High-Throughput Solid Phase Extraction for Targeted and Non-Targeted Exposomics

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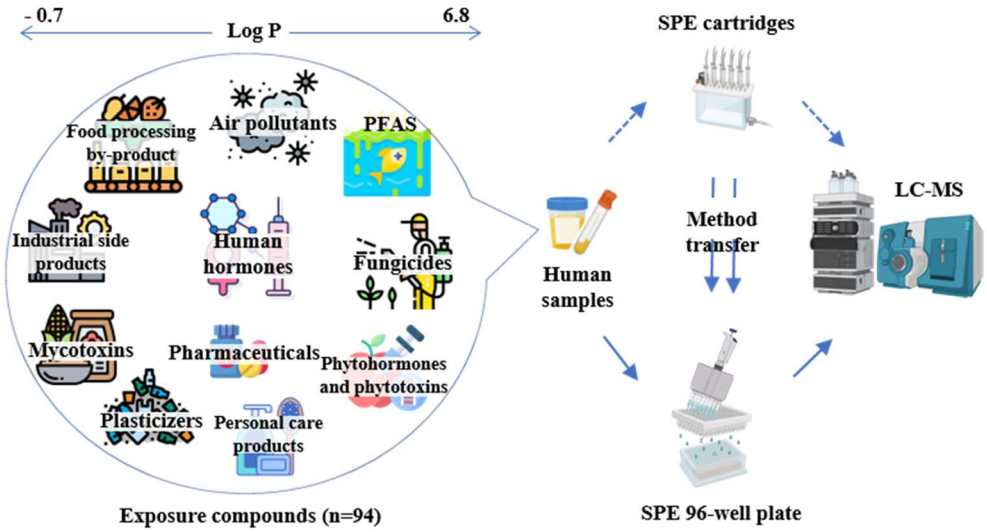
ABSTRACT: Characterizing the chemical exposome relies on advanced instrumentation including tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS), and non-targeted analysis (NTA) using high-resolution MS. However, proper sample pretreatment, balancing broad analyte coverage, method robustness, and throughput remain a major bottleneck in exposomics. Here, we developed a robust and scalable solid phase extraction (SPE) protocol in 96-well format for human urine and plasma and optimized it for a panel of 94 highly diverse environmental and food-related contaminants (LogP -0.7 ~ 6.8). Extraction recoveries (RE) and signal suppression and enhancement (SSE) were determined using targeted LC-MS/MS. Acceptable REs (60% - 140%) were achieved for >70% of all analytes, and acceptable SSE values (60% -140%) for 86% and 90% in urine and plasma, respectively. Subsequently, the method was transferred to 96-well format, significantly improving throughput to meet the capacity requirements needed for exposome-wide association studies (ExWAS). The established workflow is approximately 10× faster than routinely used metabolomics-based protein precipitation approaches when comparing the estimated total analysis time for 1000 samples. The method's applicability for NTA and suspect screening was tested and compared to a generic protein precipitation protocol using NIST standard reference materials for urine (SRM 3672) and plasma (SRM 1950). Favorable performance was shown for the protein precipitation workflow while the SPE protocol demonstrated promising results. The developed workflow is thus not only superior for future high-throughput targeted exposomics but also offers an option for NTA applications. The presented well-balanced approach is scalable and also applicable to research in the fields of pharmacology, food safety, or systems toxicology.



Humans are consistently exposed to a myriad of chemicals during their daily lives through various sources, including dietary intake and environmental pollutants[1-3]. Exposomics represents the comprehensive analysis of all environmental and food-related exposures and associated influences on various health outcomes[4]. To allow a full assessment of the totality of chemical exposures in human samples, sensitive, specific, and robust analytical techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) are required. Multi-class or next-generation human biomonitoring (HBM) methods have recently been developed to monitor a wide range of environmental chemicals and are needed to address the full complexity of chemical exposure[5, 6]. In contrast, non-targeted analysis (NTA) is an emerging technique for exposomics using high-resolution mass spectrometry (HRMS) and allows the exploration of unknown chemicals in human samples[7, 8]. Furthermore, suspect screening methods use large chemical databases and spectral libraries to prioritize detected features and annotate compound identities[9, 10]. Both targeted and non-targeted exposomic methods face significant challenges related to complex biological sample matrices[11], and ensuring repeatability and consistency across large sample sets is mandatory, especially when dealing with low-abundance analytes (i.e. pg/mL levels or lower)[12-14]. Addressing these challenges in exposomics requires sophisticated optimization of the entire analytical workflow, including sample preparation protocols. Efficient sample pretreatment before instrumental analysis can reduce interferences, separate, and concentrate analytes in diverse matrices[15, 16] and various sample preparation techniques have been optimized to analyze the exposome. Liquid extraction (LE) and protein precipitation (PPT) are widely used extraction methods for exposomics due to their comparably wide chemical coverage[6, 17]. However, these techniques are prone to preserve matrix interferences which can hamper overall method performance due to limited sample clean-up.

Recently, solid phase extraction (SPE) got more attention as an option for sample preparation in exposomics and is discussed as a promising approach for future large-scale applications, owing to its capability to reduce matrix effects (ME), enhance sensitivity, and improve consistency in high-throughput studies[18, 19]. SPE has been frequently used for the purification and extraction of various analytes from diverse sample matrices for HBM including urine[20], blood[21], and various other biological matrices[22, 23]. However, a systematic development of an exposome-scale SPE method compatible with both, targeted and non-targeted data acquisition strategies has rarely been performed. This challenge originates from the complexity of designing a balanced method with suitable method robustness and broad chemical space coverage. Most existing SPE methods focused on a limited selection of targeted analytes to obtain reliable protocols without assessing the potential coverage for NTA[24] due to the lack of certified reference standards, which restricts their utility in NTA.

In this study, a targeted high-throughput sample pretreatment method using SPE cartridges was optimized to assure the applicability of the workflow for urine and plasma samples. The predicted octanol-water partition coefficient (logP) from PubChem (pubchem.ncbi.nlm.nih.gov) was utilized to evaluate the chemical coverage in terms of analyte lipophilicity of compounds in this study. Target analytes with a wide range of physicochemical properties, including polarity, presents a central challenge for SPE methods in exposomics, due to varying analyte-sorbent interactions. This issue is a key factor that needs to be addressed during method development aiming for a comprehensive chemical coverage. Here, 94 highly diverse analytes from multiple toxicant classes including perfluorinated substances (PFAS), plasticizers, personal care products, food processing by-products, pesticides, industrial chemicals, air pollutants, disinfecting agents, mycotoxins, phytoestrogens and phytotoxins were used to optimize an SPE protocol for high-throughput applications. Optimization steps included selecting SPE sorbents and sample buffers, optimizing washing and elution steps, and improving efficiency. Subsequently, the protocol was transferred to 96-well plates, and analytical performance and throughput capacity were rigorously tested. Finally, the potential for NTA was explored using NIST standard reference materials (SRMs) for urine and plasma. The results indicate the feasibility of the approach for scaling and large-scale exposome-wide association studies.

Experimental Section

Biological samples and chemicals.

Human pooled plasma was purchased from Innovative Research (Novi, MI, USA). Pooled urine was obtained from a female volunteer who abstained from consuming food and beverages stored in plastic containers, phytoestrogens rich food, and cosmetics containing parabens for two days before sample collection[14]. Standard reference materials, including urine (SRM 3672) and plasma (SRM 1950) were obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, USA) for NTA. Detailed information on standards, including selection and spiking levels can be found in **Tables S1-S4**. The selection of target analytes and analyte classes was intended to be as broad as technically feasible with differing structures, real-life exposure levels, and toxicological modes of action. It was based on our previous studies on next-generation biomonitoring (Jamnik et al. 2022)[6].

Method development and optimization.

The sample pretreatment approach was developed and optimized in several steps. Initially, two universal SPE sorbents, a commercial hydrophilic-lipophilic balanced sorbent (HLB) from Waters and an in-house prepared mixed-mode SPE sorbent, were compared. The mixed-mode sorbent was prepared from two SPE sorbents, primary second amine (PSA) and C18 (PSA+C18), by mixing equal weights before packing. Details of the preparation are described in the **Supplementary Information** (**SI**). Packed SPE cartridges (HLB or PSA+C18) were then used to optimize the full processes including sample loading, washing, and elution. A mixture of 94 reference standards was spiked into urine, plasma, and H_2O samples both before ("prespiked") and after ("postspiked") the SPE process. Analyte loss (%), extraction recovery (RE, %), and signal suppression and enhancement (SSE, %) were calculated based on the peak areas from these "prespiked" and "postspiked" samples.

Analyte loss was defined as the fraction of an analyte that was not retained by the SPE sorbent. It was calculated as the ratio of peak areas of the effluents collected during sample loading and washing from “prespiked” samples compared to peak areas of “postspiked” effluents. Analyte loss of 0%

was achieved for complete analyte retention, whereas completely unretained analytes are characterized by a loss of 100%.

The term extraction recovery (RE) was used to describe the recovery of the full SPE process including loading, washing and elution. Thus, RE was calculated as the ratio of peak areas of a "prespiked" and "postspiked" sample. Full analyte recovery results in RE of 100%, whereas 0% indicates no analyte recovery of the SPE procedure. RE greater than 100% indicate contamination or carryover introduced during sample preparation or sample injection.

Matrix effects in terms of signal suppression and enhancement (SSE) depend on the influence of the sample matrix on the analyte's signal intensity. This was calculated as the ratio of peak areas for "postspiked" samples to a standard in pure solvent[25]. SSE values close to 100% indicate negligible effects of the sample matrix on the detector response, which is required for accurate analyte quantification.

To evaluate the method performance and provide a rationale for the individual optimization steps, the metrics described above were classified into three categories: "good," "acceptable," and "poor," as shown in **Table S5**. Noteworthy, they do not represent method validation criteria but are used for method optimization.

Subsequently, the SPE method was applied to pooled human urine and plasma samples to test applicability to analyze human samples. Sample treatment was further streamlined and transferred from SPE cartridges to 96-well plates to allow parallel sample extraction and maximize sample throughput. Furthermore, the applicability of the developed workflow for NTA and suspect screening was tested to further expand the coverage of the targeted biomonitoring assay.

SPE Sorbent Selection.

SPE cartridges with two different sorbent types, HLB and PSA+C18, were considered and the stability and robustness of the material were tested. For this purpose, loading, washing, and elution procedures were individually evaluated. In this first evaluation, analytical standards in LC-MS grade water (H_2O) were used as samples to minimize influences of the sample matrix. Analyte loss (%) for both SPE sorbents was tested using H_2O or 2% methanol (MeOH/H_2O, 2/98, v/v) for sample loading and washing. Subsequently, RE for diverse elution solvents were compared including 3% formic acid in methanol (FA/MeOH, 3/97, v/v), 3% ammonia in MeOH (NH_3/MeOH, 3/97, v/v), and pure MeOH. Details including conditioning of SPE sorbents, analyte spiking procedures and spiking levels, as well as calculations of the trapping efficiency and RE, are reported in the **SI**.

Sample Buffer Selection Before Loading.

Urine and plasma are commonly used sample matrices for HBM and, especially in the case of urine, varying concentrations of matrix components can influence analyte extraction. To ensure method reliability, commonly utilized buffers, PBS (pH 7.4, 200 mM) and NH_4AC (pH 6.0, 2.5 M), and pure H_2O were tested for sample dilution before the SPE cleanup. Based on the results of our previously published work, a final dilution ratio of 2 was used[26].

Optimization of Washing, Elution, and Reconstitution Steps.

Water was selected as the washing solvent and the required volume was optimized by comparing the SSE of either washing one or two times with 1 mL. MeOH was selected as the primary component of the elution solvent due to its widespread application in SPE processes[27]. Considering the strong anion-exchange interactions of PSA with commonly encountered acidic compounds in exposomics[9, 28], a basic MeOH solution (3% NH_3) was used as the elution solvent to compare the RE of the mixed-mode sorbent (PSA+C18). For HLB, both acidic (3% FA) and basic MeOH (3% NH_3) were evaluated due to both anion and cation exchange properties of HLB. Furthermore, MeOH and two commonly used solvents, acetonitrile (ACN) and isopropanol (ISO), and a mixture of MeOH/ACN/ISO (1/1/1, v/v/v), were evaluated as elution solvents. The solvent mixture was selected based on results obtained from urine samples. Elution volumes of 200 µL, 400 µL, and 800 µL were subsequently compared using pooled urine samples as a matrix.

A typical workflow to improve detection limits is to evaporate raw extracts and reconstitute them in a defined volume. Despite its clear benefits for enhancing the detection of low-abundant analytes, this step is rather time-consuming, especially when dealing with a large number of samples. To further enhance sample throughput and assess the effect of evaporation and reconstitution, two workflows were tested and compared. One set of SPE extracts was dried using a CentriVap Vacuum concentrator (Labconco) and reconstituted with 50% MeOH (MEOH/H_2O, 1/1, v/v). In contrast, a second set of extracts was diluted with H_2O, resulting in a final mixture of 50% MeOH for comparison. The final concentrations in the extracts of both sets were the same.

Method Transfer to 96-well Plates.

After the optimization, pooled urine and plasma were used to test extraction and cleanup using the developed workflow for SPE cartridges. Then, the method was transferred to a 96-well plate SPE packed with HLB (Waters Corporation, USA) and the following parameters: SPE plates were conditioned with 1 mL of MeOH, followed by 1 mL of H_2O. Then, 400 µL of urine or plasma sample were mixed with 400 µL of PBS. The mixture was loaded on the SPE plate and washed twice with 1mL of H_2O each. Gravity flow was selected for sample loading onto SPE and washing to minimize analyte loss. Analytes were eluted by gravity using 200 µL of MeOH twice, for a total of 400 µL, with negative vacuum by manifold before and after the elution to control the flow rate no more than 3 drops per second. RE and SSE were calculated and compared to results determined for SPE cartridges to prove applicability for high-throughput applications.

Assessing the Potential of SPE for NTA and Suspect Screening.

SPE is a promising technology to enhance method performance for targeted applications, but systematic testing about chemical coverage is a necessity before any potential application in non-targeted analysis. The coverage of other typically detected analytes in human samples and the effect on signal intensities were evaluated by comparing results of the novel 96-well-based SPE approach to results from a PPT method[29] as the reference method for non-targeted applications. SRM 1950 (plasma) and SRM 3672 (urine) were extracted with both workflows and were analyzed using LC-HRMS for NTA. Unknown compounds were annotated using publicly available spectral libraries and a set of 234 authentic analytical standards (Table S2) was spiked into samples for compound identification and to test the method for

targeted screening. Results were reported based on well-established reporting schemes, with identified compounds reported with the highest confidence level ("level 1": identified based on authentic chemical standards). Therefore, standards were only spiked into extracts from the SPE process. Spiking levels of these standards are reported in **Table S4**. After annotation, the effect of the sample preparation method on relative feature intensities was compared. Results were further investigated as a function of retention times and classifications of the annotated features. Details are outlined in the **SI**.

Data Acquisition.

Targeted LC-MS/MS.

Front-end LC separation was performed using our previously published method[6]. Identical separation gradients were used for LC-MS/MS and LC-HRMS(/MS) using an Agilent 1290 Infinity II and a Thermo Scientific Vanquish Horizon, respectively. An Acquity HSST3 column (1.8 µm, 2.1× 100 mm, Waters) was used with gradient elution using a flow rate of 0.4 mL/min and 0.3 mM ammonium fluoride in H_2O as eluent A and ACN as eluent B. Details are reported in **Table S6**. A QTrap 6500+ mass spectrometer equipped with an electrospray ionization (ESI) source (Sciex) was operated using one of our previously published multiple reaction monitoring (MRM) methods with fast polarity switching[6]. Full method parameters including MRM transitions and ESI parameters are provided in the **SI**.

Non-targeted HRMS(/MS).

An Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) was used for HRMS(/MS) measurements. Samples were analyzed using full scan mode and AcquireX was used to generate fragment spectra using a mass resolution of 120,000 at *m/z* 200, with full method parameters reported in the **SI**.

Data Analysis

Quality Control.

Several types of QC samples were used to ensure reliable results. A systems suitability test sample (SST) was measured before and after each analytical sequence to ensure favorable instrument performance (see **SI** and **Table S7**). In addition, a multi-analyte reference standard mixture consisting of 94 highly diverse chemicals in pure solvent was injected. Solvent blanks were used to monitor instrumental background and carry-over and process blanks were used to monitor background concentrations/contaminations.

Targeted Data Analysis.

RE and SSE values were evaluated for the set of 94 analytes[6] using standards in neat solvent, pooled urine, and pooled plasma. Peak integration was carried out using Multiquant (v3.0.2, Sciex) and Skyline (v21.2.2.536, McCoss Lab)[30]. Calculation of RE and SSE was performed using Microsoft Office 365. Graphics were created by Origin 2021b (v9.8; OriginLab Corporation).

Non-targeted Data Analysis.

Raw data acquired in polarity switching mode was split based on the polarity using Freestyle (Thermo). Then, raw data files were analyzed using MSDIAL (v5.2)[31]. Full data processing settings including data extraction, peak picking, compound identification, and alignment are reported in the **SI**. To evaluate the qualitative analyte coverage for NTA and suspect screening, MassBank of North America (MoNA, https://mona.fiehnlab.ucdavis.edu/) was used as a spectral library. Annotations were then classified based on the level of confidence scheme according to Schymanski et al.[32] using confidence levels 1 (confirmed by authentic standards), level 2 (confirmed by spectral library match), and level 3 (confirmed by spectral library match, but with isomeric structures). Additional data analysis and visualization was performed using Microsoft Office 365, Origin 2021b, MetaboAnalyst (v6.0), and Inkscape (version 1.2.2; Inkscape).

Results and Discussion

SPE Sorbent Selection.

Analyte loss was compared for both SPE sorbents and HLB and PSA+C18 showed comparable performance (see **Figure S1**). "Good" performance (analyte loss <10% during loading and washing) was achieved for 90% of all analytes, whereas "good" RE was achieved for 59% of all analytes using HLB compared to 39% using the mixed-mode sorbent PSA+C18.

Analyte loss >5% was observed for six analytes with LogP values between 1.7-3.2 when using the PSA+C18 for SPE, whereas analyte loss >5% was only observed for a single compound when using the HLB sorbent (see **Figure S2**). Most analytes showed sufficient RE (50%-150%) using either of the two sorbents but RE<50% were observed for 15 analytes using the mixed-mode sorbent, whereas only six analytes with RE<50% were observed using the HLB sorbent. Consequently, the HLB was selected as the sorbent material for further workflow optimization.

Buffer Selection for Sample Before Loading.

Subsequently, the buffer system used for sample loading was optimized. As shown in **Figure S3**, >95% of all analytes showed analyte losses <10% after diluting samples with PBS buffer. This fraction dropped to 80% and 87% of the analytes when using NH_4Ac and H_2O as dilution solvents. Consequently, PBS was chosen as the sample buffer for further process optimization.

Washing, Elution and Reconstitution Optimization.

Thereafter, washing, elution and reconstitution steps were optimized. Matrix effects were investigated in terms of SSE (%) and results for the two tested washing protocols were largely comparable (**Figure S4**). However, using two times 1 mL of H_2O improved SSE by approximately 5% for most analytes, especially for those with RT between 1.0 min and 7.0 min (**Figure S5**). Consequently, two washing cycles using 1 mL H_2O each were used in the final protocol.

The use of acidic MeOH solutions (3% FA) as elution solvent resulted in lower RE (<50% for most analytes), whereas better RE (50%-150% for most analytes) was achieved using pure MeOH or basic MeOH (3% NH_3) for elution. Six nonpolar compounds (LogP, 4.0 - 5.5) had surprisingly high RE values >150% using the basic MeOH solution while only a single compound had RE>150% using pure MeOH (**Figure S2 (F)**). Thus, pure MeOH was selected as the elution solvent for further optimization. In addition, it should be noted that basic solvents with high ammonia concentrations may cause issues including unstable RTs and potential damage to both the UPLC column sorbent and the UPLC system itself[33]. Different elution solvents (MeOH, ACN and ISO) were compared and elution volumes were optimized. "Good" REs were achieved for approximately 70% using MeOH for

elution, whereas this reduced to 39-62% when ACN, ISO or a 1:1:1 mixture of all solvents was used (**Figure 1**).

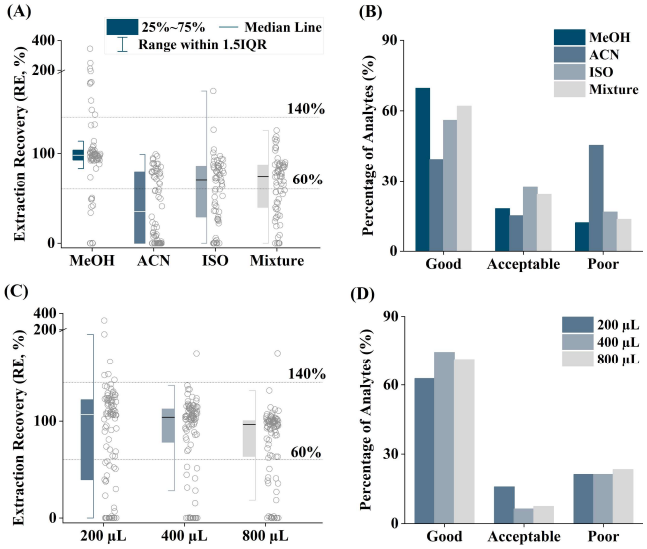


Figure 1. Extraction recovery (RE, %) for 94 analytes in pooled urine using HLB cartridges with different elution conditions (A) and classification of the performance into three categories (B) using methanol (MeOH), acetonitrile (ACN), isopropanol (ISO), and a mixture of ISO/MeOH/ACN (1/1/1, v/v/v). Panels (C) and (D) show results for the optimization of the elution volume for MeOH (200 µL, 400 µL, and 800 µL). in the following classification was used in (B) and (D): "Good": RE 60% - 140%, "Acceptable": RE 20% - 60% or 140% - 180%, "Poor": RE < 20% or RE > 180%.

Solvent volumes were optimized for MeOH and 200 µL, 400 µL and 800 µL were tested for elution (see **Figure 1 (C-D)**). Approximately 70% of compounds achieved "good" REs when using 400 µL or 800 µL of MeOH for elution, whereas this fraction dropped to 63% when eluting with 200 µL MeOH. The smaller solvent volumes (200 µL) resulted in inefficient elution for around 10% of all analytes and 400 µL (using 2 times 200 µL) was selected for further method optimization, as it represented a well-balanced compromise between good RE and low solvent consumption. Regardless of the elution conditions, certain highly polar compounds in pooled urine were poorly retained on the SPE cartridges. These compounds were primarily low-mass organic acids such as 5-hydroxymethyl-2-furanoic acid, dibromoacetic acid, dichloroacetic acid, bromoacetic acid, and p-hydroxybenzoic acid and were only weakly retained by the reversed-phase (RP-)LC column and with the used method (i.e., RT <1 min).

The effect of drying the raw extracts followed by reconstitution was investigated (see **Figure S6**), and diluting the raw extracts resulted in improved recoveries, indicating the need to consider the optimization of reconstitution steps during method development. Approximately 6% of analytes fell into the "acceptable" RE category when using drying and reconstitution, compared to the dilution method. Potential causes include short vortexing, the absence of sonication steps, or the selection of the resuspension solvent containing 10% ACN in H_2O (1/1, v/v). For example, the RE for 2-tert-butylphenol decreased significantly from 99.7% with dilution to 23% after drying and reconstitution. Furthermore, the study found an increased background signal for certain plasticizers, such as bisphenol F and S, when using drying and reconstitution. Consequently, a direct dilution approach is clearly favorable in terms of simplicity and speed, which are both required for high-throughput applications.

Transferring SPE Method from Cartridges to 96-Well Plate Format.

For maximizing sample throughput, the SPE protocol was transferred to 96-well plates (plate-based SPE) and performance was compared to the initially optimized cartridge-based SPE protocol. In total, 72% of all analytes fulfilled the criteria for "good" RE when using the cartridge-based protocol for extracting urine or plasma samples, and 86% and 90% of the analytes were classified as "good" in terms of SSE. When using 96-well plates, 71% and 60% of the analytes achieved "good" REs while 76% and 78% of all analytes obtained "good" SSE in urine and plasma, respectively. Most analytes with "good" REs using the cartridge-based workflow were also categorized as "good" using the 96-well plate workflow for the extraction of urine, whereas the performance of the 96-well plate method was reduced for 12% of the analytes in plasma. Similarly, the cartridge-based protocol outperformed the plate-based SPE method in 10% of the cases in terms of matrix-induced SSE (see **Figure S7**).

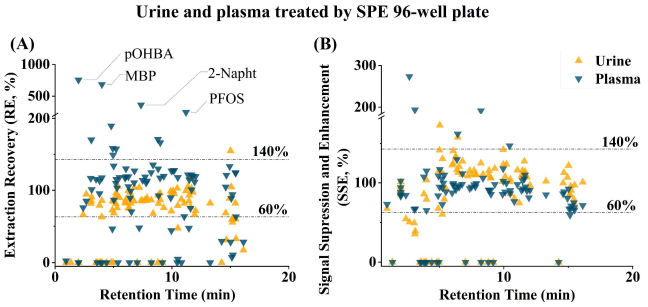


Figure 2. Extraction recoveries (A) and signal suppression and enhancement (SSE, %) (B) for 94 analytes in urine (yellow) and plasma (blue) using the optimized protocol based on SPE 96-well format. pOHBA, p-hydroxybenzoic acid; MBP, mono-butyl phthalate; 2-Napht, 2-naphthol; PFOS, perfluorooctanesulfonic acid.

In total, around 25% of the analytes were poorly extracted from plasma and 20% from urine using the plate-based protocol. The observed discrepancies might be explained by differences in the manufacturing process, including sorbent packing, or differences in sample handling when working with 96-well plates. In addition to weakly retained compounds (low-mass acids, see above), additional compounds in plasma, such as p-hydroxybenzoic acid (pOHBA), monobutyl phthalate (MBP), 2-naphthol (2-Napht) or perfluorooctanesulfonic acid (PFOS), were reported with "poor" RE. This was due to their background levels being 2 to 10 times higher than the spiked levels. In general, RE and SSE was only slightly better when using cartridges compared to 96-well plates (see **Figure 2**). Despite the slightly reduced performance in selected examples, the application of 96-well plates still holds promise for large-scale applications due to its comparable performance to the cartridges in combination with notable improvement in sample throughput, and handling. A schematic representation of the SPE workflow based on 96-well plates is presented in **Figure 3** and more details are given in **Table S8**. To assess the full potential for high-throughput applications, the total analysis time of the 96-well plate-based SPE workflow was predicted for the analysis of 1000 samples, and time estimates were compared to our routinely used PPT protocol[29]. The new plate-

based SPE protocol allows extracting 1000 biological samples within 3 days, whereas the same amount of samples would require 29 days using a routinely used PPT workflow as shown in **Table S9**.

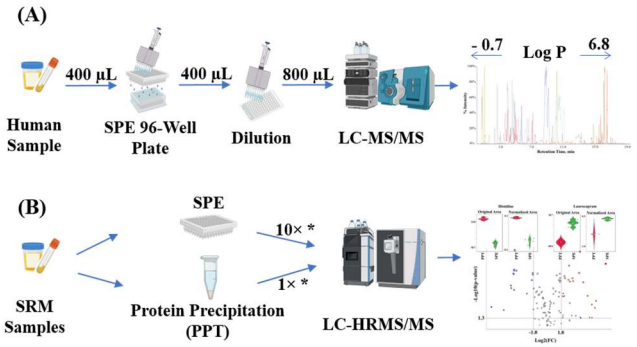


Figure 3. (A) SPE workflow for targeted analysis; (B) Illustration of the feasibility test of the established SPE method for non-targeted analysis (NTA). * The SPE protocol in 96-well formats is approximately 10× faster than a state-of-the-art protein precipitation workflow when calculated for 1000 samples. The -0.7 to 6.8 logP range indicates the workflow's ability to cover chemicals with varying polarities.

Assessing the Potential of SPE for NTA and Suspect Screening.

While systematic optimization and characterization of sample preparation methods for targeted (multi-analyte) assays can be performed easily, full-performance comparison for non-targeted applications is less straightforward. We aimed to characterize the applicability of the developed SPE method for NTA and suspect screening, by comparing performance to a well-established protein precipitation method (PPT). Thus, extracts obtained with PPT and 96-well plate-based SPE were analyzed using LC-HRMS(/MS) (see **Figure 3** (B)), and results were compared on the feature level. Extracts of NIST SRM 1950 (plasma) and SRM 3672 (urine) were analyzed on an LC-HRMS instrument. Peak picking, feature alignment and compound annotation were performed using MS-DIAL together with reference mass spectra from MassBank of North America (MoNA). Relative intensities were determined for annotated features as the area under curve (AUC), and results of the PPT and SPE workflows were compared for annotated and identified features. Values of fold change (FC > 2) and significance (p-value < 0.05) were analyzed using MetaboAnalyst[34]. Approximately 50% of the annotated features detected in SRM 1950 (plasma), showed significantly higher AUCs (FC > 2, p-value < 0.05) when using the PPT workflow (see **Table S10**). Yet, SPE extraction resulted in significantly higher signal intensities for 13% of the features, and 36% of the features were not significantly affected by the choice of sample preparation. For SRM 3672 (urine), we observed significantly higher AUCs using PPT in 32% of cases, whereas 10% were significantly enhanced using SPE, and 58% of annotated features were not significantly different (**Table S11**).

Figure 4 (A-B) shows results of the comparison of relative feature intensities as fold changes of AUC as a function of the RT, whereas results are summarized on the compound class level in **Figure 4(C)**. For SRM 1950 (plasma), higher relative signal intensities in PPT extracts were observed for polar compounds, mainly amino acids and organic acids with RT <3 min, which is well in line with the results from the targeted assay. Additionally, several nonpolar features, mainly lipids with RT > 10 min were higher in the PPT extracts. Similar results were observed for SRM 3672 urine, with polar compounds including amino acids and derivatives or organic acids with RT < 3 min showing higher AUCs in PPT extracts compared to SPE. However, some key low-abundance biomarkers were observed with significantly higher AUCs using SPE, like nicotine in urine (SRM3672) and cocaine in plasma (SRM 1950, see **Figure S8**). Interestingly, no significant differences between PPT and SPE were observed for major metabolites of nicotine and cocaine such as benzoylecgonine[35] for cocaine, and hydroxycotinine and cotinine for nicotine.

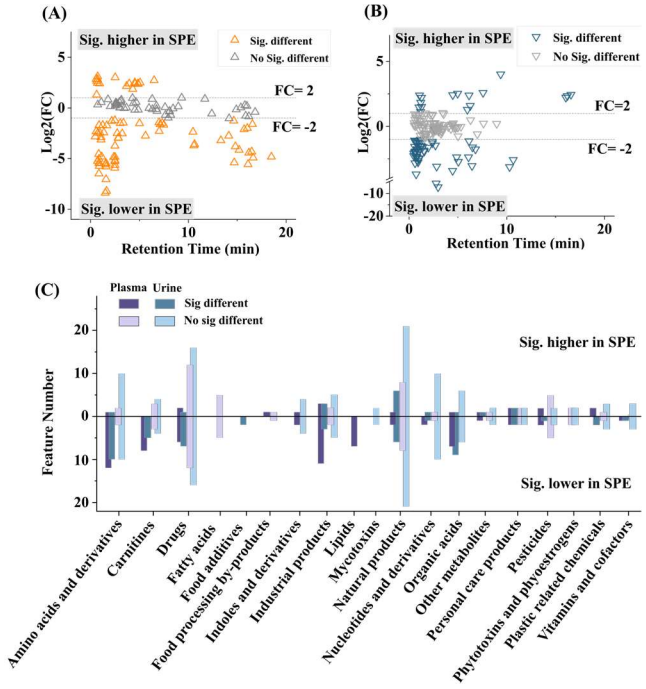


Figure 4. Evaluation of SPE performance compared to protein precipitation for non-targeted analysis (NTA). Comparison of peak areas of annotated features (confidence levels 1-3) in SRM 1950 plasma (A) and SRM 3672 urine (B) as a function of retention time as log2 fold change of the peak area, and summary of the results based on the compound class (C).

Despite these differences, comparable results were obtained for a large fraction of features and diverse compound classes including amino acids, carnitines, natural products, or synthetic compounds such as medical drugs and pesticides. For instance, 16 drugs were detected with comparable AUCs in PPT and SPE extracts of SRM 3672 urine. Similarly, twelve drug-related features were detected in the plasma with comparable AUCs using both methods.

In conclusion, the PPT workflow offers wider coverage of annotated features, but comparable results were obtained for many annotated compounds. In urine, the results were comparable for 58% of annotated features detected in extracts using both methods. However, PPT extraction provided broader feature coverage for the analysis of plasma samples, particularly endogenous polar metabolites, whereas SPE extraction improved the detection of low-abundance exogenous compounds at the cost of losing several polar metabolites.

Limitations

While the established workflow is effective for most analytes in targeted multi-class analysis of human samples and performs comparably well for a relative majority of analytes

in NTA, several limitations remain. The analytes selected for method optimization were highly diverse and yet still represent only a limited subset of the known chemical exposome. Although analytes were selected with a special emphasis on covering a wide range of physicochemical properties, coverage needs to be further expanded for exposome-scale work. A broader selection of compounds and wider chemical coverage would be needed for a truly holistic representation of the exposome in future work. Moreover, certain analytes, especially polar acidic compounds, are partly lost during the SPE process due to weak retention by the sorbent, which was expected. Although different workflows could address these specific shortcomings by targeting particular sets of analytes, conducting multiple measurements to gather sufficient information is time-consuming. Therefore, achieving favorable performance in terms of comprehensive analyte coverage, efficient cleanup, and minimal time consumption simultaneously is not always feasible and not always required[36].

Conclusion and Outlook

In conclusion, we present the development of a high-throughput SPE protocol based on 96-well format for faster, cheaper, and more robust omic-scale exposure analysis. This workflow was optimized comprehensively considering a large scale of targeted analytes, and chemical coverage was further compared with a validated PPT method to ensure sufficient analyte coverage for targeted LC-MS/MS and non-targeted LC-HRMS applications. Despite reduced method performance for very polar compounds, the comparison of SPE and PPT workflows clearly indicated that the SPE method is a suitable candidate for large-scale NTA or suspect screening applications, especially due to the outstanding enhancement of sample throughput and instrumental robustness. This is underlined by the comparable overall performance of PPT and SPE workflows for a wide range of analytes, especially when RP-LC is used as a front-end separation technique. A pragmatic balance between reduced matrix complexity and chemical coverage was achieved, and due to the high sample throughput, the presented workflow can be regarded as a suitable candidate for exposome-wide association studies (ExWAS). Future work will include the expansion to even more toxicologically relevant chemicals and testing the capacity of the developed sample preparation workflow in large cohort studies to investigate the impact on instrumental robustness and downtimes.

ASSOCIATED CONTENT

Supplementary information is provided as an Excel spreadsheet and a PDF Document with additional information regarding the methods and the results.

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