes. For instance, full-scan is used for feature discovery, DIA provides broad MS/MS coverage, and targeted MS/MS supports validation. Some studies also employ hybrid pipelines that build libraries from DDA data and then apply DIA for comprehensive screening [57].

Addressing these challenges requires standardization at multiple levels, including harmonized sample preparation, use of reference materials (e.g., NIST standards), and platform-independent identifiers such as collision cross sections (CCS) for PFAS annotation [58]. Such efforts would transform high-dimensional LC-MS data into reproducible evidence with public health relevance. Looking ahead, integrating AI and machine learning for data mining, developing automated online sampling preparation LC-MS platforms for real-time monitoring, and combining metabolomics with other omics (e.g., lipidomics, transcriptomics) will

provide a holistic view of PFAS toxicity [34,39]. Coupling LC-MS with complementary platforms like desorption electrospray ionization (DESI) and spatial mass spectrometry further enables system-level insights. This combination of technological innovation and standardization will advance PFAS metabolomics toward robust biomarker discovery and translational applications.

6. Conclusion

In conclusion, LC-MS based metabolomics has proven to be a powerful tool for discovering the molecular consequences of PFAS exposure. Its capacity to provide comprehensive metabolic profiles in diverse models has consistently highlighted perturbations in key pathways, particularly in lipid and energy metabolism. This has provided invaluable mechanistic insights and identified candidate biomarkers, thereby strengthening the translational relevance of experimental findings to human health.

The future of this field lies in the coupling of technological innovation with applied research. Continued advances in HRMS, along with standardized workflows and AI-assisted data analysis, will be critical for improving reproducibility and comparability. This will accelerate the establishment of robust biomarker panels and pathway models, ultimately empowering us to better inform PFAS risk assessment and guide precision strategies for prevention and intervention.

Declaration of interest statement

The author declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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